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Greeting from the Editor-in-Chief

On behalf of the new Editorial Board of the Acta Physiologica Hungarica let me first offer our deep appreciation to those of you represent the readership and subscribers of journal. Acta Physiologica has a long history and rich tradition in publishing high quality research papers not only from Hungarian authors but also from investigators of many other countries. The staff and the structure of the reorganized Editorial Board shall be dedicated to the traditions of this remarkable journal and will work aggressively to achieve journal of internationally recognized excellence.

Acta Physiologica Hungarica encourages submissions of original research papers in either full length, or brief rapid publications. Mini-reviews in any field of physiological sciences and letter to the Editors also will be accepted. Beyond theoretical papers based on experimental studies, the Board will specially support scientific publications which link physiology with clinics, applied medico-biological sciences, and primary prevention. We therefore, encourage our potential authors to submit publications not only in the field of experimental physiology and pathophysiology, but also those dealing with clinical and applied physiology and pathophysiology, sport and veterinary physiology, biomedical engineering, or other related applied sciences. An important new mission of the journal will be to encourage scientific publications which focus on the promotion of human and animal health and contribute to our basic knowledge related to primary prevention, and early diagnosis. The Board also encourages submission of the papers dealing with educational and historical matters of physiological sciences.

We wish to thank the members of the International Editorial Board of Acta Physiologica Hungarica, as well as the Hungarian Academy of Sciences, and the Hungarian Physiological Society for their support in this endeavor.

The history of this journal reflects also the valuable traditions and rich heritage of the Hungarian physiological sciences in both research and education (A.J.P. Adv. Physiol. Educ. 19/1: S38–39, 1998). The first Hungarian Chair of Physiology (Physiologia et Materia Medica) was founded in 1769 and the first university textbook written in Hungarian language was published in 1789 by Professor Samuel Rácz, entitled "A Brief Summary of Physiology". The book met the highest professional standards of that time (Adv. Physiol. Sci. 21: 85–203, 1981). The first Hungarian Association of Physiologists was established in 1891, lead by internationally recognized interdisciplinary scientists (Hippocrates 10: 46–56, 1993). The Nobel prize winner

Albert Szent-Györgyi was one of the founders and the first Secretary of the Hungarian Physiological Society (1931). In 1980 the Hungarian physiologists had the honor to organize – for the first time in Central Europe – the World Congress of Physiological Sciences (IUPS XXVIII) in Budapest. In 2002 the Society will be an official organizer of the International Congress of Pathophysiology (ISPIV).

It is our belief that this journal, which was born in and reflects a Hungarian melting pot of international science, will attract the submission of outstanding scientific manuscripts and that those who read this journal will decide to subscribe to Acta Physiologica Hungarica.

Budapest, January 11, 1999

Emil Monos
Editor-in-Chief

Acta Physiologica Hungarica, Volume 86 (1), pp. 3–6 (1999)

In memoriam Szilárd Donhoffer (1902–1999)

Professor Szilárd Donhoffer, the founder of the Department of Pathophysiology at the University Medical School of Pécs and Full Member of the Hungarian Academy of Sciences, died at the age of 97 on the 12th of January, 1999. He was born in Budapest on the 3rd of July, 1902 and studied medicine at the Pázmány Péter University also in the capital of Hungary. He spent one semester at the University of Vienna. In 1926 he took a job as a resident in internal medicine at the Erzsébet University of Pécs. In 1931 he spent a year in the Department of Physiology at the University of Aberdeen working under the leadership of Professor Macleod, a Nobel Laureate in Medicine. The next years found him in different positions in the Department of Internal Medicine back at his home university, his main scientific interest being in metabolic diseases and nutrition.

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Already in his early years he was active both as teacher and scientist at the Departments of Pharmacology and Basic Pathology of the University of Pécs. His well-acclaimed book on the diagnostics of internal medicine written in Hungarian and a source of relevant information for many generations of medical students and doctors alike revealed his ability to grasp essentials of a discipline and to present them in a crystal clear style and concise form.

The Department of Pathophysiology at Pécs was founded by Professor Donhoffer in 1949. Based on his experience, both in experimental medicine and in clinical practice, he was just the right person to establish not only the department itself but also the then burgeoning idea of a preclinical subject in the undergraduate medical curriculum aimed at bridging the gap between physiology and basic clinical subjects, such as internal medicine and pediatrics. Some years later he wrote the textbook of Pathophysiology, the first one in that subject. It continues to be the only one produced by a single author at the highest standard ever since in Hungarian. Although published only in two completely revised editions in the 60s, the book continued to be an essential textbook for many years to follow owing to Professor Donhoffer's visionary insight into what was going to be the logical directions of development in experimental medicine in the years to follow.

In the newly established department he was initially involved in experimental studies of nutrition, regulation of metabolic rate and food intake by thyroid hormones, goitrogens and the adrenals. A short paper published in Nature is evidence that even under the conditions of modest financial and limited political support available in Hungary in the 50s, genuine scientific material presented in faultless English such as his, could find access into the best quality international journals. In those years several other papers were published on data collected by his group on the mechanism of hyperthermia, thermoregulatory effects of hypoxia, protein deficiency and food selection, in journals such as the Experientia, Endocrinology, Brit. J. Nutr., Pflügers Arch., just to mention a few. In 1964 an additional report has appeared from his laboratory in Nature, this time on the thermogenetic role of brown adipose tissue as a site of non-shivering thermogenesis in rats just a year after the discovery of this important tissue.

Based on a long series of studies he has arrived at the conclusion that the brain can be a site of cold-induced thermogenesis, in homeothermic mammals, an idea not without opponents even in the present time. In fact, this was the topic of his monograph published in 1980 with the title "Homeothermia of the brain". The same year, largely thanks to his fame in the field, the Satellite Symposium on Thermal Physiology could be organized by the Department of Pathophysiology, of which he was still a member as a Professor Emeritus. His close collaborators were asked to edit a volume of proceedings from that symposium *(Contributions to Thermal Physiology).*

He was elected a Corresponding and later a Full Member of the Hungarian Academy of Sciences in 1964 and 1973, respectively. He served as President of the Medical Section of this institution between 1973 and 1980. His activity as an academy member was continuous up to the time of his death; his office at the Department of Pathophysiology has always been a place visited by a large number of disciples from the time of his clinical and theoretical scientific activities and also by young and older members of the academy from all around the country. Scientists from foreign countries visiting our department has always paid tribute to him by discussing various issues either in English or in German with him. He was fluent in both languages since his early time. Up to three days before his death he continued to be active spending at least five hours every day of the week in his office, many times using the public transport to and fro his home even in the harshest weather conditions.

Professor Donhoffer served as Vice-Rector and later (between 1964 and 1967) Rector of the University Medical School of Pécs. It was his concept to establish teaching hospitals in South-Transdanubia as important vehicles of practical training for medical students in an era when this was regarded by some as an unnecessary and even impractical idea in this country. In fact, the close co-operation of hospitals of neighbouring towns has been the starting point of the Faculty of Health Sciences of this university, these days attracting thousands of young people wanting to graduate in nursing, dietetics, physiotherapy and other related fields. He received the Honoris Causa Doctorate at his home university in 1996.

As, one of the founding fathers of the Hungarian Physiological Society, he held a lecture already at its second meeting organized at Pécs in 1932, evidence of a strong physiological background, which was not a very common characteristic of medical doctors at that time. As a result of his activities in experimental medicine, Professor Donhoffer presided a society meeting organized at Pécs after the war in 1953.

Professor Donhoffer belonged to the famous generation of great professors of the 50s and 60s at this medical school, an era of political and financial hardship but, at the same time, they laid firm foundations of the medical school of today. His lectures characterized by carefully formulated sentences and by the wise, succint – sometimes rather sarcastic – remarks with which he was able to clarify complicated and controversial issues at scientific meetings, will never be forgotten by those having the good fortune to be present. He was one of the very few professors who did his best – and was successful at that – in sending his young co-workers to Western physiological centers at a time, when attending even a short symposium West of the Iron Curtain was mostly in the realm of dreams or at least an extremely difficult task to achieve for various reasons.

Professor Szilárd Donhoffer had a small vineyard on the outskirts of Pécs, where he used to care for his fruit trees and some vine-stocks mostly for the sake of physical activity and having some fresh air after the long working hours spent in his laboratory and office. His other hobby was enjoying serious music, originally playing the cello himself and later listening to music, favouring mostly compositions from the classic and romantic period.

Since the death of his wife in 1990, he was living alone. She was a well-known expert in clinical dietetics and author of a success book on this topic back in the 40s. He has been survived by his beloved family: two daughters, both of them active scientists, seven grandchildren and thirteen great-grand children. Professor Donhoffer's death is a great loss for his former students, friends and colleagues. He will be remembered with great reverence by all who had the fortune to know him, a great personality leaving his impact in thermal physiology and pathophysiology as a scientist, teacher and also as an adviser in all matters of life.

Zoltán Szelényi

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Doppler echocardiographic examinations in the assessment of the athletic heart

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Doppler echocardiography is a method with the help of which flow velocity and the duration of different intervals can be estimated. The ratio between early and late peak velocities (E/A) is linearly proportional to diastolic function, i.e. to ventricular compliance. Data of 179 athletes and of 42 nontrained young healthy men indicated that the E/A quotient was higher in athletes than in the sedentary controls (2.086±0.505 vs. 1.905±0.384) in young adult age, but of the different athletes it was the only group of endurance athletes that showed a significant increase. Regular physical training seems to protect against an age-dependent impairment of left ventricular compliance, as an increased E/A ratio can be observed at the age of 31–45 years (1.77 ±0.46 vs. 1.43±0.276) as well as in men above 45 years of age (1.61±0.36 vs. 1.24±0.36). Bradycardia of the athletic heart resulted in a significantly longer duration of the cardiac cycle in athletes than in non-athletes. Different phases of the cardiac cycle, however, were not equally modified. There were periods the absolute duration of which were slightly decreased, unchanged or slightly increased, but the relative ones are strongly decreased: such as isovolumetric contraction time (ICT), acceleration of the transaortic flow (AOAT), deceleration of the transaortic flow (AODT), acceleration period of the early diastolic filling (EACC), and deceleration period of the early diastolic filling (EDT). There were periods the absolute duration of which increased proportionally to the increase of the whole cardiac cycle, while relative duration was not changed: isovolumetric contraction time (IVRT) and the atrial systole (A). There was one period that showed the greatest variability in the different subjects and both its absolute and relative duration was definitely increased in the athletes: this was the EA period, i.e. the period from the end of early filling to the beginning of the atrial systole.

Keywords: Doppler echocardiography, athletic heart, ageing, cardiac cycles

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M-mode and two-dimensional echocardiography has been quite popular to characterise athletic heart function. These methods are able to characterise, however, only the morphological variables of the heart, cardiac functions can only be estimated by some calculated volume values (cardiac output, stroke volume, ejection fraction).

Doppler echocardiography can measure flow velocities and durations of different periods of the cardiac cycle, and hence, it can give some further information to assess the functions of the athletic heart.

One of the most frequently measured parameters is transmitral flow velocity in studying diastolic function of the left ventricle. A decrease in the ratio of early and late peak flow velocities (E/A quotient) indicates an impairment of the ventricular compliance either due to ageing [3, 14, 15] or because of different diseases [20, 36, 41].

The effect of regular physical training on the E/A quotient is not equivocal in the different studies. Data of several reports are summarised in Table I. In the human studies the number of publications according to which regular training at different ages would increase E/A quotient is nearly the same as the amount of publications according to which there is no helpful effect. In animal experiments a positive effect of physical training seems to be much more prevalent.

The first aim of the present study was to investigate whether regular physical training may influence diastolic functions characterised by the E/A quotient and if so, then what kind of training is effective and which are the effects at the different ages. To this end, Doppler echocardiography was performed in a great number of athletic and nonathletic males, differences in the E/A quotients were analysed and demonstrated together with two well-known parameters of the athletic heart, namely, resting heart rate and body size related left ventricular hypertrophy.

In our recent studies [27, 28, 29] it was demonstrated that body size related echocardiographic indices are suitable to indicate real relative cardiac measures when exponents of the numerator and denominator are the same. That is why left ventricular muscle mass was related to the cube of the square root of body surface area.

Another basic parameter which can be measured by Doppler echocardiography is the duration of the various cardiac cycles.

In earlier studies different phases of systole were only measured with the help of synchronously recorded ecg, pcg and carotid pulse tracing. In some studies of other authors [1, 2, 7] and in our own works [30] some attempts were made to get information referring to the duration of different systolic periods in the athletic heart, but the method has not become a wide spread procedure in the characterisation of the athletic heart.

Table I

Effect of regular physical training on the E/A quotient in different studies

Authors	Study	Result
Shapiro, Smith 1983	different athletes	=
Granger et al. 1985	different athletes	=
Fagard et al. 1987	cycle racers	=
Missault et al. 1993	cycle racers	=
Pearson et al. 1986	weight lifters	=
Palka et al. 1997	endurance athletes	=
Matsuda et al. 1983	different athletes	+
Colan et al. 1985	different athletes	+
Douglas et al. 1986	triathlonists	+
Möckel et al. 1992	triathlonists	+
Finkelhor et al. 1986	endurance athletes	+
Spurgeon et al. 1983	animal experiments	+
Starnes et al. 1983	animal experiments	+
Tate et al. 1990	animal experiments	+
Gwathmey et al. 1990	animal experiments	+
Schulman et al. 1992	older humans	=
Fleg et al. 1995	older humans	=
Sadaniantz et al. 1996	older humans	=
Takemoto et al. 1992	older humans	+
Douglas, O'Toole 1992	older humans	+
Levy et al. 1993	older humans	+

=: physically trained hearts and non-trained hearts show similar values,

+: physically trained hearts demonstrate an increased quotient.

With the help of Doppler echocardiography phases of the diastole can also be measured, i.e. the whole cardiac cycle can be measured in its phases. In different studies [24, 33, 34, 45, 46] several phases of the cardiac cycle have been measured, mostly the time of the acceleration and deceleration of the early phase of diastolic filling, the time of atrial ejection, isovolumetric contraction time, acceleration and deceleration of the aortic flow.

In the present study we made some attempt to find some characteristics in the durations of each phases of cardiac cycles. As in the duration of the cardiac cycles no difference between males and females was expected, young adult male and female athletes were examined with Doppler echocardiography, and the data of athletes and nonathletic healthy subjects were compared.

Table II

Age and body surface area of the subjects (mean ±s.d.)

Group	N	Age (yr.)	BSA (m^2)
Young children, control	**5**	**9.0 ± 0.00**	**0.95 ± 0.03**
Young children, athletes	**34**	**9.14 ± 0.66**	**1.11 ± 0.13**
Children, control	**13**	**12.31 ± 1.03**	**1.39 ± 0.25**
Child athletes	**32**	**11.91 ± 0.73**	**1.32 ± 0.15**
Adolescent-young control	**15**	**17.00 ± 0.93**	**1.92 ± 0.18**
Adolescent-young athlete	**38**	**16.58 ± 1.08**	**1.96 ± 0.19**
Young adult control	**42**	**23.00 ± 3.28**	**1.91 ± 0.14**
Young adult strength athlete	13	22.23 ± 3.39	1.94 ± 0.22
Young adult sprinter-jumper	24	21.88 ± 2.46	2.00 ± 0.15
Young adult ball-game players	91	22.73 ± 3.24	2.09 ± 0.15
Young adult endurance athlete	46	22.72 ± 3.02	1.93 ± 0.11
Young adult athlete	**179**	**22.64 ± 3.15**	**2.01 ± 0.17**
Adult control	**22**	**38.00 ± 4.74**	**2.00 ± 0.15**
Adult athlete	**34**	**37.03 ± 4.60**	**2.07 ± 0.16**
Older adult control	**8**	**51.13 ± 3.60**	**2.01 ± 0.10**
Older adult athlete	**13**	**52.31 ± 4.85**	**1.96 ± 0.16**

BSA: body surface area.

Material and methods

E/A quotient was measured in male subjects, their data are demonstrated in Table II. According to their age. subjects were divided to 6 age-groups as follows: young children: <11 yr., children: 11–14 yr., adolescents-youngsters: 15–18 yr., the group of young adults contained the most competitors: 19–30 yr., adults: 31–45 yr., and old adults: >45 yr.

The controls were healthy males of different age without any cardiac diseases and without any medication. The athletes were competitors of different qualification.

Young child athletes were children participating in swimming, tennis or basketball training sessions 3–6 hours weekly. *Child athletes* were soccer players and swimmers. with 8–10 hours of physical training weekly. *Adolescent and young athletes* were top-level middle- and long-distance runners, cycle racers, triathlonists, waterpolo and basketball players and weight lifters.

Among the *young adults* a subdivision was made according to the types of sports. Strength athletes were top-level (members of the national teams or 1st class competitors) judoists and weight-lifters, in the sprinters jumpers' group 2nd class track-and-field athletes and top-level short track skaters were collected, ball-game players were top-level and 2nd class waterpolo and soccer players and 2nd class handball,

basketball and volleyball players. Endurance athletes were top-level road cycling racers, kayak-canoeists, pentathlonists and triathlonists, and 2nd class triathlonists and long-distance runners.

In the *adult* group several still competing top-level pentathlonists, cyclists, kayak-canoeists and some lower-level ball-game players were collected, athletes of the *older group* were leisure-time athletes with a training program of min. 3, max. 15 hours weekly.

In the second part of our study referring to the duration of the cardiac cycles altogether 15 nonathletic control subjects (2 males and 13 females, age 20.9±4.57 yr.) and 140 athletes (59 males and 81 females, age 20.59±3.90) were examined. Their age range was 18–35 yr.

Echocardiographic measurements were made by a Dornier AI 4800 type echocardiography using a 2.5 MHz transducer. Measurements were made always in the morning at absolute rest in half sinister recumbent position.

Two-dimensionally guided M-mode recordings were obtained parasternally, measurements of the left ventricular wall thicknesses and internal diameters were made by trackball on the monitor, all by the same investigator (PG).

From the several possibilities, similarly to our previous works [25, 26], left ventricular muscle mass (LVMM) was calculated by cubing the different diameters [18, 35]: $LVMM = \{(IVST+PWT+EDD)^3\text{-}EDV\}$. 1.05, where EDD is the left ventricular end diastolic diameter, EDV is the end-diastolic volume = EDD^3, 1.05 the density of the cardiac wall. According to our previous works [27, 28, 29] LVMM was related to the cube of the square root of BSA.

Transmitral flow velocity was estimated in an apical four chamber view by pulsatile Doppler measurement, transaortic flow velocity was measured by the continuous Doppler method. Peak velocities and the duration of the different phases were measured on the screen. Always several cardiac cycles were recorded, for the peak velocity values the highest of 3–5 recorded speed values were taken into account, in the duration of the phases mean values of 3–6 cardiac cycles were calculated.

Duration of the following phases were measured (the phases can be observed in Figs 1 and 2 although the order of the phases is not the same: in the figures a diastole-diastole picture is seen).

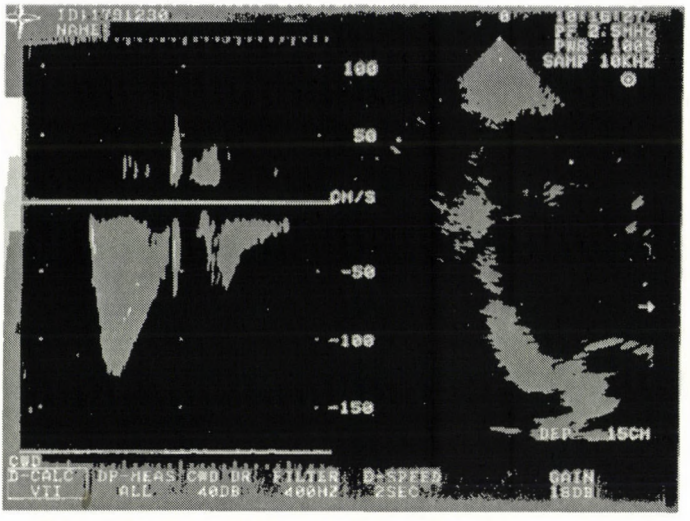

Fig. 1. Transaortal flow recorded by continuous Doppler echocardiography (downwards). AOAT: from the beginning to the peak of the atrial flow, AODT: from the peak velocity to the end of transaortic flow. ICT: from the end of the aortic flow to the beginning of transmitral flow (upwards)

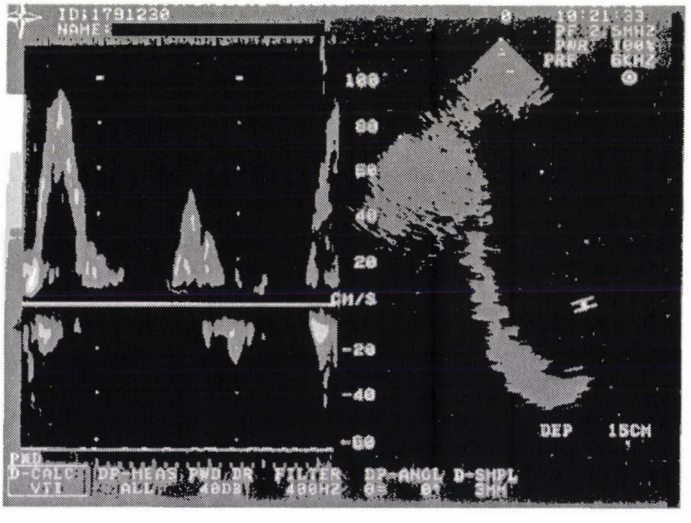

Fig. 2. Transmitral flow recorded by pulsatile Doppler echocardiography (upwards). Amplitude of the 1st wave: peak velocity of the early phase of ventricular filling (E), amplitude of the 2nd wave:, peak velocity of the late phase of ventricular filling (atrial systole A). Duration of the different phases: EACC from the beginning of E to the maximal velocity of it, EDT: from the peak velocity to the end of it, EA: from the end of E to the beginning of A, A: atrial flow, ICT: from the end of A to the beginning of transaortal flow (downwards)

Systolic phases:

ICT: isovolumetric contraction time – from the end of atrial systole (A) to the beginning of ventricular ejection,

AOAT: accelerating phase of aortic flow (= maximal ejection) – from the beginning of aortic flow until its maximal velocity,

AODT: decelerating phase of aortic flow (= decreased ejection) – from the maximal velocity of aortic flow until its end.

Diastolic phases:

IVRT: isovolumetric relaxation phase – from the end of aortic flow until the beginning of early (E) transaortic flow,

EACC: acceleration of early transmitral flow (E) – from the beginning of transmitral flow until its maximal speed,

EDT: deceleration of early transmitral flow (E) – from the maximum velocity of the transmitral flow until the end of the early phase,

EA: the period from the end of early transmitral flow (E) until the beginning of atrial systole (A) when flow is minimal,

A – atrial systole, from the beginning to the end of atrial flow.

AOAT, AODT and IVRT are seen in the transaortic picture (Fig. 1), EACC, EDT, EA, A and ICT are seen in the trasmitral picture (Fig. 2).

Athletes' data were compared with their age-matched controls by *t*-test for unpaired data. Differences with $p<0.05$ were regarded as statistically significant.

Results

E/A quotient, heart rate and left ventricular muscle mass

E/A quotient, resting heart rate and body size related left ventricular muscle mass values of non-athletes and of different athletes are indicated in Table III.

While the E/A quotient in all of the athletic groups was higher than in the sedentary controls, the difference was significant only in the endurance athletes.

The other two indicated parameters, heart rate and rel. LVMM showed more marked differences: training bradycardia was significant in all of the athletic groups, in the power athletes it was a little less marked than in the other three groups. In rel. LVMM all the athletic groups displayed a significant increase, the extent of cardiac hypertrophy showed a definite order: power athletes – sprinters jumpers – ball-game players – endurance athletes.

Table III

E/A quotient, resting heart rate and rel. LVMM of young adults (mean ±s.d.)

Group/Parameter	E/A	Heart rate, bpm	rel. LVMM, g/m^3
Control	1.905 ± 0.384	77.13 ± 14.24	73.10 ± 9.5
Power athletes	1.917 ± 0.454	***64.02 ± 12.90**	***81.49 ± 10.87**
Sprinter-jumpers	2.101 ± 0.535	**#60.90 ± 8.33**	**#85.50 ± 14.13**
Ball-game players	2.055 ± 0.442	**#58.75 ± 10.05**	**#94.72 ± 18.05**
Endurance athletes	**+2.187 ± 0.621**	**#59.24 ± 9.39**	**#100.44 ± 17.66**

E/A: ratio of the peak velocity of the early and late diastolic filling, rel. LVMM: LVMM/BSA$^{2/3}$, bpm: beat per minute, control: nontrained young healthy men, +: p<0.02, *: p<0.01, #: p<0.001. Bold numbers: significant difference from the values of the control group.

The same parameters are seen in the different age groups in Table IV.

Table IV

E/A quotient, resting heart rate and rel. LVMM in different ages (mean ±s.d.)

Group/Parameter	E/A	Heart rate, bpm	rel. LVMM, g/m^3
Young children, control	2.081 ± 0.403	82.98 ± 9.13	85.65 ± 10.90
Young children, athletes	2.038 ± 0.397	75.76 ± 11.90	84.31 ± 15.48
Children control	1.905 ± 0.335	86.33 ± 15.22	69.27 ± 9.07
Child athletes	2.021 ± 0.319	***74.06 ± 10.97**	**#86.11 ± 14.70**
Adolescent-young control	1.736 ± 0.388	71.49 ± 9.85	68.04 ± 12.76
Adolescent-young athlete	**#2.323 ± 0.587**	**#60.67 ± 9.42**	***84.51 ± 16.66**
Young adult control	1.905 ± 0.384	77.13 ± 14.24	73.10 ± 9.51
Young adult athlete	**°2.086 ± 0.505**	**#59.54 ± 9.83**	**#93.67 ± 17.80**
Adult control	1.426 ± 0.266	72.36 ± 13.08	76.27 ± 10.62
Adult athlete	***1.756 ± 0.461**	**#60.60 ± 9.66**	***88.56 ± 14.41**
Older adult control	1.243 ± 0.361	71.15 ± 6.63	82.45 ± 18.90
Older adult athlete	**°1.612 ± 0.364**	64.14 ± 8.84	85.25 ± 18.90

E/A: ratio of the peak velocity of the early and late diastolic filling, rel. LVMM: LVMM/BSA$^{3/2}$, bpm: beat per minute, control: nontrained healthy males, °: p<0.05 +: p<0.02, *: p<0.01, #: p<0.001. Bold numbers: significant difference from the control values.

E/A quotient, i.e. the proportion of the peak velocity during the early and late phase of diastolic filling, showed a marked decrease with age. In the two-child groups no significant difference was seen in the E/A quotient, in all of the other age groups the ratio was higher in the athletic groups.

Resting heart rate showed also a slight decrease with age in both groups. Lower heart rate of the athletes, i.e. the training bradycardia can be seen at all ages, so except in the young children and the older adults, the difference was significant.

Body size related left ventricular muscle mass was larger in the athletic groups. It was again the youngest and oldest groups where the difference was not significant.

Duration of the different phases of the cardiac cycle

Table V contains the absolute and relative values of the time duration of the different cardiac cycles in adult athletic and nonathletic subject of both genders.

Table V

Absolute and relative times of the cardiac cycle (mean ±s.d.)

Interval	Non-athletes		Athletes	
	Abs. (ms)	Rel. (%)	Abs. (ms)	Rel. (%)
ICT	55.3 ± 15.2	6.0 ± 1.4	**°47.5 ± 14.4**	**#4.3 ± 1.5**
AOAT	87.4 ± 9.8	9.6 ± 1.2	89.8 ± 9.8	**#8.1 ± 1.3**
AODT	200.1 ± 19.6	21.9 ± 2.0	**#218.9 ± 16.9**	***19.8 ± 2.4**
Systole	342.9 ± 33.9	37.5 ± 2.7	356.2 ± 25.3	**#32.2 ± 4.1**
IVRT	69.5 ± 12.5	7.7 ± 1.8	**#85.1 ± 17.5**	7.7 ± 1.7
EACC	86.4 ± 12.8	9.4 ± 1.2	92.6 ± 12.2	***8.4 ± 1.3**
EDT	135.3 ± 20.2	14.9 ± 2.4	**°149.3 ± 24.4**	***13.5 ± 2.5**
EA	138.0 ± 70.5	14.5 ± 6.4	**#266.4 ± 133.8**	**#22.7 ± 8.9**
A	146.6 ± 34.3	16.0 ± 3.4	**°172.3 ± 45.4**	15.5 ± 4.2
Diastole	575.8 ± 87.0	62.5	**#765.8 ± 148.3**	67.8
Total	918.7 ± 111.4		**#1122.0 ± 157.8**	

ICT: isovolumetric contraction time, AOAT: acceleratory phase of the aortic flow, AODT: deceleratory phase of the aortic flow (= decreased ejection), IVRT: isovolumetric relaxation time, EACC: acceleratory phase of the early transmitral flow (E), EDT: deceleratory phase of the early transmitral flow (E), EA: a period from the end of the early transmitral flow (E) to the beginning of the atrial systole (A) (diastasis), A: atrial systole. °: $p<0.05$, +: $p<0.02$, *: $p<0.01$, #: $p<0.001$. Bold numbers: significant difference from the control values.

Out of the very many data of the table, the following data seem to be the most interesting.

The last row of the table unambiguously indicates that there is a significant difference in the total time of the cardiac cycle. Due to training bradycardia, the cardiac cycle of the athletes is longer. In this elongation, however, the different phases of cardiac cycle have not the same share.

There is a basic difference in the elongation of the systole and of the diastole: systole is slightly, not significantly longer in athletes, while the difference in the diastole is very definite.

Different subphases can be classified into different groups as follows:

– there are periods the absolute duration of which is slightly decreased (ICT), unchanged (AOAT, EACC) or slightly increased (AODT, EDT), but the relative duration is strongly decreased: ICT, AOAT, AODT, EACC, EDT,

– there are periods the absolute duration of which is increased proportionally to the length of the whole cardiac cycle, but their relative duration is not changed: IVRT, A,

– and there is one period which shows a definite increase in absolute as well as in relative duration: E A, i.e. the period from the end of the early phase to the beginning of late filling, when flow velocity is minimal or zero.

The AOAT and the AODT together make up left ventricular ejection time (LVET), which is very often determined also by other methods, e.g. by measurements of the carotid pulse or by impedance cardiography. The LVET is also elongated a little in the athletic heart (308.7 ms vs. 287.5 ms), the relative value of the LVET is quite obviously smaller in the athletes (27.5% vs. 31.3%).

To get some information about the stability or variability of the different periods of the cardiac cycle, the ratio of the standard deviations to the mean absolute values was also calculated in the nonathletic and athletic groups, the results are indicated in Table VI.

From the two main phases, systole was much more stable than diastole. Among the subphases of the cycle it is seen that phases in which an active flow is occurring are the most stable: AOAT, AODT, EACC, EDT. It is very conspicuous that the phase from the end of E to the beginning of the A (EA period) is very variable: its coefficient of variation is above 50% in both of the nontrained and in the trained subjects.

Table VI

Ratio of the standard deviation to the mean value of different periods of the cardiac cycle in non-athletes and in athletes (%)

	s.d./mean	
Period	non-athletes	athletes
ICT	27.5	30.3
AOAT	11.2	10.9
AODT	9.8	7.7
Systole	9.9	7.1
IVRT	18.0	20.6
EACC	14.8	13.2
EDT	14.9	16.3
EA	51.1	50.2
A	23.4	26.3
Diastole	15.1	19.4

ICT: isovolumetric contraction time, AOAT: acceleratory phase of the aortic flow, AODT: decceleratory phase of the aortic flow, IVRT: isovolumetric relaxation time, EACC: acceleratory phase of the early transmitral flow (E), EDT: deceleratory phase of the early transmitral flow (E), EA: the period from the end of the early transmitral flow (E) to the beginning of the atrial systole (A).

Discussion

In our study physical training induced modifications detected by Doppler echocardiography were examined. In the first part of the study diastolic function characterised by the E/A quotient was demonstrated in male athletes of different types of sports and of different age. One regulatory and one morphologic characteristic of the heart was demonstrated as well, namely resting heart rate, and body size related left ventricular muscle mass.

Concerning the E/A quotient two questions arise. The first question is whether regular physical training induces a further improvement related to the young, healthy, nontrained sedentary persons. The second one is whether regular physical training protects against the definitely age-dependent impairment of diastolic function [3, 14, 15].

Our results are in accordance with the observations according to which regular physical training has a beneficial effect in both aspects.

Although not in childhood, but in all the other age groups the E/A ratio was significantly higher in the physically trained males than in the nontrained ones. Data of the young adult athletes, however, reveal that an improvement of the E/A quotient can only be expected in athletes whose training program contains endurance elements: the endurance group showed only significant shift in comparison with the controls, while sprinters-jumpers and ball-game players displayed only slight, nonsignificant elevations.

The athletes' higher E/A ratio was not so consistent as resting bradycardia that seemed to be independent from the type of training, as it was highly significant in all the young adult athletic groups and, except of young children and older adults, in all the other age groups.

Similarly to several earlier studies [5, 6, 10, 22, 28, 31, 32] body size related left ventricular muscle mass showed also a definite increase in athletic groups, but in this parameter a definite rank was seen according to the type of sports: hypertrophy was the highest in the endurance athletes and they were followed by ball-game players, sprinters jumpers and power athletes.

It is very probable that the demand for a larger cardiac output during an endurance physical training can be more perfectly fulfilled by a heart which is not only hypertrophied but also has an increased compliance, i.e. it is able to make room for a higher amount of blood. This results in a more effective increase of stroke volume and so of cardiac output. This idea can be supported by the study of Vanoverschelde et al. [44], in which a high correlation between maximal oxygen uptake and diastolic filling functions in endurance athletes and sedentary subjects was found.

The better compliance of the endurance athletes should be regarded as an especially positive sign, as myocardial hypertrophy was the most marked in this group, and in the different pathological cases hypertrophied hearts show a marked decrease in their compliance.

In accordance with some but not all other observations, our results suggest that regular physical training is able to decrease the age-dependent impairment of left ventricular compliance (for references Table I.) By comparing the data of other authors with our own investigations, physical activity seems to be effective when an active way of life has began at young age and has been continued during the whole life, i.e. advantage acquired during young age are maintained, conserved by the work continued at older ages.

To clarify this question, further analyses are necessary that involve a quite exact analysis of the physical activity made at different ages, a detailed examination of the E and of the A periods separately, and their dependency on heart rate. Our results suggest, however, that regular physical training, especially when it began at young age and maintained continuously, may be very useful to preserve a good left ventricular compliance.

In the second part of our investigations the duration of the different phases in the cardiac cycle was investigated.

Analysis shows that there are periods, the duration of which is relatively stable: it does not change in function of the duration of the whole cardiac cycle. The whole systole is not elongated significantly in the athletic group, and during systole it is only the deceleration period of transaortic flow that shows a slight elongation.

This stability is quite obvious: the pumping function, the ejection of the blood needs a rapid, abrupt contraction in any case, so it cannot be longer even in case of the bradycardia of the athletic heart. All that means that training bradycardia arises from the elongation of diastole, which means a more economic cardiac function: longer relaxation time, more time for recovery, and, as coronary circulation is free only during diastole, better coronary circulation.

During diastole, it is the duration also of the rapid flow, namely, of rapid filling that seems to be the most stable: rapid flow needs a rather fix absolute time, consequently its relative value is significantly decreased.

There are some periods that seem to be stable in their relative value, i.e. they change according to the whole duration of the cardiac cycle, according to the heart rate: such periods are isovolumetric relaxation and atrial systole.

Considering all cardiac phases, the greatest variability was seen in the period occurring between the end of the E period and the beginning of the A phase, i.e. during which transmitral flow is practically minimal: the period of diastasis. This period is the most variable among all of the periods. the s.d./mean ratio of the absolute length is above 50% in both groups. Thus, it seems that this is the period that can be modified to the greatest extent, training bradycardia seems to develop through elongation of this period.

Periods of the cardiac cycle seem to provide further data on the function and regulation of the athletic heart. Some data, some indices may widen the arsenal of the different signs that are characteristic to heart adapted to regular physical exercise. Among the different periods of the cardiac cicle, the EA namely the diastasis period of the diastolic filling seems to be the most characteristic of the physically trained heart.

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Effect of digoxin imprinting in adolescence on the sexual behavior of adult rats

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Four-time 3 μg digoxin treatment of male rats at puberty (in six weeks old rats) significantly increased the libido of rats (number of intromissions) and reduced the number of ejaculations, two months after the treatments (in three and a half months old rats). In female rats the Meyerson index and lordosis quotient were not significantly decreased. The experiment calls attention to the wide-ranging imprinting effect of digoxin which was also demonstrated earlier after prenatal (maternal) treatment. The experiment also supports the male sexual potency influencing effect of digoxin treatment, previously supposed in men.

Keywords: hormonal imprinting, puberty, digoxin, cardioactive glycosides, sexual behavior

Receptors develop in the presence of their ligands. The first encounter between the developing receptor and the hormone in the perinatal critical period results in the hormonal imprinting which determines the receptor-hormone relationship for life [4, 5, 7]. However, molecules similar to the appropriate hormone, as members of the same hormone family, synthetic hormones, or chemicals structurally similar to the hormone, can bind to the not completely selective developing receptor causing faulty imprinting, valid also for life [4–7, 8, 9]. The consequences of this faulty imprinting are manifested in alterations of the binding capacity of the receptors [6–8], biochemical and morphological changes [2, 13, 15, 24], and differences in the sexual behavior [13, 16, 20].

The critical period is not restricted to the perinatal age as hormonal imprinting also can be provoked in the whole life in cell producing organs, like the bone marrow and in adolescence in such organs which are developing in that time e.g. in organs of sexual apparatus and their regulators [7]. For example nandrolone – a synthetic steroid

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hormone with anabolic effect – administered at adolescence can lifelong disturb the uterine estrogen receptor's binding capacity [10].

Digoxin is a cardioactive glycoside having a steroid-like character. Perinatal digoxin treatment influences cardial parameters [12] and steroid receptor binding capacity [11] for life. Administered to rat dams three times at late pregnancy it causes alterations in the male and female offspring's sexual behavior [17]. Considering that the maturation of sexuality takes place at adolescence, in the present experiment the effect of pubertal digoxin treatment on the later sexual behavior has been studied.

Materials and methods

Animals and treatment

Six-weeks-old Wistar male and female rats of our (Charles River originated) closed breed were treated with a daily dose of 3 μg digoxin (Richter, Budapest) in four consecutive days, intramuscularly. Two months later, when the animals were adult (three and a half months old), the sexual behavior of the animals were tested.

Study of female animals

The receptivity of female rats was measured by the help of indicator (experienced) males. Two parameters were recorded for the evaluation of receptivity, the Meyerson index and the lordosis quotient. The former gives a binary answer for the appearance of the lordotic response as a result of the primary mounting by males. The latter is a ratio of the lordosis percent in ten mountings (L/M). For comparable results the females within the two-week study were screened only during estrus (the timing was made by vaginal smears).

In each group five–six animals were tested a day. During the two-week testing period one animal was tested four times as a mean.

The average of the daily data were used for evaluating significance with Student "t" and χ^2 tests.

Sixteen adult animals were tested.

Study of male animals

At first the males were studied for mounting to each other in a common cage (on two consecutive days for three hours a day). After that the male's seeking behavior were studied being alone in a cage for five minutes. The suitable males were selected.

Test females were ovariectomized in ether narcosis. The tests were done two weeks after ovariectomy. Forty-eight hours prior to the test 30 μg/animal estradiol monopropionate (Richter, Budapest) dissolved in sunflower seed oil was administered subcutaneously to the ovariectomized animals, while 4 hours prior to the test 500 μg/animal progesterone (Richter, Budapest) was injected subcutaneously. This procedure promotes receptivity.

Ten adult males were tested in a 4-week period, once a week for 30 min. Five different patterns of behavior were distinguished [14]. Males without any mounting, intromission or ejaculation within 30 min time exposed to a receptive (hormone treated) female, were taken as inactive. Others performed only mounting and some of the males had intromission, too, without ejaculation. These were the sexually sluggish (under-active) males. Males were considered as sexually active, when the full scale of male's copulation (mounting, intromission and ejaculation) appeared.

The others had the same characteristics but multiple ejaculations. Significance were evaluated as above.

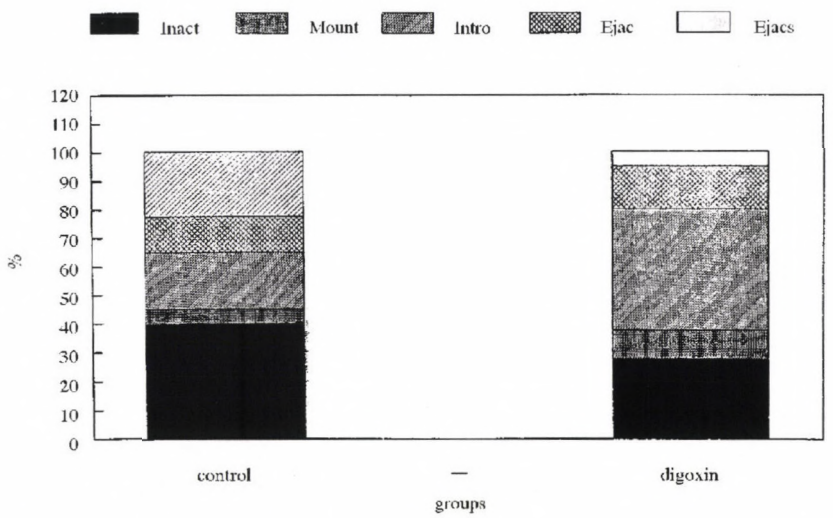

Fig. 1. Effect of four-time 3 μg digoxin treatment in adolescence on the sexual behavior of adult male rats. Number of inactive rats not significantly, and the number of ejaculations significantly ($p<0.05$) decreased, while number of mountings not significantly, intromissions significantly ($p<0.05$) increased

Results and discussion

As an effect of the previous digoxin treatment the male animal's sexual activity increased (Fig. 1). More mountings were observed, however, this value was not significant. The number of intromissions were significantly ($p<0.05$) higher, however, this did not run parallel with the quantity of ejaculations which significantly decreased ($p<0.05$). Multiple ejaculations by the same animal were enormously reduced in the digoxin pretreated group. In the case of the female animals the Meyerson index as well as the lordosis quotient decreased, however, not significantly (Fig. 2).

It is well known from the literature that chronic digoxin treatment can cause sexual dysfunction in human males [3, 21–23]. It was also demonstrated that long-term digoxin treatment (in male patients) significantly increased serum estrogen level, at the same time decreasing testosterone and luteinizing hormone levels [21]. As a result of hormonal changes, the decrease of sexual desire, sexual excitement phase (erection) and frequency of sexual relations were observed [3, 21–23]. However, contradictory results were also published [18].

In the human cases mentioned above, the statements were done *during* the digoxin treatment and by using questionnaires. In the present–animal–experiments objective observations were done and *two months after digoxin treatment.*

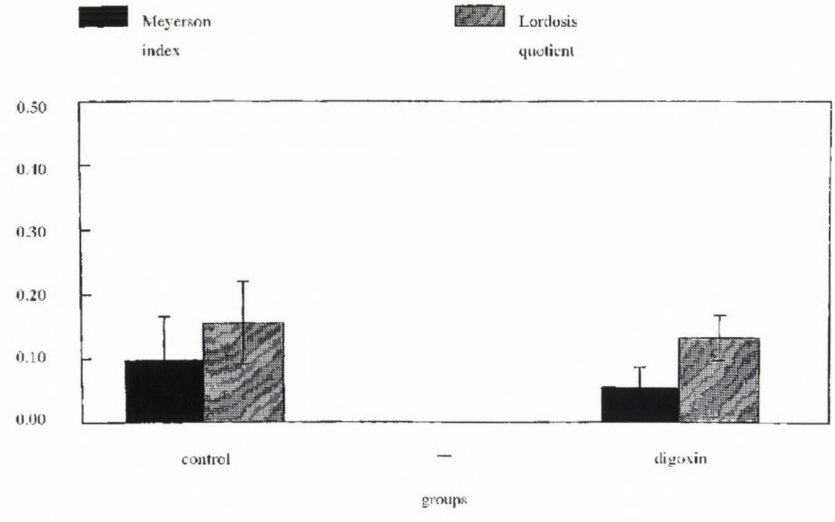

Fig. 2. Effect of four-time 3 μg digoxin treatment in adolescence on the sexual behavior of adult female rats. Both indexes are lowered in the digoxin treated group, however, not significantly

This means that in the human cases the direct effect of digoxin was measured in contrast to our experiments where the effect of pubertal digoxin imprinting has been observed two months after treatment. However, the effect of digoxin to the sexual behavior of males was clearly demonstrated. This effect was positive (and opposite to direct human experience) considering the sexual desire (mountings and intromissions) and negative (parallel to direct human experience), considering the capacity for ejaculation. This means that short-time pubertal digoxin treatment (imprinting) can have long term influence on the full scale of male's sexual behavior.

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Direct and transgenerational effect of benzpyrene treatment at adolescent age on the uterine estrogen receptor and thymic glucocorticoid receptor of the adult rat

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Hormonal imprinting develops perinatally at the first encounter between the maturing receptor and the target hormone, helping the normal accomplishment of receptor maturation. In the presence of hormone excess or foreign molecules able to bind to the maturing receptor, faulty imprinting takes place, which disturbs the normal receptor function for life. Earlier experiments demonstrated that the effect of faulty perinatal benzpyrene imprinting of the steroid hormone receptors is transmitted to the progeny generations. In certain organs which are maturing later (such as the uterus) imprinting can be executed at adolescence. In the present experiments pubertal benzpyrene imprinting caused a durable decrease in female's estrogen receptor density. The transgenerational effect of this type of imprinting was also studied. The pubertal imprinting of the parents was transgenerationally transmitted to the offspring generation in which – without further treatment – the density (B_{max}) of the uterine estrogen receptors was significantly higher than that in the controls. There were measurable effects neither in the affinity (K_d) of uterine estrogen receptors nor in the K_d and B_{max} of the male thymus glucocorticoid receptors. The experiments call attention to the profound and comprehensive imprinting effect of the environmental pollutant benzpyrene.

Keywords: hormonal imprinting, transgenerational effect, aromatic hydrocarbon, steroid receptors, glucocorticoid receptor, estrogen receptor, adolescence

The hormonal imprinting takes place in the perinatal critical period of hormone receptor development causing the life-long adjustment of the receptor-hormone relation [7, 8]. Without the presence of the target hormone in this period the receptors are not

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able to accomplish their maturation [9]. Moreover, as the discriminating capacity of receptors is not sophisticated during maturation, hormones belonging to the same hormone family, synthetic hormone analogues, or molecules having similar structure to the physiological target hormone can cause faulty imprinting [7, 8]. Synthetic steroid hormones as allylestrenol or diethylstilbestrol can reduce life-long the binding capacity of uterine estrogen [10] or thymic glucocorticoid receptors [23]. The environmental pollutant benzpyrene which also has a steroid-like structure can disturb steroid receptor development [11–13], causing faulty imprinting and malfunction of receptors, and sexual behavior [19]. The effect of neonatal benzpyrene exposure is so intense that its effect also appears in the progeny generations [6, 16, 20].

The imprintability of receptors by hormones is lost in general four days after birth [17], however in some cases it appears again at puberty [14]. In the present experiments the transgenerational effect of pubertal benzpyrene exposure is studied and compared to the direct pubertal imprinting effect of this environmental pollutant.

Methods

Six-weeks-old Wistar rats (males and females, 150 g each) of our closed breed (P generation) were treated with one dose of 300 μg benzpyrene (Sigma, St. Louis, USA). Benzpyrene was dissolved in sunflower seed oil, and was given intramuscularly in a volume of 0.05 ml. Controls received the vehicle only. Four animals were kept in one cage, and were fed with Charles-River chow and water *ad libitum*. One group of female animals was studied for uterine estrogen receptor at three months old age. Other male and female animals were mated at 3 months of age (treated with treated and control with control, randomly). The treatment did not influence fertility. The offspring of these pairs (F_1 generation) were studied in receptor assay (of thymus and uterus) when males were 6 weeks old and females were 3 months old.

Preparation of cytosol fractions

Cytosolic (soluble) fractions were prepared from thymi of males and females and uteri (of females, 8 days after ovariectomy). Organs were excised in ether narcosis. Pooled organs of four (in the case of thymus) and five (in the case of uterus) were used for one measurement.

All procedures were performed at ice/water temperature. Tissues examined were cut into pieces and homogenized in Tris-HCl containing 1.5 mM EDTA, pH 7.4 (freshly supplemented with 20 mM molybdate and 2 mM dithiotreitol) with a motor driven glass-teflon Potter homogenizer 1.5 ml/g wet weight. Homogenates were centrifuged at

100,000 g for 60 min at 4 °C and the supernatants were used for receptor assays. Protein content was estimated by the Coomassie-blue method.

Saturation analysis with ^{3}H-dexamethasone

Increasing concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40 nM) of ^{3}H-dexamethasone (Amersham, Buckinghamshire, England, spec. act. 1.5 TBq/mmol) were incubated with thymic cytosol (500 µg protein) in duplicates in a total volume of 100 µl at 0 °C for 18 h for total binding (T). Nonspecific binding (NS) was measured in the presence of 100-fold molar excess of dexamethasone acetate (Sigma, St. Louis, USA) at each radioactive concentration.

Saturation analysis with ^{3}H-estradiol

Increasing concentrations (0.312, 0.625, 1.25, 2.5, 5, 10, 20 nM) of ^{3}H-estradiol (Izinta, Budapest, Hungary spec. act. 3.5 TBq/mmol) were incubated with rat uteri cytosol (300 µg protein) in a 0.1 ml total volume, for 18 h for total binding (T). To estimate nonspecific binding (NS) there were another set of tubes containing 100-fold molar excess of unlabelled estradiol (Organon, Oss, Holland) for each radioligand concentration. All assays were performed in duplicates.

The reaction was terminated both in ^{3}H-dexamethasone and ^{3}H-estradiol saturation analysis by adding 200 µl 0.5% dextran coated charcoal suspended in assay buffer into each tube then pelleting the unbound steroid by centrifugation at 1500 g for 15 min. 200 µl aliquots of the supernatants were transferred for scintillation counting into 4 ml Optiphase "HiSafe" (Pharmacia, Lund, Sweden) and counted in a Beckman apparatus (38% efficiency).

Tubes in duplicate containing radioactivity in each concentration plus buffer alone were used to generate both total counts and blanks. The difference between T and NS was regarded as specific binding (S) at each concentration of the labelled ligand: S = T–NS.

Analysis of results

Analysis of results was carried out by a computer program written by McPherson [25] named EBDA and by a nonlinear curve fitting program modified by McPherson [26] named LIGAND. The relationship between EBDA and LIGAND: EBDA is used to process the raw data which is then expressed on the appropriate plot. The graphical representation allows initial parameter estimates to be calculated for use by LIGAND. LIGAND is used to obtain final parameter estimates. For statistical analysis of results DATAANALYSIS V.1.0. (Statistical and Design Services 1985) computer program was used.

Results and discussion

In earlier experiments pubertal benzpyrene treatment significantly reduced the thymic glucocorticoid receptor density of adult females without influencing that of the males [18]. In the present experiments uterine estrogen receptor density was reduced by similar treatment (Fig. 1). In the offspring generation – without any further treatment – the uterine estrogen receptor density was significantly elevated (Fig. 2), without any effect on female or male thymic glucocorticoid receptors (Table I). The results demonstrate that

1) the benzpyrene imprinting in adolescence has a general effect on steroid receptors;
2) this effect is transmitted to the first progeny generation, however
3) in this generation the effect is weaker, as the thymus is involved neither in males nor in females, which means that
4) the sensitivity to imprinting is dependent on the sex, and
5) the direction of the imprinting reversed in the untreated offspring.

It is noteworthy that there was also a difference in the affinity of receptors in the case of the parental (P) generation, however, this was not significant.

Table I

Affinity (K_d) and density (B_{max}) of uterine estrogen ($\times 10^{-10}$M) or thymic glucocorticoid ($\times 10^{-9}$M) receptors in rats treated or not treated with benzpyrene at adolescent age of the 1st generation and measured in the 1st or the 2nd generation

Group	Generation	Sex + n	Age in months	Organ	K_d	B_{max}
Control	P	female+4	3	uterus	9.20±0.9	6.27±1.1
Treated**	P	female+4	3	uterus	5.60±0.5	3.53±1.2
Control	F_1	female+3	3	uterus	3.26±1.2	6.88±1.4
Treated*	F_1	female+4	3	uterus	3.88±3.1	11.9±3.0
Control	F_1	female+3	3	thymus	4.10±0.5	3.69±0.8
Treated	F_1	female+4	3	thymus	4.61±0.5	4.16±0.8
Control	F_1	male+5	1.5	thymus	4.46±1.1	3.71±1.2
Treated	F_1	male+4	1.5	thymus	4.72±1.5	3.39±0.8

n = number of receptor assays of pooled organs
*: $B_{max} = p<0.05$; **: $B_{max} = p<0.02$

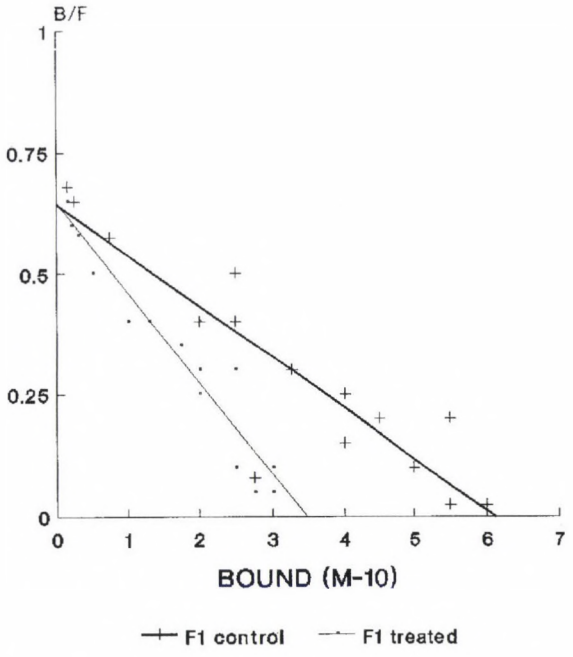

Fig. 1. Measurement of the estradiol binding capacity of the uterine estrogen receptor in the parental (P) generation. Scatchard plots of the mean values of saturation analysis data generated by EBDA

Intracellular steroid receptors form a superfamily in which steroid hormone receptors, thyroid hormone receptors, vitamin A and D receptors, peroxysome proliferator activated receptors and orphan receptors can be found [28]. Aromatic hydrocarbons also have intracellular receptor which is different from the members of the steroid receptor superfamily [27]. However, the structural similarity of a molecule to the physiological steroids is enough to execute the faulty imprinting [7, 8]. In earlier experiments the receptorial, morphological, biochemical, behavioral and genetical consequences of these faulty imprintings have been demonstrated [2–5, 21, 22, 24, 29, 20–27]. It was also shown that the perinatal imprinting effect can be transmitted to the progeny generations [6, 16, 20]. In the present experiments the transgenerational effects of pubertal imprinting were studied and justified. This means that the benzpyrene exposure in adolescent age can cause not only direct receptorial effect (in the individual exposed), but this also appears in the females of the first progeny generation.

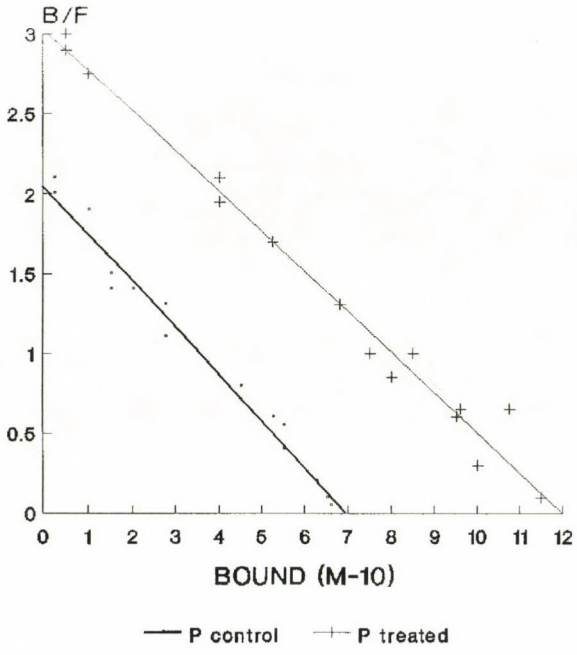

Fig. 2. Measurement of the estradiol binding capacity of the uterine estrogen receptor in the first filial (F_1) generation. Scatchard plots of the mean values of saturation analysis data generated by EBDA

In earlier experiments the transgenerational effect of *perinatal* benzpyrene imprinting was observed [16, 20]. This manifested in the reduction of the glucocorticoid receptor density of the thymus up to the third generation in males and in the F1 generation of females. However, uterine estrogen receptors were not subjected to transgenerational imprinting. In the present case (*treatment at adolescent age*) exclusively the uterine estrogen receptors were imprinted transgenerationally. Considering that the developmental peak of thymus is in the perinatal period and that of the uterus at puberty, this means that the critical time of the transgenerational imprinting runs parallel with the development of the organ. This is supported by the fact that the transgenerational imprinting of sexual behavior is also successful perinatally [20], when the adjustment of the sexual centre to the hormones is taking place [1].

While in the directly treated rats both uterus and thymus receptors were imprinted by pubertal treatment in female rats, transgenerationally the female and male thymus was insensitive. This means that the uterine imprinting is more imperishable, than that of the thymic one, in the case of adolescent and transgenerational studies.

Imprinting seems to be an obligate phenomenon, however, its direction is dependent on the time of exposure, the quantity of imprinter and the imprinted organ.

This means that the effect of imprinter on the receptor is calculable, however its direction is not and it can also be reversed. This latter happened now, when the reduced density of receptors (in the treated generation) manifested in the elevation of receptor number in the offspring. This is not unusual: the reversal of the direction of imprinting was observed earlier [15, 17].

Benzpyrene is an urban environmental pollutant. In earlier experiments its perinatal imprinting effects on the late steroid hormone binding by receptors and to the sexual behavior were demonstrated [6–9]. It was also shown that this imprinting effect is manifested in the offspring of the mothers exposed to benzpyrene during lactation [28]. Moreover, the imprinting effect of benzpyrene appeared in the offspring when the parents were treated neonatally (transgenerational effect) [10, 11]. Benzpyrene treatment in adolescence also caused imprinting [17] and the present experiments demonstrate that this type of imprinting was also transmitted to the first progeny generation. This means that benzpyrene imprinting can profoundly and expansively influence steroid hormone receptors and receptor mediated processes, threatening the progeny generations.

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Effects of cue information on response production and inhibition measured by event-related potentials

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The aim of this study was to investigate how information carried by a cue stimulus modulate event-related potentials (ERPs) to a subsequent target stimulus which either calls for an overt response (Go stimulus) or no response (Nogo stimulus). One of the cues predicted the likely appearance of the Go stimulus (Go cue) whereas the other cue predicted the likely appearance of the Nogo stimulus (Nogo cue). Our results showed that unpredicted Nogo stimuli elicited enlarged N200 component. This finding supports the notion that Nogo N200 reflects response inhibition processes, i.e., the amplitude of the N200 is a function of the difficulty of response inhibition. In other words, increased N200 to Nogo stimuli following Go cues might be related to increased efforts in activating the response inhibition system thereby interrupting preparations to respond.

Keywords: Go/Nogo paradigm, response inhibition, event-related potentials, N200, P300

For investigating response inhibition, researchers often measure event-related brain potentials (ERPs) to response-requiring targets (Go stimuli) interspersed by occasional presentations of stimuli which do not require a response (Nogo stimuli) [1, 4–8, 10, 11, 13, 14]. Findings in these studies feature conspicuous topographic amplitude differences of late positivities (P300) between Go and Nogo trials: Go stimuli typically evoke posteriorly maximal P300s whereas Nogo stimuli usually elicit anteriorly dominant P300s [1, 4, 6, 10, 11, 14]. Furthermore, the peak amplitude of the N200 component is reported to be enhanced [4, 6, 7, 8, 10] and delayed [4, 7, 14] in

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Nogo trials. These Go/Nogo effects on ERP amplitudes have been interpreted as signs of inhibitory activity in the Nogo trials which is related to the interruption of response execution [11] or cognitive task solving [10].

Some of the experiments discussed so far used warned reaction time [RT] paradigms where a cue stimulus (S1) marked the delivery of the upcoming target (S2) [4, 5, 7, 8, 13, 14]. There are only a handful of studies, however, measuring Go/Nogo ERP differences using cues that not only serve as temporal markers but also provide information about the target [4, 5, 13]. Eimer [4] and Schröger [13], for example, presented subjects with a centrally located arrow as a cue at the beginning of each trial that pointed to the likely position of the following target to which either a Go or Nogo response was required. These authors observed that cue validity modulated Go/Nogo ERPs, e.g., Schröger [13] reported an enlarged N200 to Nogo stimuli when they appeared at the validly (Exp. 4) or invalidly cued side (Exp. 3) whereas Eimer [4] showed that P300 increased if Go and Nogo stimuli, respectively, were presented at the invalidly and validly cued location.

Unlike in the paradigms used by Eimer [4] and Schröger [13], where the cue elicited a shift in *spatial* attention but did not inform about the *identity* of the upcoming target, in the present study the cue signals the likelihood of whether a Go or Nogo stimulus is to follow. It is well documented in a choice RT task [3] that S2 expectancies generated by S1-S2 probabilities are inversely proportional to the size of post-S2 N200 and P300. The present experiment investigates expectancy-related N200, P300 and late slow positive wave (SW) [2, 12] to S2 in a Go/Nogo paradigm.

Materials and methods

Subjects, stimulus presentatiaon, and procedure

Sixteen paid subjects (8 female) with the mean age of 21 (range 17–28) participated in the experiment. For half of the subjects, the Go cue was a rectangle intercepted by a horizontal line in the upper half, and was followed by the Go stimulus 88% of the time (Predicted-Go trials) and by the Nogo stimulus 12% of the time (Unpredicted-Nogo trials). In the Nogo cue the rectangle was intercepted by a horizontal line in the lower half, and was followed by the Go stimulus 12% of the time (Unpredicted-Go trials) and by the Nogo stimulus 88% of the time (Predicted-Nogo trials). For the other half of the subjects the position of the horizontal line in Go and Nogo cues was reversed. Furthermore, for half of the subjects Go and Nogo stimuli were capital Ts in normal and reversed position, respectively. The position of capital Ts as Go and Nogo stimuli was reversed for the other half of the subjects. Each cue

subtended 2° of visual arc vertically and 1.72° of visual arc horizontally. Targets subtended 0.17° of visual arc vertically and 0.29° of visual arc horizontally (from 1 m viewing distance). Stimuli in black colour with a grey background appeared in the center of a screen (VGA monitor). The stimulus duration was 100 ms for the cue and 90 ms for the target. Each target was followed by a mask (a cross) of 90 ms duration. The masking procedure was chosen to maintain attention on cue information by making recognition of the target/nontarget relatively difficult. The interstimulus interval was fixed at 1300 ms (from cue onset to target onset) and the average intertrial interval was 2400 ms (range 2100–2700 ms). A fixation point in the center of the screen was present during the trials.

The experiment consisted of 1000 trials, 880 of them were predicted and 120 of them were unpredicted. Trials were presented in ten blocks (100 trials per block) preceded by a brief practice block. The order of different trials was randomized. Subjects were instructed to press a button to the Go stimulus as fast as possible and withhold responding to the Nogo stimulus. Note that subjects were not informed about the cue-target contingencies. We measured latencies of button presses to the target (correct responses with latencies between 100 ms and 700 ms after the onset of Go stimuli were counted). After termination of the experiment subjects were required to estimate probabilities of the occurrence of Go and Nogo stimuli after each cue.

Data acquisition and analysis

Brain electric activity was measured with Ag/AgCl electrodes affixed at Fz, Cz, Pz, Oz locations all referred to linked mastoids. The electro-oculogram (EOG) was recorded from above and below the right eye (vertical eye movement) and from the two electrodes laterally to the left and right canthi (horizontal eye movement). Electrical activity (0.01–30 Hz) was digitalized at 250 Hz. Averaged ERPs were obtained separately for all four types of trials (Predicted-Go, Unpredicted-Go, Predicted-Nogo and Unpredicted-Nogo). To obtain averages of comparable signal to noise ratio, in the case of high probability trials only those which were followed by low probability trials were included in the averaging. The analysis epoch was 800 ms post-target (including a 100 ms pre-target period) referred to the mean amplitude of a 100 ms *pre-cue* baseline. Trials with incorrect responses (i.e., no button pressing between 100 and 700 ms after the onset of the Go stimulus; responding after 700 ms; and button pressing after the Nogo stimulus) were omitted from the averaging. A trial was also excluded from the analysis if its electrical activity exceeded 100 mV on the vertical, 70 mV on the horizontal eye-movement channel, or 100 mV on any other channel. For each subject, N200 and P300 components were expressed as the largest minimum and maximum

amplitude values, respectively, in the specified time window (base-to-peak measure), while SW was represented by mean amplitudes of the respective interval.

T-tests and repeated measures of analyses of variance (ANOVA) were used for detecting task-related effects on behavioral data and ERPs. ANOVAs on ERPs to S2 included electrode (Fz, Cz, Pz, Oz), S1 predictivity (Predicted, Unpredicted) and response assignment (Go, Nogo) as factors. When calculation of ANOVAs included more than two levels, the level of significance was computed according to the Greenhouse-Geisser procedure. For assessing distributional differences among the various components, interaction effects including the electrode factor were recalculated applying the scaling method suggested by McCarthy and Wood [9]. As for these interactions, the scaled data will be reported here.

Results

Behavioral results

Subjects made few errors (hit rate was 92.7% [range 83.2–98.9%]) and showed considerable precision in recognizing contingencies in post-experimental interviews (mean estimates were 86% and 14%, respectively, for the frequent and infrequent S2 after each S1). In addition, subjects reported that knowledge of cue-target associations was achieved early in the experiment (i.e., first or second trial). Furthermore, responses in Predicted-Go trials were faster than in Unpredicted-Go trials (mean RTs: 469 ms and 566 ms, $t_{15}=5.02$, $P<0.001$).

ERP results

Figure 1 presents post-target ERPs to all four types of trials. As the figure suggests, the amplitude of N200 was enhanced in the Unpredicted-Nogo trials at all sites but Oz. A three-way ANOVA on N200 data, carried out within the 260–360 ms time window, found a significant electrode × S1 predictivity × response assignment interaction ($F_{3,45}=4.21$, $P<0.01$). Subsequent two-way ANOVAs conducted at each site revealed that the S1 predictivity × response assignment interaction was significant at Fz ($F_{1,15}=10.93$, $P<0.005$) and marginally significant at Cz ($F_{1,15}=4.49$, $P=0.05$). Separate *t*-tests showed that unpredicted Nogo stimuli evoked larger N200s than predicted Nogo stimuli frontally ($t_{15}=2.18$, $P<0.05$) and centrally ($t_{15}=3.04$, $P<0.008$). These differences are illustrated in Figure 2. Finally, a three-way ANOVA conducted on N200 latencies (260–360 ms time range) found an electrode main effect ($F_{3,45}=5.91$, $P<0.008$) and a response assignment main effect ($F_{1,15}=4.72$, $P<0.05$). This latter result shows that N200 to Nogo stimuli was delayed compared to Go stimuli (301 ms vs. 290 ms).

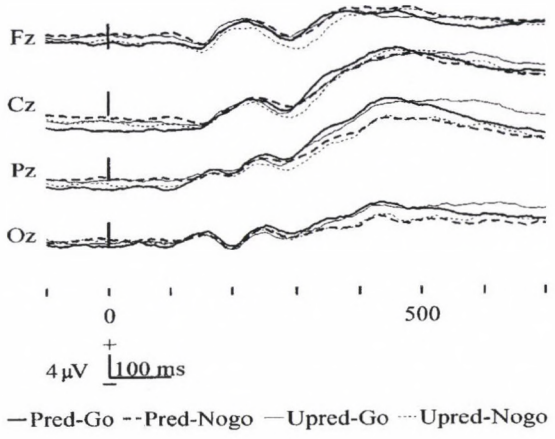

Fig. 1. Grand average ERPs after each type of trials. Pred-Go: Go cues followed by Go stimuli (88%); Pred-Nogo: Nogo cues followed by Nogo stimuli (88%); Upred-Go: Nogo cues followed by Go stimuli (12%); Upred-Nogo: Go cues followed by Nogo stimuli (12%). Shaded areas refer to latencies from which ERP data were calculated

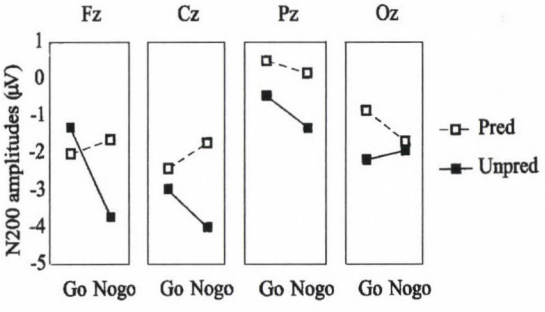

Fig. 2. Average amplitude values (mV) of N200s to predicted and unpredicted Go and Nogo stimuli at each site

Figure 1 displays a positive component peaking about 450 ms (P300) followed by another positive wave slowly returning towards the baseline (SW) 500 ms onward. As Figure 3 indicates, the scalp distribution of P300 shows some Go/Nogo differences, e.g., Go stimuli elicited parietally dominant P300s with decreasing amplitude at central and frontal sites whereas P300 to Nogo stimuli had a centro-parietal maximum. A three-way ANOVA on P300 data in the 400–500 ms latency range provided an electrode main effect ($F_{3,45}=11.33$, $P<0.0004$) and an electrode × response assignment interaction ($F_{3,45}=7.98$, $P<0.004$).

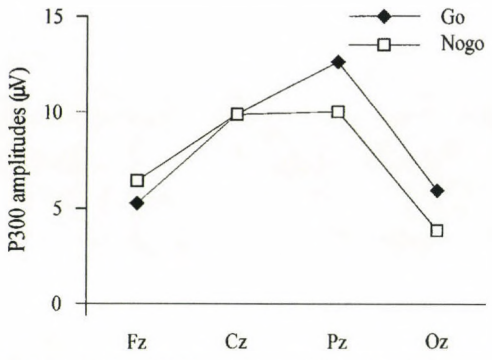

Fig. 3. Average amplitude values (mV) of P300s to Go and Nogo targets (collapsed over Go and Nogo cue presentations) at all four sites

Subsequent *t*-tests at single leads revealed that the P300 difference between Go and Nogo stimuli was significant parietally ($t_{15}=2.82$, $P<0.01$) and occipitally ($t_{15}=2.28$, $P<0.04$).

SW after Go stimuli is enlarged at posterior sites (see Figure 3). A three-way ANOVA, calculated on SW amplitudes in the 620–700 ms latency range, showed an electrode main effect ($F_{3,45}=6.47$, $P<0.01$), a marginally significant electrode x response assignment interaction ($F_{3,45}=3.22$, $P=0.057$), and an electrode x S1 predictivity interaction ($F_{3,45}=6.36$, $P<0.006$). Subsequent *t*-tests revealed that parietal SW was enhanced after Go stimuli ($t_{15}=2.89$, $P<0.01$), and it was also enhanced in unpredicted trials ($t_{15}=2.32$, $P<0.03$).

Discussion

The present data are consistent with earlier reports [1, 6, 8, 10] in that Go stimuli were associated with enlarged amplitudes of P300 and SW at parieto-occipital sites. A similar result is often found in studies where the probability of the Go stimulus is much lower than the probability of the Nogo stimulus [4, 5, 13]. Thus one might attribute the enlarged P300 and SW to the rarity of Go trials. This explanation, however, cannot account for our P300 and SW data since the number of trials with Go and Nogo stimuli was equal. Alternatively, some investigators [8, 14] attribute the increased P300 and SW, at least in part, to the CNV returning to the baseline (CNV resolution). This idea implies that the CNV, developing between S1 and S2 at a fixed interval, would overshoot before returning to the baseline and the peak of this overshoot should

temporarily approximate (and summate with) post-S2 P300 or SW. Go responses, in the present study, were highly probable after Go cues (88%) and Nogo responses were highly probable after Nogo cues (88%). Thus enlarged P300 to Go stimuli at posterior sites might have been caused by enhanced CNV coming from preceding Go cues. CNV resolution, however, cannot explain why posterior SW increased when Nogo cues were followed by Go responses (Unpredicted-Go trials).

The most important aim of this study was to investigate how probability information conveyed by the cue affects Go/Nogo ERPs. While anterior N200 to Nogo stimuli is often enhanced [7, 8], the present results showed that this effect was modulated by cue information: N200 at Fz and Cz increased only if the Nogo stimulus was unpredicted. Probability differences *per se* or summation with overlapping CNV cannot account for our N200 data. First, if N200 enhancement is connected to low probabilities then unpredicted Go and Nogo should have elicited comparable N200s since both types of trials were equally improbable. Second, if enlarged N200 to unpredicted Nogo stimuli was caused by increased CNV appearing after Go cues then similar N200s should have been found when Go cues were followed by Go and Nogo stimuli (Predicted-Go and Unpredicted-Nogo trials). Such enlarged N200s were not found.

The N200 enhancement to Nogo stimuli (Nogo N200) has typically been attributed to response inhibition processes [7, 8, 10]. According to this idea, Nogo N200 with a frontal [10] or fronto-central [7, 8] distribution should be interpreted as a real-time correlate of response inhibition which is active on Nogo, but not on Go, trials. The source of inhibitory N200 is assumed to be found in the prefrontal cortex [8]. We have found in the present experiment that inhibitory N200 could also be a function of whether or not a cue predicts the occurrence of the subsequent Nogo stimulus. This result might be explained by assuming that the N200 component to a Nogo stimulus increases in amplitude when a greater effort is required to withhold Go responses [7]. Thus enlarged N200 to unexpected Nogo stimuli in the present study might have been associated with the increased effort needed to inhibit responding when cues predict a Go response.

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Visual event-related potentials evoked by using a virtual reality display

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This study aimed at investigating whether a virtual reality display (VRD) is an appropriate tool for evoking visual event-related potentials (VEPs). VEPs evoked by VRD stimuli were highly similar in form to VEPs evoked by using a computer monitor, both having two dominant peaks, labeled P100 and N200. Monitor and VRD N200 latencies and amplitudes were highly correlated. However, peak latencies were longer and the peaks were broader when stimuli were presented on the VRD. Besides, VRD P100 amplitude was smaller, and an N75 peak could be seen usually only on monitor VEPs.

Keywords: visual event-related potential; virtual reality display

Recent advances in computer technology have permitted the development of virtual reality (VR) systems which, with the help of appropriate programs, can be used for immersing the subject into a virtual, three-dimensional (3D) world, where the visual scene changes according to his/her head movements and virtual objects can be manipulated by a "virtual hand", the movement of which imitates the subject's hand movements. Complete virtual reality devices include a helmet within which headphones and a stereo video system is mounted (typically two liquid crystal displays (LCD), one in front of each eye), and two input devices, a head movement tracker and a hand movement sensor (data glove). The use of such systems in medicine and psychology has escalated in the last some years. The most important applications include 3D visualization of human body parts, simulation of surgical procedures [11, 14], the use of

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VR in the rehabilitation process of physically disabled [5] or cognitively impaired people [8] and in behavior therapy [10]. However, brain researchers seem to show much less interest in the new possibilities provided by the VR technology than physicians. Thus far only a few research studies were published which applied VR technology. However, there are some attempts to delineate the possibilities and limitations of VR systems in special fields of research [15]. The reservations regarding the application of VRDs in experimental research might be attributed partly to the fact that VR systems, at least the cheaper ones, are in some respects less perfect than other, more conventional equipments. For example, visual stimuli can be presented with more accurate timing and with a better resolution on a standard computer monitor than on the LCD displays of a low-cost VRD. In spite of that, there may be a number of research fields where the potential advantages of VR may outweigh its limitations and one of these might be certain types of event-related potential (ERP) research. VRDs not only make possible the presentation of 3D visual stimuli or different stimuli to the two eyes, but even if the experiment does not exploit these special features of the VRD, it offers advantages that can make the experiment more comfortable for the subjects and can help in reducing eye movement artifacts. The fact that the stimuli are presented via a lightweight head-mounted display makes it unnecessary to use an uncomfortable head and/or chin support, because the relative position of the eyes and the displays does not change with head movements. In addition, VRDs ensure a symmetric visual environment and completely eliminate the effect of unwanted visual distractors.

In this study we did not exploit the possibilities of 3D graphics, we used the VR display just like an ordinary computer monitor, presenting the same two-dimensional (2D) pattern on both its displays and compared the resulting ERPs with those evoked by similar stimuli presented via a computer monitor. The VRD we tested contains two LCD screens connected to a computer and driven by normal VGA input. On a standard computer monitor the screen refresh cycle time is about 17 ms, that is, the content of the screen is updated in every 17 ms. When using our VRD, every first screen refresh occurs in one of the LCD screens and every second in the other. That is, the duration of the refresh cycle for one LCD screen is the double of that of a standard VGA monitor. This feature also implies that a new visual stimulus cannot be presented simultaneously to the two eyes, but one of the eyes receives the new stimulus with a 17 ms delay relative to the other eye. Besides, on LCD displays the offset and onset of stimuli is considerably slower than on a computer monitor.

Our preliminary study addressed the question whether – in spite of the limitations mentioned above – the VRD can be used for evoking visual ERPs of acceptable quality. We used simple, patterned visual stimuli and investigated exogenous components of the visual evoked potential (VEP), because we assumed that these must be the most sensitive to timing inaccuracies.

Materials and methods

Subjects

Five healthy subjects, 4 females and 1 male ranging in age from 22 to 42 years participated in the study. All had normal or corrected to normal vision.

Procedure

The phase reversal of a black and white checkerboard pattern was used to evoke VEPs. The two patterns were alternating at 0.5 Hz. A small red cross was continuously displayed in the center of the stimulus field and the subjects were instructed to fixate it. In each part of the experiment (see below) 150 phase reversals occurred, in blocks of 50. The first two reversals of each block were not included in the analyses. Interblock intervals were at least 15 s, and the following block was started by the subject's button press. Stimuli and triggers used to initiate recording epochs were presented by an ERTS program.

Given the profound differences between the VRD and the computer monitor in terms of size, luminance limits, contrast limits, sharpness of contrast borders and spatial resolution (see Appendix for technical details) and, consequently, the subjective experience they produce we did not attempt to perfectly match the monitor and VRD stimuli. The characteristics of stimuli presented via the monitor were similar to those commonly used in VEP studies. However, because of the limitations of the VRD, the luminance and the contrast of VRD stimuli was lower, and the size of the checks was greater than the respective values in most VEP studies. Because of the great check sizes, the observed evoked potentials were mainly due to the changes in local retinal luminance for both VR and monitor stimuli [6]; (for review, see [9, 12]).

The VEP recordings were carried out in a dimly lit room. The subjects sat in a reclining chair with their head leant against a head support. Two IBM-compatible computers were used to control the experiment, one for stimulus presentation and the other for EEG data acquisition. In the first part of the experiment a VGA computer monitor viewed binocularly from a distance of about 1.2 m was used to present the stimuli. The size of the screen was 240×180 mm which resulted in a stimulating field of 11° 20'×8° 30', and each check subtended 50'. The luminance of black and white checks was less than 0.1 cd/m^2 and 48 cd/m^2, respectively, producing a mean luminance of 24 cd/m^2 and a contrast of 100%. In the second part of the experiment a virtual reality device (i-glasses!, Virtual i.O) was put on the subjects' head and the stimuli were presented quasi-simultaneously (with one of them lagging behind the other by one screen refresh cycle,16.7 ms) on the two 30°×25° 30' screens of the device. The

individual check size was 2° 8'. The mean luminance of the stimulus field was 9.1 cd/m^2 and the contrast was 94% (black squares: 0.5 cd/m^2, white squares: 17.6 cd/m^2).

Pattern reversal VEPs were recorded by three occipital electrodes (Oz, Ol, O2), with Cz as the reference electrode. Although in most VEP studies occipital-Fz leads are used, we chose Cz instead of Fz in order to minimize the effect of eye movements on the EEG records. An electrode on the forehead served as ground. The vertical electro-oculogram (EOG) was recorded from electrodes located above and below the left eye. The horizontal EOG was recorded from electrodes positioned at the outer canthus of each eye. Electrode resistances were kept below 5 kOhms. The silver-silver chloride recording electrodes (Medicor) were applied to the scalp with electrode cream (EC2, Grass Instruments). Electrodes were connected to the amplifiers of a 16-channel EEG recording system (Elsoft). The amplifier bandpass was set at 0.1–300 Hz and the signals were AD converted with 12 bit resolution at a sampling rate of 2000 Hz (Scientific Solutions Lab Master AD PGL board; DataSponge, BioScience Analysis Software).

For each epoch the analysis time was 600 ms (prestimulus time 100 ms, poststimulus time 500 ms). Prior to averaging each epoch was automatically scanned for EOG and EEG artifacts. Whenever the absolute value of any measured point on any channel exceeded 100 μV or the difference between the greatest and the smallest voltage value exceeded 100 μV an epoch was rejected. Baseline correction and lowpass digital filtering of VEPs with a Butterworth filter (cutoff frequency: 100 Hz, order: 12, no phase distortion) was applied off-line to maximize the accuracy of peak measurement. The two most consistent peaks, labeled P100 and N200 were identified by determining the greatest/smallest value in the time windows 60–160 ms and 160–260 ms, respectively. Peak latencies were determined and amplitudes were measured from the baseline (–100–0 ms). All analyses were done by a MATLAB program written by ourselves.

Results

Preliminary analyses suggested no remarkable differences between the recording channels relevant to the comparison of monitor and VRD VEPs, thus here only the Oz channel data will be analyzed. Mean latencies and amplitudes of the monitor and VRD VEPs are presented in Table I. Figure 1 shows the grand averages of monitor and VRD VEPs and two characteristic individual averages are given in Figure 2.

Table I

Monitor and virtual reality display VEP latency and amplitude means (SDs in parentheses)***

	Monitor		Virtual reality display	
	Latency (ms)	Amplitude (μV)	Latency (ms)	Amplitude (μV)
N75	74.5 (5.4)	–7.0 (4.7)	–	–
P100	102.9 (4.4)	11.8 (3.0)	120.7 (4.2)	8.8 (3.3)
N200	207.5 (23.2)	–6.0 (5.7)	214.4 (17.9)	–9.1 (5.6)

* Amplitudes measured from baseline

**n=5

The form, peak latencies and amplitudes of averaged monitor VEPs closely corresponded to those found in other studies using the same electrode placements [1]. The form of VRD and monitor grand averages were highly similar. However, while an early negative peak (N75) could be clearly seen on most individual monitor VEP averages, this peak was barely discernible by visual inspection on VRD VEPs. Therefore the amplitude and latency measures of this peak are given only for monitor VEPs. The additional main differences were that the VRD P100 latency was longer and P100 amplitude was smaller than the respective values of the monitor VEPs.

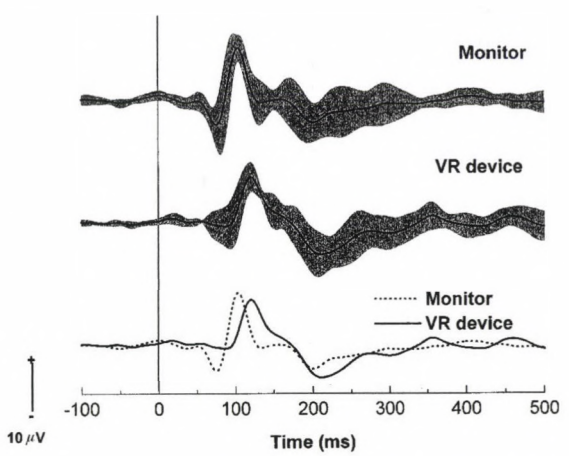

Fig. 1. Top: Grand average and standard deviation of monitor VEPs. Middle: Grand average and standard deviation of virtual reality display VEPs. Bottom: Monitor and virtual reality display VEP grand averages superimposed. Lead: Oz-Cz

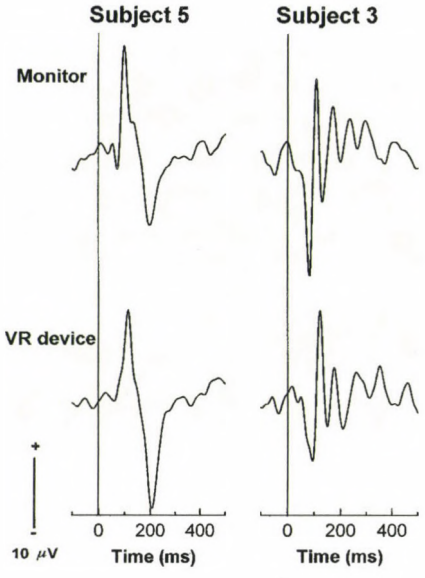

Fig. 2. VEP averages of two subjects for monitor (top) and virtual reality display (bottom) stimuli. Lead: Oz-Cz

When analyzed with one-sided, paired *t*-tests, these differences turned to be significant at the $p<0.02$ level. VRD N200 latency was also longer than monitor N200 latency ($p=0.046$), but for N200 the amplitude difference was reversed: the VRD N200 peak was greater than the monitor N200 peak ($p=0.016$). The most prominent P 100 peak could be invariably seen on the individual averaged VRD VEPs. However, even VRD P100 was less clearly defined as compared to the monitor P100. As it can be seen on Figure 1, the well-distinguishable small deflection on the descending limb of monitor P100 almost disappears on VRD P100, and VRD P100 is definitely broader than monitor P100. VRD N200 was also broader than monitor N200.

The correlations between the latencies and amplitudes of monitor and VRD VEP peaks were computed in order to examine whether a linear relationship exists between the parameters of monitor and VRD VEPs. Table II displays the correlation coefficients and the significance values. In spite of the small number of subjects, the amplitudes and latencies of VRD and monitor N200 are closely, positively related, while no such relationship was found for P100.

Table II

Correlations between latencies and amplitudes of the respective peaks of monitor and virtual reality display VEPs***

	Latency		Amplitude	
	r	*p*	r	*p*
P100	0.50	0.391	0.77	0.130
N200	*0.97*	*0.005*	*0.93*	*0.024*

* Amplitudes measured from baseline

**n=5

Discussion

The main finding of this preliminary investigation was that even a low-cost VRD is capable of presenting visual stimuli fast and accurately enough to evoke event-related potentials very similar in form to VEPs evoked by a computer monitor. It seems also encouraging that VRD N200, which was the longest latency component examined was nearly perfectly correlated with the monitor N200, both in terms of latency and amplitude and that the average latency difference between monitor N200 and VRD N200 was only 7 ms, as compared to the 18 ms difference between monitor P100 and VRD P100. Thus, it seems that longer latency components are less susceptible to the different physical characteristics of monitor and VRD stimuli. If this finding could be repeated in a greater sample and for endogenous components, too, the conclusion could be drawn that the VRD is a promising candidate for investigating cognitive components of event-related potentials.

A number of physical attributes of VRD and monitor stimuli known to influence amplitude and latency (luminance, brightness contrast, check size, stimulus field size) [2, 3, 7, 13; for review, see 12] were different in this study. Thus, the comparison of peak latencies and amplitudes serves only the purpose to assess the capabilities of the VRD as compared to those of a standard monitor. It is unquestionable that the differences between VRD and monitor VEPs are at least partly due to the physical differences between monitor and VR stimuli. In other studies where the stimuli were more similar to those produced by our VRD, the VEPs were not very much different from those found in this study. For example in a study of Cant et al. [2] where the stimulus field was 28°, the luminance of the white checks was 10 cd/m^2, and the check size was 50', the mean P100 latency turned to be 116.8±7 ms; in Tobimatsu et al.'s study [13], where the stimulus field was 16°, the luminance of the white checks

11 cd/m^2 and the check size 50', the mean amplitude of the P100 was 7.5 μV. However, it is most probable that the latency increase and the less sharp form of both VRD VEP components and the amplitude decrease of VRD P100 are partly attributable to the facts that VRD stimulus onset is definitely slower and there is an about 17 ms time delay between stimulus onsets for the two eyes. Because of these constrictions the use of the VRD for investigating early, exogenous components of VEPs in vision research or in clinical settings cannot be recommended unless the normative values for the latencies and amplitudes of the VRD VEP early components are reliably determined. However, in studies using a repeated measures design this problem seems to be less crucial, and VRD might be an appropriate choice.

Appendix

i-glasses! specifications

Optics[1]

Field of view: 30° each eye
Fixed focus at 11'
Requires no interpupillary distance adjustment
Can be worn with eyeglasses

Displays[1]

2 full-color 0.7" liquid crystal displays
Resolution: 180,000 pixels per LCD panel

Luminance limits

The maximal luminance attainable by i-glasses! was measured by 1° viewing angle luminance meter (MFKI MOD. 612) and was found to be 17.6 cd/m^2. For comparison, the maximal luminance of the computer monitor was 130 cd/m^2. Minimal VRD luminance was 0.5 cd/m^2, while minimal monitor luminance was less than 0.1 cd/m^2. Thus, the maximal contrast i-glasses! can produce is 94%.

[1] Data specified by the manufacturer.

Speed of luminance change

In Fig. 3 and Fig. 4 the speed of luminance change is demonstrated when using i-glasses!. A calibrated photoresistor (TESLA 65037 1k5) was used to continuously record the luminance change of the center of the LCD display while the color of the whole screen changed from black to white and from white to black. As it can be seen, the time required to attain 50% of the maximal luminance change is about 60 ms when the display becomes white (Fig. 3, bottom), and about 20 ms when the display darkens (Fig. 4, bottom). For comparison, 50% of the maximal luminance change on the monitor is attained in both cases within one screen refresh cycle, that is, the respective values are less than 16.6 ms (Fig. 3 and Fig 4, top).

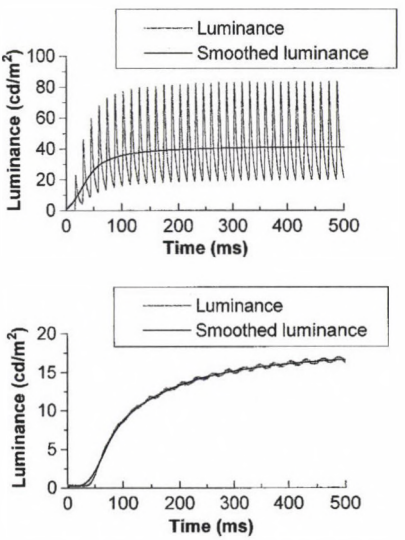

Fig. 3. The speed of luminance change of the monitor (top) and the virtual reality display (bottom) when the screen turns from black to white. Sampling rate: 10,000 Hz. Note the luminance oscillations corresponding to every screen refresh. An 1000-point Fast Fourier Transform smoothing was performed on the luminance data, and the smoothed values are shown, too

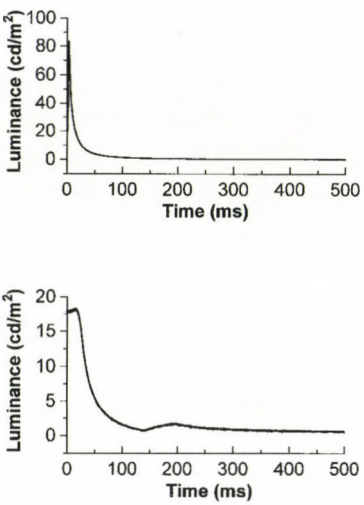

Fig. 4. The speed of luminance change of the monitor and the virtual reality display when the screen turns from white to black. Sampling rate: 10,000 Hz

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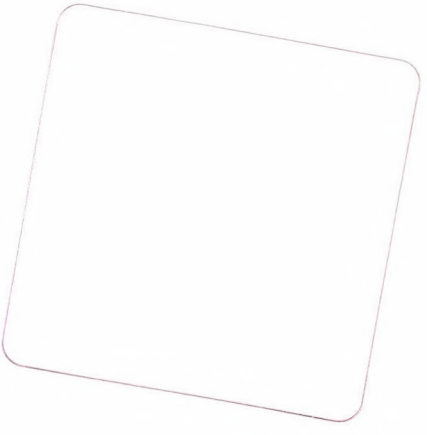

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Experimental hypertriglyceridaemia and hypercholesterolaemia in rats

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Lipid disorders and cardiovascular diseases have been related in many studies. We here studied the influence of acute ingestion of a long chain triglyceride (LCT) emulsion (rich in triglycerides) on plasma triglyceride (TG) and total cholesterol (TC) levels in laboratory animals (Wistar rats), comparing it with the induction of hypertriglyceridaemia and hypercholesterolaemia by Triton WR-1339 injection. The results show that Triton would be suitable for inducing hypercholesterolaemia but not hypertriglyceridaemias similar to those in humans. The LCT emulsion intake, however, provoked transitory hyperlipaemia with values similar to those often found in hyperlipaemic subjects, and would thus be suitable for testing possible antilipaemic treatments.

Our study also presents a model of hypertriglyceridaemia, hypercholesterolaemia and obesity in experimental animals, provoked by a chronic intake of an LCT emulsion. This model may be useful in investigating the mechanisms of the pathogenesis of atherosclerosis and the pharmacological treatment of obesity and dyslipaemias.

Keywords: hypertriglyceridaemia, hypercholesterolaemia, Wistar rat

Hyperlipidaemia, generally regarded as a problem of affluent socities, is one of the major risk factors for the development of atherosclerotic heart diseases [1]. The continual ingestion of a high proportion of fats seems to be directly related to obesity and hyperlipaemia. There have for some time been attempts to develop experimental dyslipaemia in animals that are similar or comparable to the values in humans. Various authors [2, 7, 9] have described the induction of dyslipoproteinaemias in laboratory animals. The advantages of experimental dyslipaemias are the production of atheromatous lesions in a relatively short space of time, adequate control of dietary and

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environmental factors, the possibility of studying the reversibility of atherosclerotic lesions, and the preclinical trial of hypolipaemic substances [3]. In most studies on humans, the appearance of atheromatous lesions is intimately related to a dysfunction in cholesterol metabolism (raised total plasma levels, augmented LDL and diminished HDL). For that reason, almost all attempts to produce dyslipoproteinaemia in animals have been based on a massive intake of cholesterol-rich foods, with the aim of producing a maintained hypercholesterolaemia. Currently, without calling into question the importance of total cholesterol (TC), there is a discussion about the importance of the possible role of triglycerides (TG) in cardiovascular risk [6], with growing support for the attribution of a potentially atherogenic role to high plasma TG levels [19].

In the present work we study the production of hyperlipaemia in rats receiving different doses of Triton-WR-1339 (oxyethylated tertiary octylphenol formaldehyde polymer), an agent already used by other investigators [18, 26], and compare the results with those obtained in another group of rats ingesting high calory content fat emulsions (LCT emulsion) at a high concentration for a brief period of time, seeking in both cases high TG and TC levels similar to those found in hyperlipaemic humans.

Our results lead to a model of hypertriglyceridaemia, hypercholesterolaemia and obesity in experimental animals, provoked by the chronic ingestion of an LCT emulsion, that could be used for the study of dyslipaemia, atherosclerosis, or obesity, since the high levels of plasma TG and TC, as well as the increase in weight of the model animals, are long-lasting.

Material and methods

Animals and diet

Female Wistar rats (Hannover origin) were housed in the University Animalarium in an air-conditioned room at 24±2 °C, with artificial light from 8:00 until 20:00, continuous air flow at a rate of 13 renewals per hour, 55% regulated humidity. A standard diet (B.K. Universal CB, S.L., Barcelona, Spain) was used. Food and fluid intake were measured daily.

Triton-induced hyperlipaemia

Rats of body, weight range 200–230 g were divided into two groups and injected intraperitoneally with Triton-WR-1339 (Sigma Chemical Co., St. Louis, Mo, USA).

The first group (n=10) was injected once with Triton (400 mg/kg body wt). Blood samples were taken before the Triton injection (basal value), and at 24, 48 and 72 h.

The second group (n=10) was injected with Triton (200 mg/kg per day) on four successive days. A blood sample was taken before each Triton injection, and 24 h and 72 h after the last injection (4 and 6 days, respectively after the basal extraction).

All samples (0.2 ml) were taken from the tail vein, with the animal conscious and unfasted, and plasma TG and TC levels were determined.

LCT-emulsion-induced hyperlipaemia

The LCT emulsion used was Intralipid 20% (Kabi Pharmacia AB, Sweden).

Rats of body weight in the range 200–230 g were divided into two groups. The first group (n=10) was fasted (water *ad libitum)* for 22 h and then allowed free access to 20% LCT emulsion for 2 h (from 8:00 to 10:00) without supplying food. Blood samples were taken after the 2 h ingestion of the LCT emulsion (0 h), and 2 h and 24 h after removing the emulsion and supplying food and water *ad libitum.*

The second group (n=10) was fasted for 22 h, allowed free access to 20% LCT emulsion for 2 h (from 8:00 to 10:00) without supplying food, fasted again for 22 h, and again allowed free access to 20% LCT emulsion for 2 h (from 8:00 to 10:00) without supplying food. Blood samples were taken after the 2 h ingestion of LCT emulsion on the second day (0 h), and 2 h and 24 h after withdrawing the emulsion and supplying food and water *ad libitum.*

All samples (0.2 ml) were taken from the tail vein, with the animal conscious and unfasted, and plasma TG and TC levels were determined.

Model of induction of hypertriglyceridaemia, hypercholesterolaemia and obesity

Two groups of female Wistar rats were formed: Group A (n=10) drank *ad libitum* a 5% LCT emulsion from birth; the Control Group (n=10) drank water. The 5% emulsion given to the animals was prepared weekly and stored in a refrigerator until one hour before administration. For 4 months, food and fluid intake was measured daily, and each animal was weighed and a blood sample taken (0.4 ml) from the tail vein weekly, with the animal unfasted.

Plasma analyses

All blood samples were immediately centrifuged (2500 r.p.m., 10 min, room temperature) and analysed in a Coulter CPA analyzer at 37 °C. The enzymatic methods were: for triglycerides, GPO-POD; for total cholesterol, CHOD-POD.

In animals serving models for induction of hypertriglyceridaemia, hypercholesterolaemia and obesity, the plasma creatinine levels and the plasma activities of the

transaminase enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also determined. The analyses were carried out using Coulter CPA analyzer, with the following enzymatic methods: for creatinine, the Jaffe method (picric acid, NaOH, phosphate); for AST activity, the AST-MDH method; for ALT activity, the ALT-LDH method.

All the reagents used were "Reagents for Coulter CPA analyzer", CCS, Coulter Científica, Madrid, Spain.

Statistical analyses

Groups of data are expressed as mean ±SD. The differences were considered to be statistically significant if $p<0.05$ as evaluated by the Friedman and Kruskal-Wallis tests.

Results

Induction of hypertriglyceridaemia with Triton-WR-1339

Figure 1 shows the plasma TG and TC levels obtained in a group (n=10) of rats 24, 48 and 72 h after the intraperitoneal administration of a single dose of Triton (400 mg/kg body wt). As it can be seen, there is a statistically significant rise in the TG levels at 24 h (22.46±9.54 mM, $p<0.0001$), with respect to the basal levels (0.76±0.28 mM). At 48 h, these levels have fallen back, approaching normal values (1.93±0.40 mM, $p<0.001$ vs 24 h levels, but still higher than the basal values, $p<0.05$) and at 72 h they have returned to normality (0.80±0.24 mM). The TC levels rise significantly from a basal value of 1.71±0.23 mM, to a value at 24 h of 7.29±2.43 mM ($p<0.005$), followed by a decline to within normal limits at 48 h (1.97±0.88 mM), and 72 h (1.82±0.19 mM).

As one sees in Fig. 2, with a Triton dose of 200 mg/kg body wt per day over 4 days, one obtains progressively higher levels of TG and TC, with stabilization after the third Triton administration. The TG levels were (n=10): basal, 1.22±0.85 mM; 24 h, 16.59±13.84 mM, $p<0.05$; 48 h, 28.33±10.99 mM, $p<0.005$; 72 h, 52.24±24.76 mM, $p<0.005$; 96 h, 49.99±23.71 mM, $p<0.005$ (all vs basal values). The TC levels were: basal, 1.97±0.41 mM; 24 h, 5.33±2.43 mM, $p<0.05$; 48 h, 10.01±3.23 mM, $p<0.005$; 72 h, 14.07±3.85 mM, $p<0.005$; 96 h,14.22±3.57 mM, $p<0.005$ (all vs basal values). Both levels return to their normal values 72 h after the last (fourth) Triton injection (the TG and TC levels were 1.48±0.85 mM, and 2.02±1.03 mM, respectively).

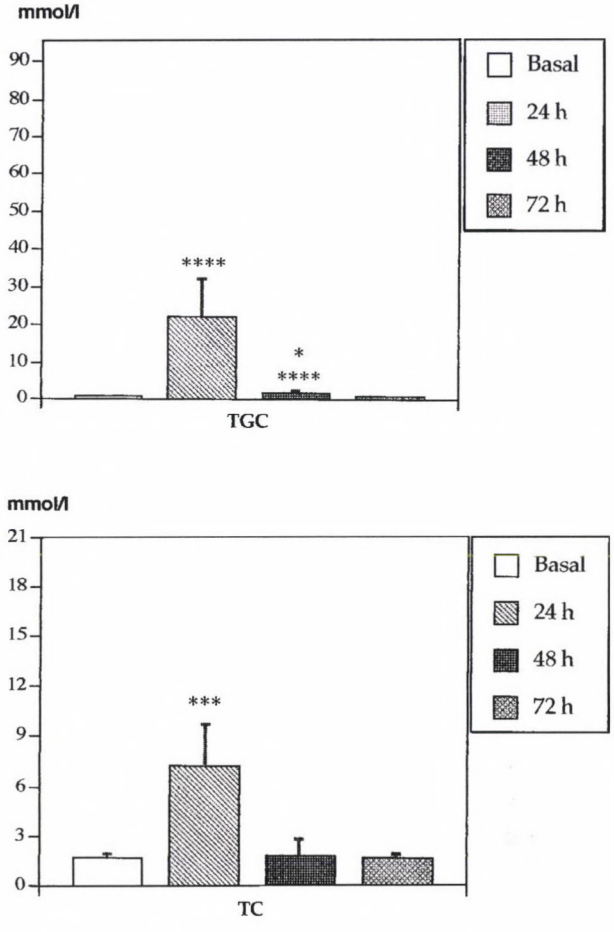

Fig. 1. Plasma triglyceride (mM) and total cholesterol (mM) levels after intraperitoneal administration of one dose (400 mg/kg body wt) of Triton to a group (n=10) of rats. Times are measured from the administration of the first dose. TG: ****$p<0.001$, 24 h vs basal *****$p<0.001$, 48 h vs 24 h, $p<0.05$ vs basal TC: ***$p<0.005$, 24 h vs basal

Induction of hypertriglyceridaemia with LCT emulsion

Figure 3 shows the plasma TG levels of the group of rats subjected to a 24 h fast and then allowed free access to a 20% LCT emulsion for 2 h. The average intake of LCT emulsion in these conditions was 7±4 ml (n=10). The figure also shows the plasma TG levels of the other group of rats that underwent this same protocol twice for a total of 48 h. The average LCT emulsion intake in this group was 7±3 ml (n=10) the first day

and 11±2 ml (n=10) the second day. One observes that, after 2 h access to oral LCT emulsion, the plasma TG levels were significantly higher in both groups relative to their basal values (0.61±0.24 mM, n=10 and 3.15±1.81 mM, n=10, $p<0.05$ in the group that fasted 24 h; 0.49±0.20 mM, n=10 and 7.81±2.83 mM, n=10, $p<0.001$ in the group that

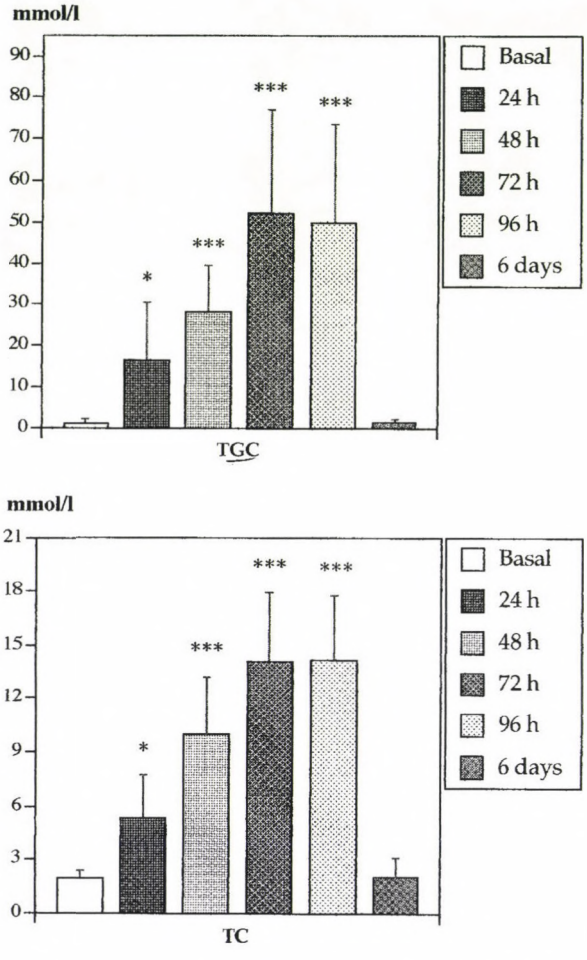

Fig. 2. Plasma triglyceride (mM) and total cholesterol (mM) levels after intraperitoneal administration of a sequence of four doses (200 mg/kg body wt) of Triton with 24 h separation between each to a group (n=10) of rats. Times are measured from the administration of the first dose. TG: *$p<0.05$, 24 h vs basal ***$p<0.005$, 48 h vs basal, 72 h vs basal, 96 h vs basal TC: *$p<0.05$, 24 h vs basal ***$p<0.005$, 48 h vs basal, 72 h vs basal, 96 h vs basal

fasted 48 h). At 2 h after terminating ingestion of the LCT emulsion, the TG levels were 1.06±0.34 in the group that fasted 24 h, $p<0.05$ vs basal values, and 5.67±2.47 in the group that fasted 48 h, $p<0.001$ vs basal values. The hypertriglyceridaemia was more marked in the group of animals that underwent 48 h of fasting, with the levels returning to normal 24 h after the experiment in both groups: 0.79±0.25 mM (the group that fasted 24 h, n=10), and 0.87±0.14 mM (the group that fasted 48 h, n=10).

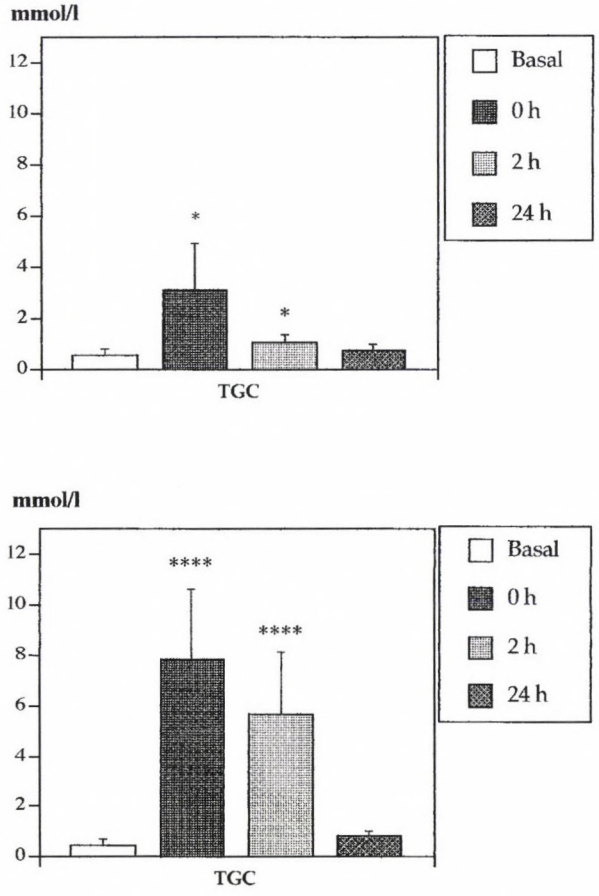

Fig. 3. Plasma triglyceride (mM) levels of : a group (n=10) of rats fasted for 22 h followed by free access to a 20% LCT emulsion for 2 h. Times are measured after ending the LCT-emulsion drinking period. – a group (n=10) of rats fasted for 22 h followed by free access to a 20% LCT emulsion for 2 h, fasted again for 22 h, and again allowed free access to 20% LCT emulsion for 2 h. Times are measured after ending the last LCT-emulsion drinking period. *$p<0.05$, 0 h vs basal, 2 h vs basal ****$p<0.001$, 0 h vs basal, 2 h vs basal

The plasma TC levels in the same two groups of animals: in the group that fasted 24 h, the basal values, and the values after 2 h access to the LCT emulsion, 2 h after stopping the ingestion of the LCT emulsion, and 24 h after, were 1.84±0.52, 1.76±0.47, 1.68±0.36 and 1.78±0.39 mM, n=10. In the group that fasted 48 h, the respective values were 1.81±0.18, 1.78±0.34, 1.55±0.44 and 1.68±0.28 mM, n=10. There numbers show that there was no change in the TC values due to the emulsion intake, either in the group that fasted 24 h, or in the group that fasted 48 h.

Model of induction of hypertriglyceridaemia, hypercholesterolaemia and obesity

Table I lists the weights, plasma TG and plasma TC levels 2 and 4 months after the beginning of the study and two weeks after ending the study. Also this Table lists the results for the plasma creatinine levels and the plasma activities of the transaminases AST and ALT after 4 months of treatment.

Table I

Weights, plasma TG and TC levels 2 and 4 months after the beginning of the study of the influence of chronic LCT emulsion ingestion and two weeks after ending the study. Plasma creatinine levels and plasma activities of the transaminases AST and ALT after 4 months of treatment

	Group A			Control Group		
	2 months after birth (n=10)	4 months after birth (n=10)	2 weeks after ending (n=10)	2 months after birth (n=10)	4 months after birth (n=10)	2 week after ending (n=10)
Weights (g)	****	*	*			
	204±18	298±33	309±40	176±12	272±12	274±13
TG (mM)		*	**			
	0.86±0.49	1.72±0.55	2.45±0.63	1.21±0.41	1.27±0.29	1.23±0.38
TC (mM)	***	****	**			
	2.44±0.26	2.76±0.36	2.74±0.21	1.91±0.34	1.76±0.28	1.84±0.32
Creatinine (mg/dl)	–	1.0±0.4	–	–	0.9±0.3	–
AST (U/L)	–	55±8	–	–	50±6	–
ALT (U/L)		*				
	–	104±9	–	–	85±16	–

*p<0.05: Group A vs Control Group

**p<0.01: Group A vs Control Group

***p<0.005: Group A vs Control Group

****p<0.001: Group A vs Control Group

Discussion

Our objective was to seek an experimental model that allows one to obtain elevated cholesterol and triglyceride levels in blood that are similar to those found in hyperlipidaemic humans. We think that, while Triton is not a good hypertriglyceridaemia inducing agent, the blood TG levels being excessively elevated and not comparable to those found in hyperlipaemic subjects, it would be suited to testing hypotriglyceridemic agents, since the TC levels are very similar to those observed in hypercholesterolaemic humans.

The hypertriglyceridaemia induced with LCT emulsion is similar to that which appears in most hyperlipaemic subjects (high blood TG levels due to the abundant consumption of fat-rich foods).

Induction of hyperlipidaemia with Triton-WR-1339

Most studies have shown Triton to induce structural changes in high-density lipoproteins (HDL) caused by a progressive displacement of the A-1 apoprotein of the HDL surface. It is also capable of breaking the HDL molecules down into micelles, leading to a release of triglycerides and cholesterol into the blood supply [25]. Our results (Fig. 1) with this agent agree with those described by Schurr [21] where parenteral administration of a dose of Triton-WR-1339 to adult rats produced a hyperlipidaemia in which maximum plasma TG and TC levels were reached at 20 h followed by a decline to normal values.

Since the lipid levels obtained by administering Triton at a dose of 400 mg/kg are not comparable to those observed in hyperlipaemic subjects, we decided to reduce the dose to 200 mg/kg and administer it daily over a longer period (4 days) seeking rises in plasma TG and TC concentrations that are less abrupt and reach levels that are comparable to human hyperlipaemia. This system, too, yielded TG values that were far higher than those found in subjects with abnormal lipid metabolism, but the values of cholesterolaemia were similar to those found in hyperlipaemic patients, and in agreement with those reported by other workers who use Triton to induce hyperlipaemia [14].

Induction of hyperlipaemia with LCT emulsion

With this protocol of ingestion of large volumes of 20% LCT emulsion in short periods of time, we found a more marked hypertriglyceridaemia in the group of animals that underwent 48 h of fasting than in those who ingested the emulsion after only 24 h of fast. The levels returned to normal 24 h after the experiment in both cases. These results

are consistent with those of Moran [16] who describe hypertriglyceridaemic effects of the intravenous administration of LCT emulsion followed by a decline due to the activation of the serum lipoprotein lipase system. Such major activation of plasma LPL was also reported by Hallberg [8] after intravenous LCT emulsion administration as causing TG levels to fall back to normal values after 24 h. The reduction in serum TG levels observed 24 h after the oral administration of LCT emulsion confirms those earlier findings, and leads us to the conclusion that we have not attained a truly hyperlipaemic state, but rather, as in the case of using Triton, high transitory levels of TG in the blood of the experimental animals, which situation could be useful in itself. However, with this model we did not obtain elevated TC levels either in the 24 h fasted group or the 48 h fasted group.

Model of induction of hypertriglyceridaemic, hypercholesterolaemia and obesity

Obesity is one of the most worrisome clinical entities in the more developed societies. Several studies [23] confirm that, in the obese fed high-calory diets, the hepatic TG production rate is significantly higher than in slim subjects. Our experimental model of a hyperlipaemic state and obesity verified that the prolonged ingestion of high-calory foods, specifically, rich in long chain TG, produces obesity in the animal at the same time as a sustained hypertriglyceridaemic and hypercholesterolaemic state.

In this model, the animals ingest a Long Chain Triglyceride (LCT) emulsion as this is metabolized more slowly than the Medium Chain Triglyceride (MCT) emulsions [13], and because its administration does not disturb glucose metabolism [5]. Both the LCT and MCT TG are hydrolyzed *in vitro* by the enzymes lipoprotein lipase and hepatic lipase, but the reaction is faster with MCT [20].

Since the addition of lipids to intravenous feeding formulas, animal and human studies have shown impairment of the reticuloendothelial system due to slow rates of clearance and gradual accumulation of long chain TG in the liver. Although medium chain TG accumulate only minimally in the liver and appear not to impair the reticuloendothelial system, studies are still inconclusive [10]. It seems that the chronic parenteral administration of fat-rich emulsions in rats is followed by histopathological alterations [12, 22]. In the present study, with ingestion of four months duration, we found no more than a slight rise in plasma AST activity, so that we may deduce that, from oral administration, any liver damage that might be caused would not be severe. No animals died during the study, and none presented any apparent disease.

As can be seen in Table I, the treated animals had a much greater weight gain than the controls. This finding coincides with numerous reports in the literature that giving an LCT emulsion has advantages over MCT emulsions: Chanez [4] found that

animals that drank LCT emulsion weighed significantly more; Hwang [11] found an increase in the weights of perirenal and epididymal adipose tissue pads in rats fed LCT emulsion; Lynch [15], in a 30-day study, also found that provision of dietary energy as the short-chain TG instead of LCT resulted in lower weight gain and fat deposition.

The plasma TG levels found in the present study showed that at two months there was no statistically significant difference between the 5% emulsion ingesting group and the controls. This seems to indicate that the TG degrading enzyme system is functioning perfectly, in agreement with Nordenstrom [17], which would explain the correlation between plasma TG levels and the lipoprotein lipase activity.

Swift [24], in a 6-day protocol, observed no changes in plasma TC levels. However, we found that the plasma TC levels had risen at two months, remaining significantly high throughout the study.

Until recently all workers in the field related atherosclerotic processes to high TC levels in the blood. Now there are studies [19] which relate atherosclerosis to plasma TG levels. We believe that the model of hyperlipaemia and obesity which we achieve with the chronic LCT emulsion ingestion is suitable for attempts to elucidate the role of chronic hypertriglyceridaemia in the genesis of atheromatous plaques. Similarly, it could be an optimal experimental model for the study of possible new treatments in both obesity and hyperlipaemia.

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Experimental data proving the presence of inhibitors of amylase activity in biliary and pancreatic juice

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Two parallel studies on albino male rats are performed. In the first study, there is a group which underwent resection of the proximal third of the small intestine. While the other group despite resection of the same segment also has a ligated common biliary and pancreatic duct. In the second study, one group of the experimental animals is only with ligated pancreatic duct and in the other group the same duct is implanted in the initial part of the ileum. On the 15th day after the surgical interventions the amylase activity and the absorption of glucose in the small intestine are studied by the method of turned sacs "in vitro". It is established that the glucose transport does not change after the four surgical interventions. However, the amylase activity increases about twice times after resection of the upper third of the small intestine and more than 4 times after resection of the same segment with simultaneous ligature of the common biliary and pancreas duct. Only at ligating the duct, the amylase activity is decreased in the jejunum and is significantly increased in the ileum, while its implantation in the initial part of the ileum does not change its activity in both studied segments of the small intestine. It is concluded that there are unknown inhibitors for the amylase activity in the biliary and pancreatic juice. The discussed issue is why they inhibit only the enzymatic compensatory processes without influencing the transport systems of the small intestine.

Keywords: amylase activity, glucose transport, resection of the small intestine, ligated duct, implanted duct, inhibitors, intestinal adaptation

It is known that after surgical removal of a portion of the small intestine, structural changes occur in the remaining functioning segment, which lead to compensatory strengthened functions [1, 8, 10]. Simultaneously with this the authors point out that after vast resection of the intestine, the contraction of the orbicular

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musculature is decreased, the nitric balance is deteriorated and the body weight of the experimental animals is decreased.

By using morphometric and proliferative methods it is stated that after resection of a part of the small intestine, the processes of proliferation in the intestinal membrane are strengthened [2]. It is assumed that the hormone of weight strengthens the trophic processes in the intestine after resection. It is supposed that the intestinal adaptation after severe intestinal resection is due to humoral as well as to luminal and cellular mechanisms [4].

In relation to the functional characteristics of the small intestine after resecting a part of it, it is stated that primarily inhibition of the nutritional characteristics in the remaining sections appears, and later on, after recovery, a strengthening of the most functions occurs [3, 5]. It is pointed out that the reserve functional possibilities of the ileum after resection of the jejunum are much greater than those of the jejunum after resection of the ileum.

Interesting compensatory reactions in the functions of the small intestine are also observed after ligating the common biliary and pancreatic duct of experimental animals. As a result of the absence of the main pancreatic enzymes in the cavity of the small intestine an induction is registered not only of the intestinal gamma amylase activity necessary to compensate the functions of the pancreatic alpha amylase, but also in the activity of the other intestinal carbohydrases [9]. Despite this an increase in the activity of some intestinal proteases is established as well as intensified absorption of some monomers [6].

Nowadays there are also unclear issues on a number of aspects of the intestinal adaptation. That is why the findings presented show that the experimental-surgical methods are a good possibility for studying the mechanisms of the functional compensatory reactions of the small intestine in the modelled conditions.

Material and methods

We conducted two independent studies on albino male wistar rats weighing 180 g. In the first study despite intact controls we used 2 experimental groups of animals. The first group was with resection of the proximal third of small intestine and the other despite resection of the same segment was with ligated common biliary and pancreatic duct. In the second study we compared the functional characteristics of the small intestine in experimental animals with ligated common pancreatic duct and in animals in which the duct was implanted in the beginning of the ileum. In the second group after testing some operational variations the most suitable was that in which the

implantation of pancreatic duct is made by using a part of the intestinal wall around its labia as the defect in the duodenal wall was recovered by the most correct possible way.

In both studies the amylase activity and the glucose transport in the small intestine were studied by using the method of the turned sacs by Wilson–Wiseman [11]. By using this method the transport of substances through the entire small intestine wall is read. For this goal the turned segments of small intestine with length of approximately 1 cm are incubated in special chambers in which 10 mmol solutions of glucose and 0.2% solution of soluble starch respectively are placed. The incubation is performed as a $t° = 37$ °C for 60 minutes at continuous aeration of the media with oxygen. In the cavity formed by the serous part of the intestine. One ml Ringer's solution is placed, in which the portion of glucose, which passed through the intestinal wall after a period of incubation is registered. We read the amylase activity by the increase of glucose passed in the serous incubate as a result of the substrate hydrolysis of starch. We determined the quantitative analysis of glucose by classical methods [7]. The principle of the method is based on the oxidation of the reducing sugars with copper reagents with subsequent recovery by arsenic-molibdenum reagent, which mixed with glucose gives bluish-green colouring. Colorimetry is made up 10–15 minutes after the appearance of staining on spectrophotometer at wave length of 525. The results obtained from the studies are expressed in mmol/l and they are processed statistically with the methods of variation and correlation analysis.

Results and discussion

On the 15th day after the surgical interventions in the experimental animals we did not register changes in the transport of glucose in the rest two-thirds of the small intestine in both experimental groups (Fig. 1). However, the amylase activity was compensatorily increased about twices after resection of the proximal segment of the small intestine and more than 4 times after its resection with simultaneous ligature of the common biliary and pancreatic duct.

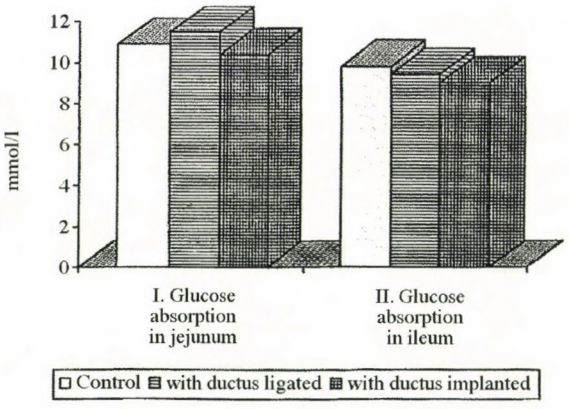

Fig. 1. Glucose absorption and amylase activity at small intestinal resection and pancreatic ductus ligated

		n	x±Sd	p
Glucose absorption				
1	Control	6	11.1±0.73	–
2	With intestinal resection	6	11.9±0.87	>0.05
3	With intestinal resection and pancreatic ductus ligated	6	11.5±0.94	>0.05
Amylase activity				
1	Control	6	4.8±0.72	–
2	With intestinal resection	6	9.1±0.98	<0.01
3	With intestinal resection and pancreatic ductus ligated	6	18.4±0.98	<0.001

These results enable the implication that at resection of the intestine and free access of the pancreatic and biliary juice in the lumen of its remaining part, compensatory processes are developed leading to increase in the amylase activity. At interruption of their access to the small intestine, the power of the compensatory processes in the remaining two-thirds of it, particularly in relation to the gamma amylase activity, are more strongly expressed (Fig. 2).

The absence of changes in the glucose absorption, after applying surgical interventions show that the glucose transport systems are not influenced by them and do not react with any compensatory overadjustments either in changes of the functional loading of small intestine or in excluding the pancreatic and biliary excretion from the digestive process.

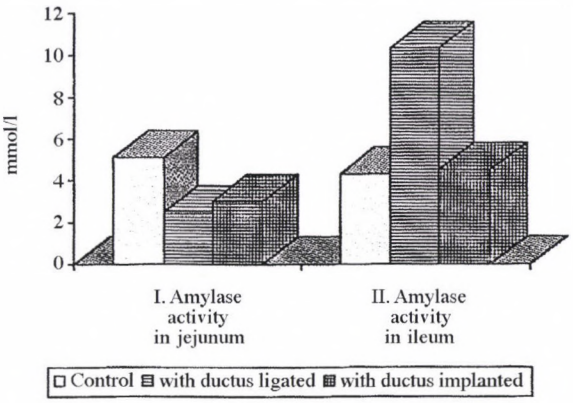

Fig. 2. Amylase activity at pancreatic ductus ligated and pancreatic ductus implanted

		n	x±Sd	p
Amylase activity in jejunum				
1	Control	6	5.1±0.64	–
2	At pancreatic ductus ligated	6	2.5±0.59	<0.025
3	At pancreatic ductus implanted	6	3.0±0.81	>0.05
Amylase activity in ileum				
1	Control	6	4.3±0.54	–
2	At pancreatic ductus ligated	6	10.3±0.91	<0.001
3	At pancreatic ductus implanted	6	4.5±0.65	>0.05

The data of our study show that the increased enzymatic activity only after resection of a part of the small intestine is an adequate compensatory reaction of its remaining part in these conditions. Concerning the significant increase of the gamma amylase activity after ligating the common pancreatic duct at the background of proximal resection of the small intestine, it is suggested to be due to the presence of unknown inhibitors in the biliary and pancreatic juice with access, interrupted after the surgical intervention. This is confirmed completely by our second study in which we studied the amylase activity in experimental animals with ligated common pancreatic duct or its implantation in the beginning of the ileum (Fig. 2). It is stated that the amylase activity at ligating the duct was decreased in the jejunum and increased significantly in the ileum whereas its implantation did not change statistically significantly its activity in both examined segments of the small intestine. Similarly to our first study in the second no one we established that in the glucose absorption change in the jejunum or in the ileum in any of the surgical interventions occurred (Fig. 3).

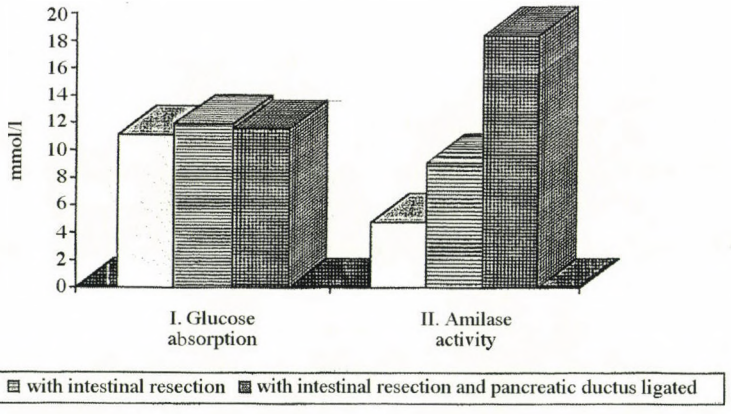

Fig. 3. Glucose absorption at pancreatic ductus ligated and pancreatic ductus implanted

		n	x±Sd	p
Glucose absorption in jejunum				
1	Control	6	10.9±0.86	–
2	At pancreatic ductus ligated	6	11.5±0.85	>0.05
3	At pancreatic ductus implanted	6	10.4±0.79	>0.05
Glucose absorption in ileum				
1	Control	6	9.8±0.77	–
2	At pancreatic ductus ligated	6	9.4±0.8	>0.05
3	At pancreatic ductus implanted	6	9.0±0.82	>0.05

The data obtained from the second study show that when excluding the luminal hydrolysis of starch in the jejunum and at its storage in the ileum, compensatory reactions in the distal segments of the small intestine are not developed. When comparing these data with the results of the first study in which the interruption of the access of pancreatic and biliary juice induces powerful increase of the amylase activity in the distal intestinal segments here the stated hypothesis is confirmed. Doubtlessly in the biliary and pancreatic juice there are substances inhibiting the compensatory reactions of the intestinal nutritional enzymes. There is no definite position on the origin of these substances and the mechanism of their influence. It is worth noting that these substances selectively inhibit the enzymatic systems without influencing the transport functions of the small intestine. Whether this substance or a group of substances is contained in the biliary and pancreatic juice and whether they influence only the amylase activity or other enzymes is an object of future studies.

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Regulation of the ryanodine receptor calcium release channel of the sarcoplasmic reticulum in skeletal muscle

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In striated muscle contraction is under the tight control of myoplasmic calcium concentration ($[Ca^{2+}]_i$): the elevation in $[Ca^{2+}]_i$ and the consequent binding of calcium to troponin C enables, while the decrease in $[Ca^{2+}]_i$ prevents the actin-myosin interaction. Calcium ions at rest are stored in the sarcoplasmic reticulum (SR) from which they are rapidly released upon the depolarisation of the sarcolemmal and transverse (T-) tubular membranes of the muscle cell. The protein responsible for this controlled and fast release of calcium is the calcium release channel found in the membrane of the terminal cisternae of the SR. This review focuses on the physiological and pharmacological modulators of the calcium release channel and tries to draw an up-to-date picture of the events that occur between T-tubular depolarisation and the release of calcium from the SR.

Keywords: ryanodine receptor, skeletal muscle, calcium release, sarcoplasmic reticulum

The identification of the calcium release channel protein was done utilising its high-affinity binding site for the plant alkaloid ryanodine (from *Ryania speciosa* Vahl) and it is thus referred to as *ryanodine receptor* (RyR). Since the first description of its amino acid sequence RyRs have been found not only in skeletal muscle but in several other tissues with three different isoforms (RyR1, RyR2 and RyR3) identified in mammals. Due to their wide tissue and species distribution together with the pivotal role they play in intracellular calcium homeostasis RyRs gained a lot of attention recently (for reviews see [38, 60, 72]).

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The functional channel has been shown to be a homotetramer with each subunit comprised of 5037 amino acids (for RyR1) having a molecular weight of ~560 kDa [18]. This makes it the largest ion channel described so far. The large molecular weight not only implies a large size, the dimensions of the tetramer are 29 nm×29 nm×12 nm (length × width × height), but also looms ahead the possibility of having several endo- and exogenous regulators. In fact RyR is known to be regulated by calcium, magnesium, ATP, inorganic phosphate and several attached proteins, just to name the most important present in a muscle cell. Subsequent sections will try to shed light on this intriguing molecule and its intertwining regulation.

Time course of SR permeability increase during excitation

Before the RyR was identified as the calcium release channel of the SR membrane pioneering work with calcium sensitive dyes explored the changes in $[Ca^{2+}]_i$ in isolated muscle fibres [31]. These calcium transients were in turn used to determine the flux of calcium through the SR membrane [19] during muscle excitation (*calcium release rate*). Although depletion of calcium in the SR does occur during a long stimulus [54] the calcium release rate essentially reflects the depolarisation-induced increase in SR permeability [9], that is, the opening of the RyRs. This method thus provides a convenient tool for studying RyR function *in situ* (Fig. 1).

Abbreviations used in this paper:
$[Ca^{2+}]_i$, myoplasmic calcium concentration;
CaMKII, calmodulin dependent protein kinase;
CICR, calcium-induced calcium release;
DHPR, dihydropyridine receptor;
ECC, excitation-contraction coupling;
EC_{50}, half activatory concentration;
FKBP, FK506 binding protein;
IC_{50}, half inhibitory concentration;
jTT, junctional T-tubule tetrad;
MH, malignant hyperthermia;
P_i, inorganic phosphate;
PK, protein kinase;
RyR, ryanodine receptor;
SR, sarcoplasmic reticulum;
T-tubule, transverse tubule.

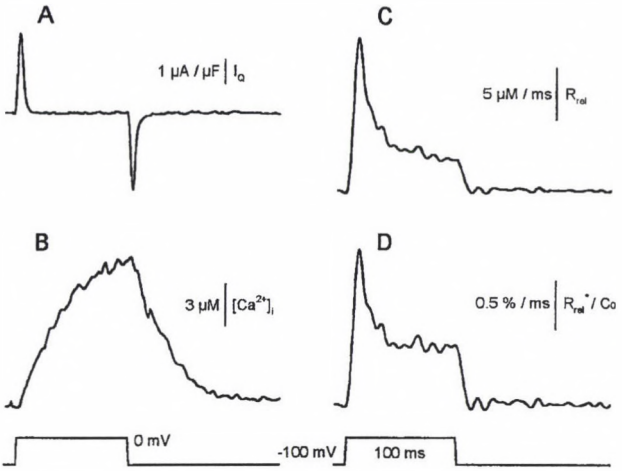

Fig. 1. Steps in ECC measured on a voltage clamped isolated skeletal muscle fibre mounted into a double Vaseline-gap system. (A) Non-linear capacitive current representing intramembrane charge movement (I_Q). Upward deflection from baseline (first 10 ms) corresponds to positive current. Ionic currents through the surface membrane were blocked using non-permenant ions – tetraethylammonium and methane sulphonate in the extracellular while Cs^+ and glutamate in the intracellular solutions – and ion channel blockers. Capacitive currents accompanying depolarising and hyperpolarising pulses were measured and the latter was subtracted from the former after appropriate scaling. (B) Changes in $[Ca^{2+}]_i$ associated with a depolarisation to 0 mV for 100 ms. The metallochromic calcium indicator Antipyrylazo III and the fluorescent calcium probe Fura-2 were introduced into the myoplasmic space. The fibre was transilluminated using red (λ>600 nm) and epi-illuminated with near UV light (λ=358 or 380 nm). The changes in fibre absorbance and fluorescence associated with calcium binding were measured simultaneously at 510, 720 and 850 nm. (C) The calculated calcium release flux (R_{rel}) through the SR membrane. First the parameters of the intracellular calcium binding sites and the transport properties of the SR Ca^{2+}-ATPase were determined from the declining phase of the calcium transient following repolarisation. This in turn enabled the determination of the amount of calcium leaving the SR, the first derivative of which gave the release flux. Note that the slow decline near the end of the pulse is due to the depletion of calcium in the SR. (D) Changes in SR permeability (R_{rel}*/C_0). The record in C was corrected for depletion (R_{rel}*) and normalised to the calcium content (C_0) that was present in the SR prior to the pulse to give the actual permeability of the SR membrane to calcium. The values were expressed as $\%ms^{-1}$, indicating what percent of the calcium stored in the SR left it in one millisecond

Using 100 to 200 ms long depolarising pulses SR permeability was shown to undergo a rapid rise followed by a fast decline to a quasi-maintained steady level [42]. The early increase represents the depolarisation-induced opening of the RyRs, while the fast decline is the inactivation of the calcium release channels [53]. To understand the functioning of the RyRs in a working muscle the mechanisms that connect the depolarisation to channel opening, as well as its regulation by endogenous ligands – including proteins, ions and small molecules – must be examined in detail.

In the followings first the details of this control are discussed and then the current understanding of the steps involved in excitation-contraction coupling (ECC) are outlined.

How does excitation lead to the opening of RyR?

With the intense research many of the molecular interactions of ECC, a term introduced almost 50 years ago for the events between membrane excitation and the production of force, are now clearly understood. One of the few still not completely resolved mysteries is how the depolarisation of the surface and T-tubular membranes leads to the opening of the calcium release channels located in the adjacent but morphologically distinct SR membrane.

There are, however, clues. Shortly after the idea of *gating charge* for voltage gated ion channels was established it was suggested that a molecule with permanent charges in the T-tubular membrane should serve as a *voltage sensor* for ECC, as well. A current that might reflect the conformational change of the voltage sensor was indeed found [52] and the term *intramemb rane charge movement* was introduced. A large number of studies have since linked intramembrane charge movement to the initiation of events subsequent to membrane depolarisation [26, 49]. Pharmacological investigations also demonstrated the dihydropyridine sensitivity of intramembrane charge [47].

The final identification of the molecule serving as the voltage sensor came from genetic studies. A lethal mutation in mice – *muscle dysgenesis* – was shown to result from the lack of transmission of information from the T-tubular membrane to the RyR. Dysgenic myotubes in primary culture do not contract upon electrical stimulation and have a reduced amount of intramembrane charge. Further investigations revealed that the $\alpha 1$ subunit of the L-type calcium channel (*dihydropyridine receptor*, DHPR) is missing in these animals [64]. Furthermore, the injection of cDNA-s encoding the $\alpha 1$ subunit into dysgenic myotubes restored ECC in parallel with intramembrane charge [1]. The involvement of a voltage gated calcium channel in the early stages of ECC – hence the similarity with cardiac cells – raised the possibility of calcium ions being the link between the T-tubular depolarisation and the opening of RyR in skeletal muscle, too.

Calcium-induced calcium release (CICR)

Work in the late 1960s demonstrated that raising $[Ca^{2+}]_i$ induces the release of calcium from the SR of skinned (sarcolemma removed) skeletal muscle fibres [15, 16]. This observation has since been confirmed by several other groups, using a number of

different techniques, including isolated SR vesicles. Although the $[Ca^{2+}]$ required to induce release is too high compared to the global $[Ca^{2+}]_i$ in the myoplasm during physiological activation the local calcium in the restricted space between the T-tubular and SR membranes (junctional space) might easily reach the necessary level.

Calcium entering through the DHPR-s functioning as voltage gated channels has, however, been ruled out in skeletal muscle [2]. Not only is the opening of the channel too slow to account for the fast activation of calcium release but removing extracellular calcium with EGTA or blocking its entry with inorganic cations (Cd^{2+}, La^{3+}) did not alter the ability of skeletal muscle fibres to contract.

The idea that calcium ions might regulate channel opening under physiological conditions, nevertheless, remained open. We have shown that the microinjection of strong calcium buffers – BAPTA or Fura-2 – alter the kinetics of calcium release [9]. Others have since confirmed that if the concentration of the introduced buffer reaches several millimolar the rise in $[Ca^{2+}]_i$ during a depolarising pulse is less than 0.1 μmol/l and the amount of calcium released from the SR is suppressed, indicating the presence of CICR [27]. Although the magnitude of the suppression was questioned [25], the idea that calcium ions leaving the SR open additional RyRs gained wide acceptance.

Mechanical coupling

Together with the description of intramembrane charge movement a simple model was proposed for the interaction of the voltage sensor and the calcium release channel: a mechanical plunger [5]. In this framework the depolarisation would induce such a conformational change in the molecule responsible for voltage sensing that it simply moves away from the pore of the RyR.

Electron microscopic measurements provided a structural basis for the mechanical coupling. Electron dense structures (*feet*) were shown to bridge the gap between the T-tubules and the SR at the level of the triads [18] (Fig. 2). These were later identified with the cytosolic parts of the RyR. Studies on the swimbladder muscle of the toadfish revealed structures with fourfold symmetry both in the T-tubular and in the SR membranes. The former proved to be four DHPRs organised into a, so-called, *junctional T-tubule tetrad* (jTT) while the latter is the RyR with its homotetrameric assembly. Furthermore, comparing the distance between adjacent jTT-s in the T-tubular wall to that between neighbouring RyRs in the SR membrane revealed a clear pattern: every other RyR was facing a jTT [18].

This observation, supported by data from microinjections (see above) and recent description of the calcium dependence of the elementary calcium release events (*sparks*), led to the proposal of a *dual control* for the activation of SR calcium release [48].

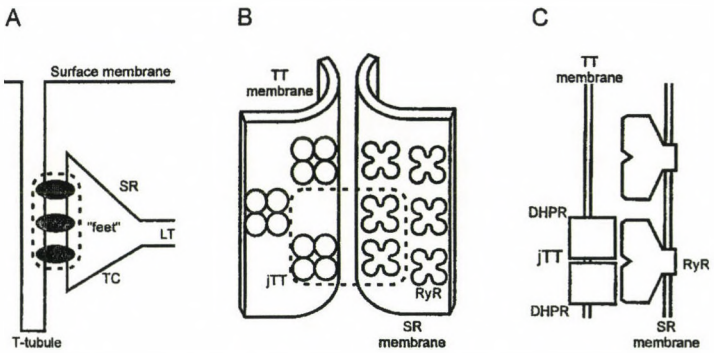

Fig. 2. Structural basis for the "dual control" hypothesis of ECC in skeletal muscle. Areas circled with dashed lines are shown enlarged on the consecutive panel. (A) Cross-section of a skeletal muscle fibre showing the localisation of the "feet"-structures. (B) The relative distribution of the jTT-s (four assembled DHPRs) and the RyRs in the triad. (C) The close association of the DHPR and RyR complex bridging the gap between the T-tubular and SR membranes and thus forming the "foot". Note that only every second RyR faces a jTT

In this framework RyRs facing jTTs would be opened by a direct mechanical interaction with the DHPR-s whereas non-coupled RyRs would be activated by calcium ions released through neighbouring channels. Molecular biological studies have since identified the putative domains on the DHPR that interact with the RyR (see section on DHPR below).

It should be noted, however, that most of the evidence supporting the dual control came from measurements on amphibians. Studies on mammalian skeletal muscle, although much less numerous, do not seem to unquestioningly support such a mechanism.

RyR isoforms in skeletal muscle

RyR isoforms have been identified in a number of species including invertebrates [59]. The proteins must have appeared early in the evolution since they have been described (using Western analysis, [^{3}H]ryanodine binding or through effects of ryanodine on function) form Echinoderms and Nematodes through Mollusks to Insects and Crustaceans. However, the homology between invertebrate and vertebrate RyRs is relatively low (less than 50% similarity of *Drosophila* RyR with mammalian isoforms). Furthermore, the regulation of RyRs, expressed in a number of invertebrates, appears to be different from those in vertebrates. The subsequent sections thus focus on RyRs present in the striated muscle of vertebrates in which four different RyR isoforms have been cloned and sequenced.

Distinct role for RyR1 and RyR3 in mammalian skeletal muscle

The major RyR isoform in mammalian skeletal muscle is RyR1. While adult fast twitch muscles contain RyR1 developing fibres also express RyR3 [66]. In slow twitch muscles and in the diaphragm substantial RyR3 expression remains after development. Although the exact role of RyR3 in muscle development remains to be elucidated certain characteristic features might be linked to the presence of RyR3.

In cardiac muscle, where CICR is the mechanism for calcium release channel opening, elementary calcium release events, *calcium sparks*, were identified [6]. Interestingly, similar events were absent in adult skeletal muscle [56] but were readily observed in developing skeletal muscle [7]. Furthermore, myotubes of RyR3 knock-out mice did not show calcium sparks unless the cDNA encoding RyR3 was injected and RyR3 expressed. These preliminary observations imply that RyR3 might be involved in CICR in skeletal muscle.

There are indications that a number of RyR related proteins are also expressed in these tissues [59]. Some might be alternative splice variants (a second transcription initiation site has been described on the *ryr1* gene) or their connection to RyRs remains to be established (as for a 106 kDa protein isolated from skeletal muscle that forms calcium channels and binds ryanodine).

The α and β isoforms of lower vertebrates

Most non-mammalian skeletal muscles of vertebrates express two RyR isoforms, termed α-RyR and β-RyR, at similar levels [59]. Their amino acid sequences resemble to RyR1 and RyR3, while their regulation with calcium to RyR1 and RyR2, respectively. In contrast, only a single isoform, identified as α-RyR, was found in muscles capable of very fast contractions (e.g. toadfish swimbladder, toadfish extraoccular, rattlesnake rattler muscle) [44].

Together with two RyR isoforms calcium sparks were also observed in amphibian striated muscle. Interestingly, the general characteristics of these elementary calcium release events did not differ from those observed in cardiac muscle. Nevertheless, their occurrence was under the tight control of membrane voltage indicating that a voltage sensitive and a subsequent calcium sensitive mechanism might be the underlying phenomena giving further support for the dual control of SR calcium release in these animals.

The above observations, together with the close resemblance of α-RyR and RyR1, the isoform of mammalian fast twitch muscles, suggest that faster contractions favoured a single RyR isoform during evolution.

Proteins that alter RyR function

The observation that two RyRs in the SR are 32 nm-s apart (centre to centre) hardly leaves space for anything else in the triadic junction. Nevertheless, the number of identified proteins localised to this area is still increasing. Although some are only structural, many have been shown to interact in one way or another with RyRs or may have important regulatory functions as glycolytic enzymes that could produce ATP (see below for the action of ATP on RyR). The most important, or most extensively studied junctional regulatory protein of RyR is, undoubtedly, the DHPR.

The T-tubular DHPR

As mentioned above both morphological and functional evidence support that the T-tubular DHPRs and RyRs interact in skeletal muscle. Experiments on transgenic mice have placed the possible interaction site on the intracellular loop connecting the II and III repeats (II–III loop) of the $\alpha 1$ subunit of the skeletal DHPR.

Synthetic peptides with the amino acid sequence identical to parts of the II-III loop have been shown to interact with the RyR under a number of different experimental conditions. The first such peptide containing 126 amino acids (from Glu^{666} to Leu^{791} of the II–III loop) was shown to increase the open probability of the isolated RyR reconstituted into planar lipid bilayers [35]. The effect seemed to be specific for RyR1 since RyR2 was not affected. Subsequent experiments revealed that the phosphorylation of Ser^{687} interfered with the ability of the peptide to activate RyR1. The actual binding sites were localised to two subfragments of this original peptide, to peptide A (Thr^{671} to Leu^{690}) and peptide C (Glu^{724} to Pro^{760}). While the former activates RyR1 the latter inhibits the activating effect of peptide A [13]. It was thus proposed that in a resting muscle peptide C interacts with RyR and prevents its opening. Upon depolarisation the inhibition is removed and the activation of the II–III loop comes into prominence.

However, attempts to chemically cross-link DHPR and RyR have not been successful so far. This observation initiated a number of investigations to find a protein that would interact with both DHPR and RyR and thus serve as a link between the two (see Cytosolic and SR associated proteins).

FK506 binding protein

FK506 and rapamycin are well-known immunosuppressant drugs. In skeletal muscle a 12 kDa protein has been identified as their cytosolic binding site (FK506 binding protein, FKBP). FKBP was shown to be closely associated with RyR in a 4:1

molar ratio suggesting that each monomer has a bound FKBP [38]. The dissociation of FKBP from RyR with FK506 results in the appearance of distinct subconductance states. The presence of FKBP on RyR prevents the occurrence of these substates [67] and also blocks the "reverse" (from cytoplasm to lumen) flow of calcium without affecting the movement in the physiological (from lumen to cytoplasm) direction [37]. FKBP thus acts as a stabiliser of the calcium release channel.

Cytosolic and SR associated proteins

Triadin is a major integral protein of the SR membrane at the level of the triads. It was demonstrated to bind to both RyR1 and the α1 subunit of the DHPR [4]. This led to the proposal that triadin might be the link between these two key proteins of ECC. Recent studies with fusion protein affinity chromatography revealed that triadin interacts with calsequestrin [22], the major calcium binding protein in the lumen of the SR. It was thus postulated that rather than coupling DHPR to RyR triadin anchors calsequestrin to RyR. This interaction would bring the calcium stored in the SR close to its release site.

Calsequestrin is a moderate affinity calcium storage protein with the capability of binding up to 40 mol of Ca^{2+} per mol of protein. Since the release of calcium from the SR is preceded by an increase in intraluminal $[Ca^{2+}]$ it has been suggested that RyR activation induces the release of calcium from calsequestrin [20]. The concept of interaction was further strengthened with demonstrating that the presence of calsequestrin activates the isolated RyR [61]. Phosphorylation of calsequestrin interfered with its ability to activate the channel providing a possible regulatory step [61].

A cytosolic calcium binding protein, *calmodulin*, has also been described to interact with RyRs. In the presence of micromolar $[Ca^{2+}]_i$ calmodulin reduces calcium release by decreasing the mean open time of the channel [58]. Since the inhibition occurred in the absence of ATP and between isolated proteins it involves a direct calmodulin-RyR1 interaction. Indeed, several putative calmodulin binding sites have been identified on the calcium release channel protein. Calmodulin, however, might also modify channel activity via calmodulin dependent protein kinases (CaMKII).

Protein kinases

Phosphorylation of ion channels is a widely spread mechanism to regulate their gating behaviour. Several exogenous protein kinases (PK), including PKA, PKC, PKG and CaMKII have been shown to phosphorylate the isolated RyR1. Phosphorylation increases channel activity that seems to be very specific since dephosphorylation by

protein phosphatase 2A reduces channel activity to pre-phosphorylated levels. Although several putative phosphorylation sites have been identified on RyR1 the serine residue at position 2483 seems to be the primary target for all protein kinases [38]. At the moment there is no consensus on the level of RyR1 phosphorylation *in situ* or if endogenous kinases play any role in the regulation of channel activity. PKC, however, is associated with junctional T-tubular membranes making it a possible candidate for regulating RyR function under physiological conditions.

Endogenous regulators of RyR

The number of different ions and molecules that alter RyR function is too large to include them all into this short overview. Only the most important or best understood effects are listed in this and in the subsequent section. For further information several recent reviews should be consulted [60, 72].

The effects of Ca^{2+} and Mg^{2+}

The physiologically occurring Ca^{2+} and Mg^{2+} have major importance in the regulation of RyR [41]. Ca^{2+} is thought of as the "physiological" activator since other ligands cannot activate the channel in its absence or they require calcium to exert their maximal effect. The calcium concentration dependence of channel activation is bell-shaped: micromolar [Ca^{2+}] (in the absence of Mg^{2+} and ATP) activate the channel, concentrations higher than 0.1 mmol/l inhibit channel opening [29, 40]. To explain these observations the concerted action of two independent calcium binding sites on the RyR have been proposed. A high affinity Ca^{2+} binding site (EC_{50} of approximately 2 μmol/l and Hill coefficient close to two) would be responsible for the activatory, while a low affinity site (IC_{50} around 150 μmol/l and Hill coefficient of >1) for the inhibitory effects [40]. Although the actual calcium binding sites have not yet been identified several putative regions have been suggested [71]. It should be noted that the different mammalian RyR isoforms have different affinities for calcium, the low affinity binding site on RyR2 has been reported to have at least an order of magnitude higher IC_{50} than that on RyR1.

Apart from data on isolated RyR, Ca^{2+} was shown to modify the calcium release channel *in situ*. In myotubes expressing RyR1 and the cardiac isoform of the α1 subunit of the DHPR calcium influx through the surface membrane can induce the release of calcium from the SR [65]. Furthermore, as we have shown, introducing high affinity calcium buffers into the myoplasm suppresses calcium release.

Unlike calcium, Mg^{2+} has only inhibitory effects on the isolated RyR [3, 29, 40]. IC_{50} was found to be around 20 μmol/l in the presence of 1 μmol/l, and around 100 μmol/l in the presence of 10 μmol/l $[Ca^{2+}]_i$ with a Hill coefficient greater than one, indicating a co-operative binding [39]. Not only calcium alters the affinity of RyR for Mg^{2+} but depolarisation also increases IC_{50} by more than 10-fold [33]. On the molecular level Mg^{2+} is believed to bind to both Ca^{2+} binding sites [8]. It seems to mimic the effect of Ca^{2+} on the low affinity inhibitory site, whereas its binding to the activatory site keeps the channel closed. At physiological $[Mg^{2+}]_i$ an approximately 10 μmol/l calcium concentration is needed to out-compete Mg^{2+} on the activatory site.

Small molecular weight compounds that affect RyR

ATP, with an EC_{50} around 1 mmol/l and a Hill coefficient close to two, is a potent activator of RyR [3, 40]. In fact both Ca^{2+} and ATP are required for maximal activation of the channel. It is thus believed that the presence of ATP is essential for RyR1 to function at the high $[Mg^{2+}]_i$ found in skeletal muscle. The effect of ATP is not exerted through phosphorylation since other adenine nucleotides, including non-hydrolysable analogues, can activate RyR while non-adenine nucleotides are ineffective [39]. An endogenous metabolite of nicotinamide-adenine dinucleotide, *cyclic adenosine diphosphate-ribose*, can also mobilise calcium from intracellular stores in many cell types [34]. However, a direct action on RyR1 under physiological conditions seems unlikely [23].

Endogenous polyamines such as spermine, spermidine and putrescine inhibit SR calcium release [45]. From the three polyamines spermine was found to be the most potent with an IC_{50} between 10–100 μmol/l. Single channel studies suggest that polyamines enter the channel pore and compete with the current-carrying ions. Since the IC_{50} value is close to the actual concentration of spermine in the cytosol this modulation of calcium release might have physiological relevance [70].

Long chain *acyl carnitines* such as palmityol carnitine induce SR calcium release [14]. This activation has an EC_{50} around 10–15 μmol/l rendering the physiological implications uncertain [12]. On the other hand the sphigolipid derivative *sphingosine* inhibits calcium release in skeletal muscle.

Pharmacological modulators of RyR

The exogenous ligand that should be considered first is *ryanodine*. It was first marketed as an insecticide (*Ryania* wood) and is still available for such purposes. Ryanodine and related compounds have complex effects on RyRs [60]. In low

concentration (<1 μmol/l) ryanodine activates the channel while in higher concentration it inhibits calcium release. The high affinity (EC_{50} between 1 and 10 nmol/l) binding occurs to the open conformation of the channel and in most cases locks the channel in a permanently open, half conducting state. These properties of ligand binding have proven useful in studying RyRs. The former permits [^{3}H]ryanodine to be used as an index of channel activation while the latter is a signature of a ryanodine-modified channel thus identifying it as an RyR. The low affinity binding has no state preference and produces a closed state of the channel. At present there is no consensus on the relationship of the two binding sites.

Methylxanthines are a large group of chemically related compounds that enhance calcium release. The most extensively studied and recognised representative of this group is undoubtedly *caffeine* [24]. Caffeine at low (0.5 to 2 mmol/l) concentrations sensitises RyR to calcium resulting in a dramatic increase in the open probability of the channel at micromolar $[Ca^{2+}]_i$. Higher concentrations (>5 to 10 mmol/l) of the drug can stimulate the channel even in the presence of picomolar $[Ca^{2+}]_i$, explaining its ability to cause a contracture. Channels activated by caffeine retain their ryanodine and ATP sensitivity and can be blocked by Mg^{2+} or ruthenium red. In intact fibres the effect of low concentrations of caffeine was to increase the calcium release flux from the SR [30]. Although this augmentation was present on both the early peak and on the maintained steady component, experiments carried out in our laboratory indicated a relatively more pronounced increase in case of the peak [32].

Several anions, including *inorganic phosphate* (P_i), perchlorate, thiocyanate, nitrate and vanadate but not sulphate or arsenate are capable of activating skeletal RyR. Their EC_{50} is in the millimolar region and due to similarity in their actions they are believed to interact with a common anion binding site [9]. Although the concentration in which P_i is present in muscle fibres is comparable with its EC_{50} the physiological significance of the effects of P_i on RyR awaits further studies. From among these anions only the effects of *perchlorate* on ECC were studied in depth. It was originally shown to affect intramembrane charge movement by shifting its voltage dependence to more negative voltages [36]. In explaining this effect of the drug a direct action on the DHPR and an alteration of the allosteric interaction between RyR and DHPR have been proposed [21]. In any case, perchlorate potentiates the contractile response in intact fibres by increasing the amount of calcium released from the SR.

Several polyamines and/or cationic proteins affect the RyR (see above for endogenous polyamines). The most commonly used representative of this group *ruthenium red,* a polycationic dye with 14 amino acids, is an effective inhibitor (IC_{50} in the range of 20–100 nmol/l) of calcium release [57]. It dramatically reduces the open probability of the channel by favouring long-closed events. The inhibition is practically irreversible and develops even on the ryanodine modified channel. *Aminoglycoside*

antibiotics were also found to inhibit calcium release from the SR [3, 45, 68]. In skeletal muscle neomycin and gentamicine display the highest affinity with an IC_{50} in the order of 50 and 200 nmol/l, respectively. Other members of the group include streptomycin, clindamycin and kanamycin with half inhibitory concentrations in the micromolar range.

Local anaesthetics, procaine and tetracaine, have been used in early experiments to block CICR. They have since been shown to block calcium release with an IC_{50} around 1 and 0.1 mmol/l, respectively, by reducing the open probability [69]. The reduction was the consequence of the appearance of long-closed events. Like perchlorate, *tetracaine* was originally shown to alter intramembrane charge movement by suppressing the delay or "hump" component [26]. Later work by others and us have linked this effect of the drug to the inhibition of SR calcium release [10]. A detailed analysis of tetracaine action on calcium release in intact fibres carried out in our laboratory revealed that tetracaine preferentially inhibits the peak component in amphibian striated muscle while it equally affects the peak and the steady part in mammalian muscle [11, 50].

As a postsynaptic muscle relaxant *dantrolene* is the drug used primarily in the treatment of malignant hyperthermia. It inhibits calcium release in a concentration close to the therapeutic range (10 μmol/l) [16]. However, its effects on isolated calcium release channels have been contradictory, and recent reports favour a dantrolene binding site that interacts with but is distinct from the RyR.

Activation of SR calcium release: a possible course of events

Based on the current knowledge of the regulation of RyR considered in the preceding sections we can now try to summarise the events that connect the depolarisation of the T-tubular membrane to the increase in $[Ca^{2+}]_i$.

At rest calcium release is suppressed

In an unstimulated fibre the resting $[Ca^{2+}]_i$ is 0.1 μmol/l, the concentration of free ATP is 0.5 mmol/l, while that of free Mg^{2+} is 1 mmol/l (the values are approximate). This calcium concentration, taken together with the concentrations of the other two ligands, is too low to activate the RyRs to a considerable degree since the activatory calcium binding sites are essentially calcium free. There is, nevertheless, a small but constant leak of calcium from the SR under these conditions which is less than 1% of the maximal flux achieved during excitation. The leak is compensated by the reaccumulation of calcium into the SR via the Ca-ATP-ase.

It should be noted that since the T-tubular membrane is polarised, the resting membrane potential is around –85 mV, the DHPRs are in their resting state. Therefore, the inhibiting part of the II–III loop (presumably in the region of Glu^{724} to Pro^{760}) is attached to the facing RyR. This interaction keeps the affinity of RyR for Mg^{2+} sufficiently high and thus contributes to the low open probability of the calcium release channels. Under physiological conditions the inhibitory effect of the DHPR together with the high $[Mg^{2+}]_i$ is enough to prevent spontaneous contractions from occurring in striated muscle. Lowering $[Mg^{2+}]_i$ below 0.1 mmol/l results in oscillatory contractions even in the absence of electrical stimulation [28].

Depolarization initiates the release of calcium from the SR

Upon excitation the T-tubular membrane is depolarised and the DHPR changes its conformation (this conformational change can be detected as intramembrane charge movement). The inhibitory part of the II–III loop, attached to the adjoining RyR at rest, is replaced by the activatory region (Thr^{671} to Leu^{690}) and the calcium release channel is opened. The underlying sub-molecular changes probably involve both a direct activatory effect of the DHPR and a decrease in the affinity of RyR towards Mg^{2+}. Recent evidence from our laboratory suggests that the conformational change of the DHPR does not inevitably result in the opening of the adjacent RyR [32]. Since each of the monomers of RyR binds an FKBP under physiological conditions sub-conductance states, presumably, do not occur.

Calcium ions leave the SR through the open channels and appear in the junctional space. The $[Ca^{2+}]$ increases rapidly reaching several tens of micromol/l in a couple of milliseconds. Due to the increased $[Ca^{2+}]$ calcium will bind to the regulatory sites on the RyRs. This binding could have several effects depending on which binding site – activatory or inhibitory – becomes occupied and whether calcium is bound to an already open or a still closed RyR. Let us first consider the case when calcium binds to the activatory site (this in fact happens earlier due to the higher affinity and faster kinetics of these sites).

If the calcium release channel is closed the binding of calcium to the activatory site should induce the opening of the channel and initiate additional calcium release (CICR). Although this mechanism seems straightforward, only circumstantial evidence support its existence in skeletal muscle (see above). Nevertheless, the presence of two RyR isoforms in lower vertebrates together with the peculiar organisation of jTTs and RyRs led to the suggestion that calcium released through DHPR-gated channels (presumed to be α-RyR) would activate the neighbouring, non-coupled RyRs (dual control). There is no evidence yet to decide whether calcium binding to a DHPR-activated channel would further increase the permeability of the channel, or not.

Similarly, it awaits further investigation to prove if CICR is functional in striated muscles with a single RyR isoform as fast mammalian skeletal muscle fibres.

Not only CICR functions as a positive feed-back in ECC. Several laboratories, including ours, presented evidence that calcium ions released from the SR modify intramembrane charge movement [10, 63], presumably by binding to the DHPR itself. If this calcium binding occurs to DHPRs which have not yet changed their conformation it results in additional charge movement and subsequent opening of calcium release channels. This charge movement component, which is most prominent at intermediate depolarisations, was termed "hump" due to its peculiar shape evident on the current records [26].

Termination of calcium release is due to the deactivation and/or inactivation of RyR

Whether CICR is present or not, all events in the triadic junction discussed so far serve a single goal: to rapidly release calcium from the SR. Simple economic reasons, on the other hand, require calcium release to be terminated if enough calcium is released to initiate a twitch. Two independent mechanisms work together to stop the release of calcium from the SR.

Under physiological conditions the depolarisation – the action potential – is short. Repolarisation of the T-tubular membrane induces a conformational change in the DHPRs and the events that resulted in the opening of the adjacent RyRs are reversed. The DHPR-gated calcium release channels close, *deactivate*, which in turn results in the lowering of the $[Ca^{2+}]$ in the junctional space due to the diffusion of calcium away from the release sites. This, finally, decreases SR permeability to the resting value.

When a train of action potentials is delivered or a long-depolarising pulse is used the DHPR-gated calcium release channels stay open and the $[Ca^{2+}]$ remains high in the junctional space for a longer period. It is now time to consider the events that occur when calcium binds to the slow, inhibitory sites on RyRs. If these sites on RyR are occupied the channel enters an inactivated state. While the rate of inactivation is relatively fast, it occurs in around 10 ms, the recovery from inactivation might take hundreds of milliseconds. It should be noted that recent evidence question such simplified view of inactivation. Not only are the inactivation sites close to fully saturated by magnesium but inactivation seems to be directly linked to activation, e.g. only those channels can inactivate that were previously open.

As demonstrated in Fig. 1C & D SR permeability does not decline to zero during constant depolarisation indicating that some of the channels remain open even in the presence of high junctional $[Ca^{2+}]$. The physiological significance of this sustained release is to maintain $[Ca^{2+}]_i$ and, consequently, the force production during a tetanus despite the steady removal of calcium by the SR Ca-ATP-ase. It is yet unclear whether

the steady release is due to a discrete population of channels that never inactivate (e.g. those gated by the DHPRs) or it reflects a dynamic equilibrium.

SR permeability displays two distinct kinetic components

As demonstrated in Fig. 1 SR permeability first rises to an early peak and then declines to a maintained steady level during a depolarising pulse. After repolarisation SR permeability returns to its resting value. These kinetic components reflect the molecular events detailed above, the opening of RyRs by the DHPRs (and calcium?), the inactivation caused by binding of calcium to the inhibitory site and, finally, the deactivation of the channels. Examining the record in Fig. 1D reveals that the deactivation is faster than both the activation or inactivation of calcium release. Fitting single exponentials to the corresponding parts of the transient gave a time constant of 2 ms for deactivation while 5 and 8 ms for activation and inactivation, respectively, at 18 °C.

The time constants for activation and inactivation show clear voltage dependence, they both decrease with increasing depolarisation. The former simply arises from the voltage dependent rate of conformational change of the DHPRs. To understand the voltage dependence of the rate of inactivation one has to consider the fact that the calcium concentration in the triadic junction is greater at more positive voltages due to the activation of more RyRs. Assuming a simple model for inactivation where calcium first binds to the inhibitory site on the release channel which then changes its conformation and thus enters the inactivated state we were able to estimate the dissociation constant of the binding site and the rate of conformational change *in vivo* [51]. The obtained values, 150 μmol/l and 4 ms^{-1}, were close to corresponding data from isolated channels.

A convenient measure of the fraction of channels that underwent a calcium dependent inactivation is the ratio of peak to steady SR permeability. For a large depolarisation this calculates to be around three, indicating that at least two-thirds of the channels are inactivated by the end of a 100 ms long pulse. Since the junctional $[Ca^{2+}]$ is a monotonic function of the membrane potential one would expect the peak to steady ratio to be monotonic, as well. This, in fact, is not the case for amphibians where the ratio has a distinct peak at intermediate voltages [55]. On the other hand, fast twitch mammalian fibres have constant peak to steady ratio in line with expectations [55, 62]. This characteristic difference has been suggested to reflect the presence of the two RyR isoforms in amphibian skeletal muscle.

The two components of SR permeability might be liked to alternative activation pathways

Considering the morphological evidences for the dual control and the possibility that the peak and steady permeabilities might reflect the activation of different channels an interesting hypothesis emerges. The steady component of SR permeability could reflect the opening of those channels that are linked to DHPRs, while the peak is generated by channels that are opened by the released calcium [48]. Although there is no direct evidence to support the theory several observations are consistent with this idea.

As mentioned earlier, the injection of strong calcium buffers suppresses SR permeability. We have shown that this suppression preferentially eliminates the peak component with essentially no effects on the steady part [9]. Pharmacological differences between the two kinetic components have also been described. Tetracaine, although affects both components, suppresses the peak at lower concentrations resulting in a decreased peak to steady ratio [46, 50]. Cardiac glycosides, on the other hand, increase the ratio by preferentially augmenting the peak component [51].

It should be noted, however, that the above effects were derived from measurements on amphibian skeletal muscle. In fact, tetracaine has no effect on the peak to steady ratio in mammals at concentrations where the suppression of calcium release is already 50% [11]. Recent evidence from our laboratory indicates a substantial potentiation of calcium release by perchlorate in mammalian fibres without any significant alterations in the peak to steady ratio [32]. However, caffeine together with increasing SR permeability increased the peak to steady ratio, as well [32].

Taken together, these data suggest that the DHPR gated calcium release channels contribute primarily to the steady component, but some contribution in amphibians and a large contribution in mammals to the peak should also be considered.

RyR associated diseases

Two inherited skeletal muscle disorders, malignant hyperthermia and central core disease, have been linked to alterations in RyR1 [43]. *Malignant hyperthermia* (MH) is characterized by uncontrolled contractures, hypermetabolism, hyperkalemia, cardiac arrhythmias and hyperthermia. The symptoms are triggered by volatile anaesthetics, as halothane, and can be fatal if not treated immediately (usually with dantrolene). The underlying reason for the above symptoms is an abnormal sensitivity of RyR to stimuli that induce the release of calcium from the SR (in clinical tests for MH susceptibility the threshold concentration for caffeine and halothane induced contractures is determined).

Although relatively few data are available from humans a similar disease in pigs, the porcine MH syndrome, has been studied in detail. This revealed a point mutation on RyR1 (Arg615Cys) associated with altered Ca^{2+} dependence of channel gating. Similar mutation has been found in humans (Arg614Cys), however, it only accounts for approximately 5% of mutations found in families with MH. Other mutations on RyR1 have been described and these probably represent additional forms of MH.

Central core disease is associated with the formation of discrete zones devoid of oxidative enzymes resulting in muscle hypotonia and weakness. Genetic analysis has revealed at least four different mutations on RyR1.

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Distribution of cadmium in selected organs of mice: Effects of cadmium on organ contents of retinoids and β-carotene

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Cadmium was administered to 32 adult ICR mice i.p. in two single doses (0.25 and 0.5 mg $CdCl_2$ per kg of b.w.). After 48 hours concentrations of cadmium in kidneys, liver, spleen, muscle (*m. quadriceps femoris*), ovaries and testes and the concentration of retinyl palmitate, retinol and β-carotene in kidney, liver and testes were determined. Significantly higher cadmium concentration was found in liver, kidney and ovary in both experimental groups in comparison with the control group ($p<0.001$). In muscle, spleen and testis the cadmium level was higher, however not significantly. No significant differences in the concentration of retinyl palmitate, retinol and â-carotene in liver were found. Concentration of â-carotene in kidney and testis was significantly decreased in both groups administered with cadmium ($p<0.001$). Concentration of retinyl palmitate was significantly lower in testis in the group with higher cadmium level ($p<0.001$) and the concentration of retinol significantly decreased in kidney and testis of mice after an administration of 0.5 mg $CdCl_2$/kg b.w.

Keywords: cadmium, accumulation, retinol palmitate, retinol, β-carotene, mice

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Concern about the constantly increasing environmental levels of heavy metals has stimulated interest in the study of toxic substances on life systems. Cadmium, one of these substances is a toxic, environmental pollutant that serves no biological function, and is therefore totally unwanted. Cadmium is chemically similar to zinc and occurs naturally with zinc and lead in sulfide ores. Some cadmium has been found in all natural materials that have been analyzed. High concentrations in air, water and soil are, however, commonly associated with industrial emission sources, particularly non-ferrous mining and metal refining [4].

Exposure to cadmium, via air and food, leads to renal tubular dysfunction. This is primarily a reabsorption defect in the proximal tubules and the critical effect of cadmium [4, 5, 13, 14]. There are also various effects on reproduction, causing follicular atresia [10], edematization of uterus [12] as well as degenerative alterations in testes [18]. Cadmium mainly accumulates in kidneys and liver [4, 9, 15, 16].

It is estabilished that vitamin A and cadmium are preferentially accumulated in the liver in high concentrations and that the metabolism of the vitamin is controlled in the liver [6]. This suggests the possibility of an effect of cadmium on vitamin A metabolism.

The aim of our work was to investigate the distribution of cadmium (kidney, liver, muscle, spleen, testes, ovary) after an intraperitoneal (i.p.) administration and determine concentration of retinyl palmitate, retinol and β-carotene in liver, kindey and testes in mice.

Materials and Methods

The experiments were conducted with 32 adult randombred ICR mice (Velaz Prague, Czech Republic) kept in plastic cages. Animals were divided into three groups (A, B, K). Mice in group A (11) were treated with a single i.p. cadmium dose of 0.25 mg $CdCl_2$ (Sigma Chemical Company, St. Louis, MO, USA) per kg body weight, and were killed 48 hours later. Animals (11) in group B received a single i.p. dose of 0.5 mg $CdCl_2$/kg b.w and were killed 48 hours after cadmium administration. The control group (K) consisted of 10 untreated mice. From all animals kidneys, liver, spleen, muscle (*m. quadriceps femoris*), ovaries and testes were collected. Concentrations of cadmium were determined by electrotermic atomic absorption spectrophotometry (Ekologické a veterinárne laboratóriá, Spišská Nová Ves, Slovak Republic, Unicam Solar 929). Aspiration method [4] of the ash solution with 1% HNO_3, and then read at 228.8 μm wavelength, following the Unicam AAS methods manual (1991) was used.

Concentrations of retinyl palmitate, retinol and β-carotene were measured in all (32) animals. Retinoid content (retinyl palmitate, retinol) of kidney, liver and testes was extracted by *n*-hexane containing antioxidant (50 mg/l BHT). The extract was injected onto Si-100 S 10 CN column (BST Ltd., Budapest, Hungary). The elution parameters were as follows: mobile phase *n*-hexane: methanol (99.5:0.5), flow rate 1.51 ml/min at 50 bar. The UV detection was carried out at 325 nm. Concentrations of β-carotene were measured in a similar arrangement but the detection was carried out at 450 nm. The peaks were identified by standard compounds: retinol (Sigma Chemical Company, St. Louis, MO, USA), retinyl palmitate (NBC, Cleveland, USA) and β-carotene (Merck, Darmstadt, FRG). The concentrations were determined from the peak-heights obtained by measuring the standard dilutions. From final data, basic statistical characteristics were calculated (mean, s.d.) and an analysis of variance by Sheffe's test was completed for each variable.

Results

After a single intraperitoneal administration cadmium mainly accumulated in the liver. This accumulation is dose dependent (Table I), and proved to be significant in comparison with the control group ($p<0.001$). In the kidney cadmium levels significantly increased similarly to the liver. When evaluating muscle (*m. quadriceps femoris*), the cadmium concentration was in control 0.125 mg/kg, higher in group A 0.144 mg/kg and the highest in group B 0.636 mg/kg. Low levels of cadmium were found in spleen (0.026–0.414 mg/kg). In ovary, cadmium concentration in control animals was under the detectable limit of 0.01 mg/kg, but a highly significant ($p<0.001$) increase after cadmium administration (1.835; 2.134 mg/kg, respectively) is reported. Very similar accumulation of cadmium has been observed in testis, and is dose dependent.

Determination of concentration of retinyl palmitate, retinol and β-carotene is listed in Table II. No significant differences were found in the liver, and the concentration of retinyl palmitate was 16.96–18.18 μg/g. In kidney, the level of retinyl palmitate was 15.05–16.81 μg/g. Retinol concentration significantly decreased only in the group with higher cadmium concentration – group B ($p<0.001$). Level of β-carotene was significantly decreased in kidney and testis in both Cd-treated groups ($p<0.001$). In testis all parameters were affected. Significant decrease of retinyl palmitate and retinol was found in group B ($p<0.001$) in comparison with the control group. Analysis of β-carotene showed significant decrease ($p<0.001$) in both groups with cadmium administration (0.85 and 0.41 μg/g, respectively) in comparison with control animals (1.48 μg/g).

Table I

Distribution of cadmium (mg/kg wet tissue) in mice

Organ	Group K	Group A	Group B
Liver	0.052 ± 0.008	4.196 ± 0.357*	8.360 ± 0.711*
Kidney	0.355 ± 0.030	2.382 ± 0.202*	2.709 ± 0.230*
Muscle	0.125 ± 0.011	0.144 ± 0.021	0.636 ± 0.145
Spleen	0.026 ± 0.002	0.026 ± 0.004	0.414 ± 0.035
Ovary	<0.010	1.835 ± 0.156*	2.143 ± 0.182*
Testis	0.333 ± 0.028	0.450 ± 0.038	0.738 ± 0.063

Group K – control, untreated mice (n=10); Group A – single i.p. dose 0.25 mg $CdCl_2 \cdot kg^{-1}$ (n=11); Group B – single i.p. dose 0.5 mg $CdCl_2 \cdot kg^{-1}$ (n=11)
*P<0.001, n = 32

Table II

Concentrations of retinyl palmitate, retinol and β-carotene in mice liver, kidney and testis (in μg/g)

Organ	Liver	Kidney	Testis
Group K			
RP	18.18 ± 1.85	15.05 ± 1.55	6.52 ± 0.78
ROL	26.39 ± 2.97	22.95 ± 1.98	8.21 ± 0.45
BC	1.95 ± 0.23	12.95 ± 1.33	1.48 ± 0.12
Group A			
RP	16.96 ± 1.55	16.81 ± 1.77	6.48 ± 0.78
ROL	15.61 ± 1.64	22.72 ± 2.23	8.27 ± 0.99
BC	3.19 ± 0.04	7.71 ± 0.77*	0.85 ± 0.09*
Group B			
RP	17.55 ± 0.97	16.44 ± 1.98	3.03 ± 0.54*
ROL	13.24 ± 1.35	16.95 ± 1.55*	5.24 ± 0.26*
BC	3.24 ± 0.22	4.94 ± 0.58*	0.41 ± 0.05*

RP = retinyl palmitate, ROL = retinol, BC = β-carotene (all values are expressed in μg/g)
Group K – control, untreated mice (n=10); Group A – single i.p. dose 0.25 mg $CdCl_2 \cdot kg^{-1}$ (n=11); Group B – single i.p. dose 0.5 mg $CdCl_2 \cdot kg^{-1}$ (n=11)
*P<0.001; n = 32

Discussion

Cadmium mainly accumulates in liver and kidney [2, 3, 4, 8, 9, 19, 20]. In control animals we have found higher cadmium concentration in kidney (0.355 mg/kg) than in liver (0.052 mg/kg). After single i.p. administration of cadmium we have observed higher cadmium levels in liver (4.196 and 8.360 mg/kg) than in kidney (2.382 and 2.709 mg/kg). This single exposure experiment has shown that initially, a very high proportion of the dose is found in the liver. With time there is a redistribution of cadmium from the liver to other tissues, particularly the kidneys as we found it in our earlier study in rabbits [4] and as it has been described by others [9]. This is probably due to an efficient metallothionein synthesis in the liver. Cadmium bound to metallothionein may subsequently be released into plasma, filtered through renal glomeruli and reabsorbed in the tubuli. This is of significance for human and animals in high exposure situations. Even if the daily exposure ceases, the renal cadmium concentration may be maintained for a long time or even increase if sufficient amounts of cadmium are stored in the liver.

We report dose dependent accumulation of cadmium in testis as well as alterations in retinyl palmitate, retinol and β-carotene concentration in testis. It has been reported that cadmium causes degeneration of the seminiferous epithelium [1, 7, 18]. All biochemical and physiological changes are known to occur in the testis at late time intervals following cadmium treatment are secondary to ischaemia rather than due to a direct effect of cadmium. Higher cadmium concentration also inhibits the motility of spermatozoa [11] and causes structural alterations of spermatozoa [7].

In this study significant decease of retinol (group B) and β-carotene (both experimental groups) in kidney and retinyl palmitate (group B), retinol (group B) and β-carotene (group A and B) in testis is reported. No significant differences were found in liver. β-carotene is a provitamin of vitamin A. Cadmium in a long-term study has been describe to induce significant decrease of serum vitamin A, while food intake and body weights remained unchanged [17]. Absorption of vitamin A from the intestine, the release of newly absorbed vitamin A from the liver to serum, and the conversion of vitamin A to water soluble metabolites in the liver were not influenced by cadmium. These findings suggest that cadmium interfered with the release of vitamin A, especially stored vitamin A, to serum [17].

There may be many ways to interfere with the excretion mechanism of vitamin A to the serum. For example, the direct binding of cadmium with specific proteins essential for vitamin A excretion as retinol binding protein, prealbumin or vitamin A hydrolase, or the decrease in these proteins by cadmium and the destruction of excretion canals of cell organelles.

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The influence of sound stimulation during hatching on the mortality of ducks

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In the work the influence of the artificial sound stimulation during incubation on the speed of the Pekin duck breed hatching as well as on their mortality during that period was observed. The set eggs were hatched in four hatcheries. The eggs of the first two hatcheries (control groups Ka and Kb) were not sound stimulated. In the other two groups the set eggs were, from the very first hour of hatching, stimulated by the "knocking" sound from an electronic sound generator (experimental groups "a" and "b"). For the stimulation, the acoustic signal with intensity of 25 dB and frequency 5.68 Hz was applied. The fastest hatching process was recorded in the groups with sound stimulation. The "a" experimental group was the fastest, the ducks were hatched after 640.75±10.15 hours, in the "b" experimental group the ducks were hatched after 656.50±1.70 hours of incubation.

In both sound stimulated experimental groups (a, b) the increased embryonic mortality resulted in the decrease of the percentage of hatchability - to 68.09±1.77 % and 76.44±2.68%, respectively. This is statistically evident ($P<0.01$) when compared with the respective control groups which reached the 88.81±1.86% and 89.35±0.81% hatchability.

Keywords: hatching, sound stimulation, ducks, mortality

Beakclapping is the first instinct that birds show. The number of sound signals produced by birds at beakclapping has been exactly determined. The problems of the application of a sound stimulation of the bird embryos was discussed by several authors [5, 7]. In experiments with the influence of a sound stimulation, the various authors have used variable time for beginning of stimulation. Martin and Moses [6] observed influence of a sound stimulation in the first three days of incubation. Glazev [4] started

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stimulation after 18.5 days of incubation, Veterány et al. [16] started stimulation after 19th day of incubation, Vince a Frances [18] after the 20th day of incubation. The usual way of stimulation was to use the tape recorders with recorded signal of mothers calling her youngs for food or the sounds of the youngs themselves. Some experiments showed that the youngs from the sound stimulated embryos beakclap from the eggs faster and their yolk sac gets absorbed faster as well, which decreases the number of infectious diseases [13]. Orcutt [8] show that the application of sound during hatching shortened the time needed for beakclapping. Vince [17] found that the sound stimulation shortened the incubation time in comparison with the groups, which were not sound stimulated during hatching.

The aim of our experiment was to determine the influence of the artificial sound stimulation from an adjustable electronic generator on the Pekin breed duck embryos. The eggs were stimulated by the "knocking" sound with intensity of 25 dB and frequency of 5.68 Hz (on oscillograph sound with amplitude of power 1250 mV and time interval 176 ms).

Materials and Methods

The Pekin duck breed of the parental group aged one year and of the fourth month of laying were used in the experiment. The two groups were created. In the first control group (Ka) and experimental group (a) were included the eggs weighing 81–85 g, while in the second control group (Kb) and experimental group (b) the weight of eggs was 86–90 g. The set eggs were hatched in the four BIOS MONO-06 type hatcheries. The eggs in both control groups were not stimulated by sound, while the eggs in the experimental groups were stimulated by acoustic signal from an electronic generator with intensity of 25 dB and frequency of 5.68 Hz. The sound stimulation in the experimental groups was applied immediately during the first hour of incubation and it lasted until the end of the process of hatching. Beginning of duck incubation was stated from inserting of eggs into temperatured hatchery (37.6 °C). As the end of incubation was stated time, when young duck completely leave the egg shell. The electronic sound generator loudspeakers were placed asymmetrically at the sides of the hatcheries. During hatching the following data were observed in the thirty-minute intervals: the beginning of beakclapping, the whole group beakclapping time, the whole group hatching time, hatchability and embryonic mortality.

The results given in the tables are based on four experiments. The results served for the calculation of the basic variative-statistical indicators. The differences between experimental and control groups as well as between experimental groups themselves were tested by the Student's *t*-test.

Results

The total results of the experiment with sound stimulation indicate that the ducks first started to beakclap after 629.00±9.92 hours in the experimental group "a", in which the set eggs weighing 81–85 g were applied (Table I). The whole group beakclapping time (11.75±1.68 hours) and the whole group hatching time (640.75±10.15 hours) were the shortest in this group as well. The differences were evident ($P<0.01$) when compared with control group "Ka", without sound stimulation, in which ducks started to beakclap after 649.00±1.62 hours, the whole group beakclapping time took 17.50±0.87 hours and ducks were hatched after 666.50±1.84 hours of incubation. Also in the second experimental group with sound stimulation, "b", the beakclapping started sooner than in the respective control group, i.e. after 641.63±1.60 hours of incubation ($P<0.01$), the beakclapping time was 14.88±1.29 hours, the hatching time was 656.50±1.70 hours, ($P<0.05$). The differences became evident when compared with control group "Kb", the weight of ducks being the same, i.e. 86–90 g, in which the beginning of beakclapping was recorded after 649.75±1.44 hours, the whole group beakclapping time was 16.38±0.96 hours, and ducks were hatched after 666.13±1.39 hours of incubation. In both experimental groups, e.g. in experimental group "a" the hatchability was 68.09±1.77% (in control group "Ka" being 88.81±1.86%), in group "b" the hatchability was 76.44±2.86% (in control group "Kb" it was 89.35±0.81%). The differences reached the force of evidence ($P<0.01$) when experimental groups were compared with respective control groups. A considerable increase of embryonic mortality as well as the occurrence of deformed ducks were recorded in the sound stimulated groups (Table II). The highest percentage of dead embryos with developed yolk sac blood circulation (5.64±1.81%), with alantochorionic blood circulation (3.43±2.66%), with an unresorbed yolk sac (6.88±3.69%), were recorded in the 81–85 g set egg weight experimental group. Also the highest number of dead embryos in the reverse position (4.43±2.63%) was recorded in this experimental group. The highest number of dead embryos in the irregular position (7.52±1.24%) was recorded in the 86–90 g incubated egg weight experimental group.

Discussion

The total results of the experiment with sound stimulation indicate that the duck first started to beakcleap the whole group beakclapping time and the whole group hatching time were the shortest in groups with sound stimulation. The observed data correspond with results of Orcutt [8] who found out that the sound stimulation

shortened the time needed for beakclapping of the whole group of chickens. Vince [17] also managed to shorten the incubation time in comparison with the groups which were not sound stimulated during hatching.

With the artificial sound stimulation the hatching process began sooner when the set eggs with lower weight were applied (81–85 g). This also confirms the findings of Sergejeva [11] who showed that under the same conditions of incubation the ducks from smaller eggs hatch faster. On the other hand, in the groups with no sound stimulation the time of hatching was for both weight groups very similar, the ducks from the heavier set eggs hatched, though, a little bit sooner than the other ones. This is confirmed also by the results arrived at by Smirnov et al. [14], Wilson [19], and Burke [1]. While the artificial sound stimulation had a very positive effect for the hatching of chickens (faster resorbtion of yolk sacs, considerable decrease of the infectious diseases of navel), as it is confirmed by the observations of Zajančkovskij [20], Veterány and Hluchý [15], in the case of ducks the artificial sound stimulation during hatching had a stressful effect [3, 12].

Table I

Indicators of duck eggs hatching

Indicators	Units	Ka 1st control group	a 1st experimental group	Kb 2nd control group	b 2nd experimental group
Weight of eggs	gram	81–85	81–85	86–90	86–90
Amount of incubated eggs	pieces	236	236	236	236
Average number of incubated eggs per each experiment	pieces	59	59	59	59
Beginning of beakclapping	hours	649.00 ± 1.62	629.00 ± 9.92^{++Kaa}	649.75 ± 1.44	641.63 ± 1.60$^{++Kbb+ab}$
All group beakclapping time	hours	17.5 ± 0.87	11.7 ± 1.68^{++Kaa}	16.38 ± 0.96	14.88 ± 1.29^{+ab}
Hatching time	hours	666.50 ± 1.84	640.75 ± 10.15^{++Kaa}	666.13 ± 1.39	656.50 ± 1.70$^{+Kbb+ab}$
Hatchability	percentage	88.81 ± 1.86	68.09 ± 1.77^{++Kaa}	89.35 ± 0.81	76.44 ± 2.86$^{++Kbb+ab}$

+P<0.05
++P<0.01

Table II

Indicators of mortality at ducks hatching

Indicator	Units	Ka 1st control group	a 1st experim group	Kb 2nd control group	b 2nd experiment
Weight of eggs	gram	81–85	81–85	86–90	86–90
Amount of incubated eggs	pieces	236	236	236	236
Average number of incubated eggs per each experiment	pieces	59	59	59	59
Infertile eggs	percentage	4.26±0.82	1.75±1.19	5.10±1.10	2.09±0.56
Dead embryos with developed yolk sac blood circulation	percentage	1.30±0.77	5.64±1.81	1.67±2.04	2.63±1.06
Dead embryos with alantochorionic blood circulation	percentage	1.25±1.38	3.43±2.66	0.50±0.87	2.05±1.83
Dead embryos with unresorbed yolk sac	percentage	2.68±2.20	6.88±3.69	1.18±1.27	5.76±2.84
Prolapse of cerebrum	percentage		0.76±1.31	0.42±0.72	1.42±1.64
Dead embryos in the reverse position	percentage	1.30±0.77	4.43±2.63	0.88±0.90	2.09±1.30
Dead embryos in the irregular position	percentage	0.42±0.72	$7.27\pm3.29^{+Kaa}$	0.50±0.87	$7.52\pm1.24^{+Kbb}$
Cyclop	percentage		0.92±0.92	0.42±0.72	
Shortened upper beak	percentage		0.83±1.43		

$+P<0.05$

While in our experiment we found out that the highest embryonic mortality occurred in the embryos of ducks in irregular position, Reddy et al. [10] observed the highest mortality for embryos in the reverse position with the head turned to the obtuse end of the egg. The results of the hatchability in both groups without sound stimulation correspond with the results given by Peter et al. [9]. The higher biological quality of eggs wears in higher hatchability in control groups [9].

The given data indicate that the artificial sound has a more negative effect on the hatching of ducks from the lower weight set eggs. In the higher weight set eggs the distribution of individual egg components is changed in favour of the white and shell, which results, in our opinion, in the greater sound isolation of the embryo and thus weaker perceptivity for the sounds coming from the outer environment.

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Changes in rat muscle with compensatory overload occur in a sequential manner

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The present study was initiated to determine the time course of changes in the profile of selected skeletal muscle myofibril proteins during compensatory overload. Whole muscle isometric contractile properties were measured to assess the physiological consequences of the overload stimulus. Compensatory overload of plantaris muscle of rats was induced by surgical ablation of the synergistic soleus and gastrocnemius muscles. Myosin light chain (LC) and tropomyosin (TM) compositions of control (CP) and overloaded plantaris (OP) muscles were determined by electrophoresis and myofibrillar ATPase assays were performed to assess changes in contractile protein interactions. Within one week of overload decreases in the α:β TM ratio and myofibrillar ATPase activity were observed. Following 30 days of overload, a transition in type II to type I fibres was associated with an increase in slow myosin LC_1. Interestingly, after 77 days of overload, the TM subunit ratio returned to one resembling a fast twitch muscle. It is proposed that the early and transitory changes in the TM subunits of OP, as well as the rapid initial depression in maximum tetanic isometric force and myofibrillar ATPase activity may be explained as a result of muscle fibre degeneration-regeneration. We propose that alterations in protein expression induced by compensatory overload reflect both degenerative-regenerative change and increased neuromuscular activity.

Keywords: muscle contraction, tropomyosin, myosin, myofibrillar ATPase, muscle damage

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Chronic changes in muscle function are accompanied by altered gene expression which results in a remodelling of muscle contractile protein constituents [5, 28]. Compensatory overload, following ablation of the synergistic gastrocnemius muscle, results in the eventual adaptation of the fast-twitch rat plantaris muscle to one which is more slow-twitch in character. The adaptation, which affects both metabolic and contractile systems, is assumed to reflect the increased role of the overloaded muscle in ambulation and postural maintenance [13].

Similar adaptive responses have been reported following chronic electrical stimulation of skeletal muscle in which it has been observed that the muscle transformation occurs in a sequential fashion [16, 28]. Early changes in oxidative enzyme activities and sarcoplasmic reticulum function are followed by alterations in the regulatory proteins and finally appearance of the slow myosin isoform [28]. Whether the selective increase in neuromuscular activation with compensatory overload is also accompanied by a sequential shift in the composition of various myofibrillar proteins is unknown. The degree to which changes in muscle composition represent an initial response as opposed to a chronic adaptation is also unclear. In the present investigation, an assessment of muscle contractile function, myosin and tropomyosin composition and myofibrillar interaction was employed to evaluate the time course of muscular adaptation to compensatory overload.

Materials and Methods

Subjects and experimental design

Male Sprague Dawley rats with initial body weights of approximately 250 grams were randomly assigned to eight groups including two control groups and six groups which underwent unilateral ablation of gastrocnemius and soleus muscles. The experimental protocol was approved by the University Council on Animal Care at the University of Western Ontario and animals were maintained according to the Principles of Animal Care, the Canadian Council for Animal Care. The six groups of animals on which surgery was performed were sacrificed at 2, 5, 7, 15, 21, and 30 days post-myectomy. One non-operated control group was euthanized at the start of the experimental period (0NC) and a second non-operated control group 30 days later (30NC). The two unoperated control groups were employed to account for the effects of animal growth over the initial 30 days of the experimental period. In addition, selected experimental procedures designed to examine the long-term adaptation of the muscle to overload were conducted 77 days postmyectomy. Rats were housed in individual wire mesh cages, maintained on a 12:12 h dark/light cycle and fed and watered *ad libitum*.

Surgery

Rats were anaesthetized with sodium pentobarbitol (60 mg/kg i.p.) for all surgical procedures. Supplemental doses were administered as necessary. Compensatory overload of the plantaris muscle (OP) was induced by complete ablation of the ipsilateral gastrocnemius as described by Ianuzzo and Chen [15]. The contralateral limb (CP) was subjected to a sham operation and served as the internal experimental control. Age matched rats in the 2 day and 30 day NC were not subjected to any surgical procedure.

On the appropriate day, rats from the designated group were anaesthetized and either prepared for contractile property measurements or killed immediately. Following death, the plantaris muscles were excised, trimmed of excess fat and connective tissue, weighed and prepared for subsequent analyses. For animals on which contractile properties were to be measured, both OP and CP muscles were exposed, freed from surrounding muscle and connective tissue and recordings were obtained sequentially and in random order. Each hind leg of the rat was fixed to an aluminum frame by a drill bit through the femur with a clamp on the tibia such that when the plantaris muscle was attached to the force transducer (Gould UC2), it was approximately perpendicular to the femur. Both the hind legs and the sciatic nerves were submerged in mineral oil maintained at 37°C.

Isometric contractile properties

Isometric contractile characteristics were measured according to Pettigrew and Gardiner [29]. Following a 10-minute temperature equilibration the plantaris muscle was stimulated to contract at 1 Hz by a supramaximal square wave stimulus (4 V with a duration of 0.1 ms) through the sciatic nerve. Muscle length was adjusted in small increments until maximal isometric twitch tension was achieved. The muscle length at which maximum isometric twitch tension was achieved was defined as the optimal length for force development (Lo). Once Lo was determined, and following a 3 minute rest period, the plantaris was stimulated with a 1 s train at 200 Hz. Maximal isometric tetanic force (N) was calculated from the peak amplitude of the 200 Hz tetanic contraction (Po-200) and normalized by muscle weight (N/g).

Myofibrillar ATPase activity

Skeletal muscle myofibrils were prepared using a modification of the Baldwin technique [3]. The modification included: 1. initial suspension of muscle in 20 rather than 10 volumes of homogenizing solution which contained 50 μM EGTA rather than 5 μM EGTA and 2. the initial centrifugation was at $10,000 \times g$ rather than $1000 \times g$. Isolated myofibrils were suspended in 150 mM KCl and 50 mM Tris at pH 7.4 and brought to a final concentration of 6 mg/ml. Protein concentrations were determined by the Lowry method [20].

Myofibrillar ATPase assays were performed according to Goodno et al. [8] with modifications as outlined by Roy et al. [32]. All ATPase assays were completed on the same day that the myofibrils were isolated. Samples containing 0.3 mg of myofibrillar protein were pre-incubated in the reaction medium (0.25 mM $CaCl_2$, 40 mM $MgCl_2$, 50 mM KCl, 2 mM Tris, 2 mM NaN_3) at pH 7.4 for 5 minutes at 30°C. Myofibrillar ATPase reactions were initiated by the addition of 0.1 ml of 30 mM NaATP and quenched with 2% SDS, 50 mM Tris (pH 7.2) and placed in an ice bath. The myofibrillar ATPase activity was determined from the amount of inorganic phosphate liberated during a 2 min reaction period [30].

Electrophoretic analyses

Electrophoretic separation of myosin light chains was performed on 12% SDS gels loaded with 75 μg of myofibrillar protein according to the procedure of Laemmli [18]. Gels were stained overnight in 1% Coomassie Brilliant Blue R-250, 25% (v/v) methanol, and 10% (v/v) acetic acid under the condition of mild agitation, followed by destaining in 40% methanol and 10% acetic acid. Densitometric analyses of the light chain composition were performed with the use of a LKB 2202 ultrascan laser densitometer. The proportion of each myosin LC isoform was expressed as a percentage of the total light chain content.

Analysis of the TM subunit composition was accomplished using muscle homogenates according to the procedure of Heeley et al. [11]. Following isoelectric focusing (pH 4–6), the TM isoforms were resolved in the second dimension on a 9–19% SDS linear gradient separating polyacrylamide gel with a 4% acrylamide stacking gel. The 2-D electrophoretograms were stained as described above. The amount of Coomassie Blue bound to the TM was assessed employing a procedure described by Ball [4]. Briefly, a 5 mm^2 area of the gel containing the protein band of interest was excised and the protein extracted in 50% isopropanol (v/v) and 3% sodium dodecyl sulphate (w/v) for 24 hours at 37°C. The relative protein content was spectrophotometrically determined at 595 nm.

Histochemical analyses

To estimate the per cent alkaline-labile (Type I) and alkaline-stable (Type II) fibres, muscle cross-sections of 10 μm thickness were stained for myofibrillar ATPase following an alkaline pre-incubation at pH 10.3 [9, 27].

Fibre-type populations were estimated by visually counting fibres from fasciculae in each of eight regions evenly distributed throughout the muscle cross-sections [14]. Dark-staining fibres were classified as alkaline-stable while light-staining fibres were counted as alkaline-labile. Fibres with an intermediate staining intensity for myofibrillar ATPase were recorded as alkaline stable.

In order to examine the gross morphology of the muscle, muscle cross-sections of 10 μm thickness were stained with Harris hematoxylin and eosin [12].

Statistical analysis

Statistical comparisons between groups were performed using both one and two-way analyses of variance. Upon determination of significant differences ($p \leq 0.05$) a post-hoc Student-Newman-Keuls test was applied.

Results

Muscle weight and maximum isometric force

As a result of maturation, the plantaris exhibited significant growth (0 NC vs 30 NC). Compensatory overload was accompanied by a further increase in plantaris wet weight by 15 days following overload (OP relative to CP). With the age-associated increase in muscle mass, both CP and OP muscles were capable of generating greater tetanic isometric forces (Po-200). The increase in muscle mass following overload was accompanied by an increased Po-200 which resulted in significant differences between CP and OP by 21 days postmyectomy and thereafter. When Po-200 was normalized for muscle weight (N/g), CP muscles did not differ over the time course of the study. When CP and OP were compared, significant differences were observed at day 2 when OP exhibited a deficit of 34% compared to CP and 44% when compared to muscles from Day 0 NC (Table I).

Table I

Plantaris muscle weights, fibre composition and tetanic tensions following compensatory overload

Days post-overload	Condition	Muscle weight (g)	Alkaline-labile fibres (%)	Po-200	
				(N)	(N/g)
0	NC	0.220	–	3.18	14.30
		±0.007		±0.44	±1.44
2	CP	0.228	8.0	2.79	12.22
		±0.004	±0.7	±0.20	±0.87
	OP	0.251	7.1	2.06[b]	8.12[ab]
		±0.009	±0.5	±0.27	±0.95
5	CP	0.253	6.4	3.95	15.69
		±0.008	±0.8	±0.14	±0.81
	OP	0.272	6.2	3.55	12.37
		±0.013	±0.8	±0.20	±1.26
7	CP	0.254	7.2	3.89	15.68
		±0.011	±1.3	±0.24	±1.19
	OP	0.278	8.0	3.51	12.6
		±0.015	±1.0	±0.24	±0.66
15	CP	0.375[b]	6.7	5.19[b]	13.84
		±0.008	±0.8	±0.37	±0.93
	OP	0.443[ab]	7.0	6.39[ab]	14.02
		±0.012	±0.7	±0.29	±0.80
21	CP	0.386[b]	8.2	5.13[b]	13.27
		±0.011	±2.1	±0.27	±0.43
	OP	0.517[ab]	11.9	6.61[ab]	13.11
		±0.011	±4.9	±0.30	±0.51
30	CP	0.378[b]	10.4	5.78[b]	15.32
		±0.008	±2.2	±0.28	±0.70
	OP	0.552[ab]	21.3[a]	8.50[ab]	15.19
		±0.016	±7.9	±0.55	±0.62
30	NC	0.392[b]	–	5.48[b]	14.12
		±0.016		±0.22	±0.74
77	CP	0.538[b]	9.5	8.22[b]	15.23
		±0.013	±2.4	±0.54	±0.77
	OP	0.895[ab]	16.0[a]	9.99[ab]	11.22
		±0.039	±4.8	±0.35	±0.51

Values represent mean ±SEM. Po-200 – isometric tetanic force of muscle stimulated at 200 Hz, CP – control plantaris, OP – overloaded plantaris, NC – plantaris muscle from age-matched rats on which no sham operation had been performed. a – Significant difference ($p<0.05$) between CP and OP. b – Significant difference ($p<0.05$) from day 0 – NC.

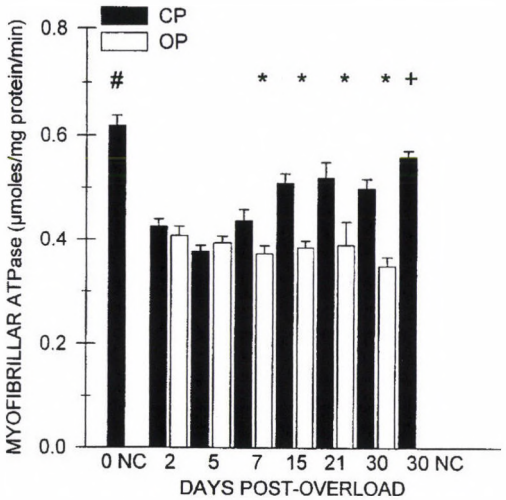

Fig. 1. Myofibrillar ATPase activities (at 30°C) for control and overloaded rat plantaris. Values represent mean ±SEM (n=6–12 per group). CP – contralateral control plantaris; OP – overloaded plantaris; NC – non-operated control plantaris. * represents a significant difference between OP and CP at $P<0.05$. + represents a significant difference between 30 day NC and day 2, 5, and 7 CP and all OP values at $P<0.05$. # represents a significant difference between 0 day NC and all other values except 30 day NC at $P<0.05$

Myofibrillar ATPase activity

When compared to NC, myofibrillar ATPase activity (Fig. 1) was depressed in both the CP and OP muscles. An initial decrease in myofibrillar ATPase activity of approximately 32% for both the CP and OP muscles was noted. Thereafter the myofibrillar ATPase activity of CP increased with time (from day 15 the values were significantly higher than those at days 2–7). By 7 days postmyectomy and thereafter, the myofibrillar ATPase activity of OP was significantly less than CP.

Electrophoretic analyses

An increase in the per cent alkaline-labile fibre composition of the overloaded muscle was observed by day 30 (Table I). Changes in the composition of slow myosin light chain (SLC_1) and fast myosin light chain three (FLC_3) for muscle from operated animals are presented in Fig. 2. Presumably because of low molecular weight degradation products, no assessment of the myosin light chain composition could be conducted until 5 days postmyectomy. Significant increases in myosin SLC_1 and significant decreases in myosin FLC_3 of OP as compared with CP were noted by 21 days postmyectomy.

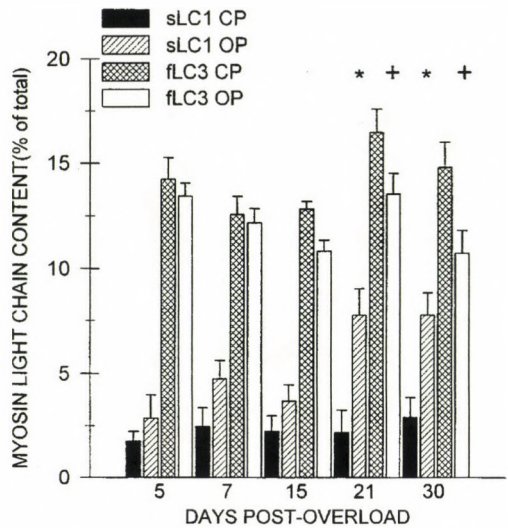

Fig. 2. Relative content of myosin slow light chain 1 (sLC1) and fast light chain 3 (fLC3) in control and overloaded rat plantaris. CP – contralateral control plantaris; OP – overloaded plantaris; Values represent mean ±SEM (n=6 per group). * represents a significant increase in sLC1 in OP vs CP at $P<0.05$. + represents a significant decrease in fLC3 in OP vs CP at $P<0.05$

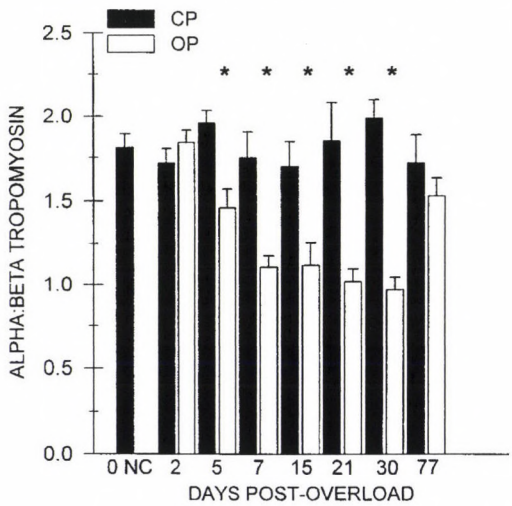

Fig. 3. Alpha to beta tropomyosin ratio in control and overloaded rat plantaris muscle. Values represent mean ±SEM (n=7–10 per group). * represents a significant difference between OP and CP at $P<0.05$

The plantaris muscle of rats which did not undergo surgery exhibited an α:β TM ratio of 1.81 (Fig. 3). A similar α:β ratio of 1.84 was demonstrated in the CP muscle of

the 2 day animals. At no time was the α:β ratio in CP different from that of NC. The content of β tropomyosin, as indicated by shifts in the α:β ratio was significantly increased in OP muscle over the time course 5–30 days but by 77 days of overload the TM isoform ratio was not different from CP.

Histochemical analyses

Hematoxylin and eosin stained cross-sections from 2 day CP muscle presented a normal histological appearance (Fig. 4a). However, by 2 days postsurgery, OP muscle was characterized by several indices of focal damage including the presence of widened interstitial spaces between the muscle fibres and apparent mononuclear cell infiltration into the interstitial spaces (Fig. 4b). Concomitant with these signs of muscle fibre damage were the appearance by 5 days, peaking by 7 days, and persisting up to 15 days of small fibres characterized by central nuclei (Fig. 4c). Although minor focal damage persisted up to 21 days, examination of muscle which had been overloaded for 30 days or longer revealed a normal histological appearance (Fig. 4d).

Discussion

Ablation of the synergistic gastrocnemius and soleus muscles of the rat triceps surae removes approximately 70–80% of the musculature involved in plantar flexion of the foot. As a result, the plantaris muscle is extensively recruited even during normal ambulation with a doubling of muscle fibre involvement for a standardized walking task by 15 days postmyectomy [7]. This increased recruitment is reflected in long-term changes in muscle size and force-generating capacity [14, 17], motor unit composition [25] and contractile protein expression [26, 34]. In addition, Roy et al. [32] showed that long-term overload of plantaris muscles results in the development of contractile properties that are more slow-twitch in character. Similar adaptive responses have been noted following chronic electrical stimulation of mammalian fast-twitch muscles [22, 28]. Morever, during electrical stimulation, muscle adaptation appears to occur in a sequential fashion [22, 28]. Results reported herein indicate muscle overload induced by ablation of synergists is also accompanied by a sequential change in markers of myofibrillar protein composition and function.

Slow-twitch skeletal muscle and large slower contracting hearts are associated with a lower α:β TM ratio [6, 19], lower myofibrillar ATPase activity [32], increased slow myosin content [34] and an increased percentage of alkaline labile muscle fibres [32].

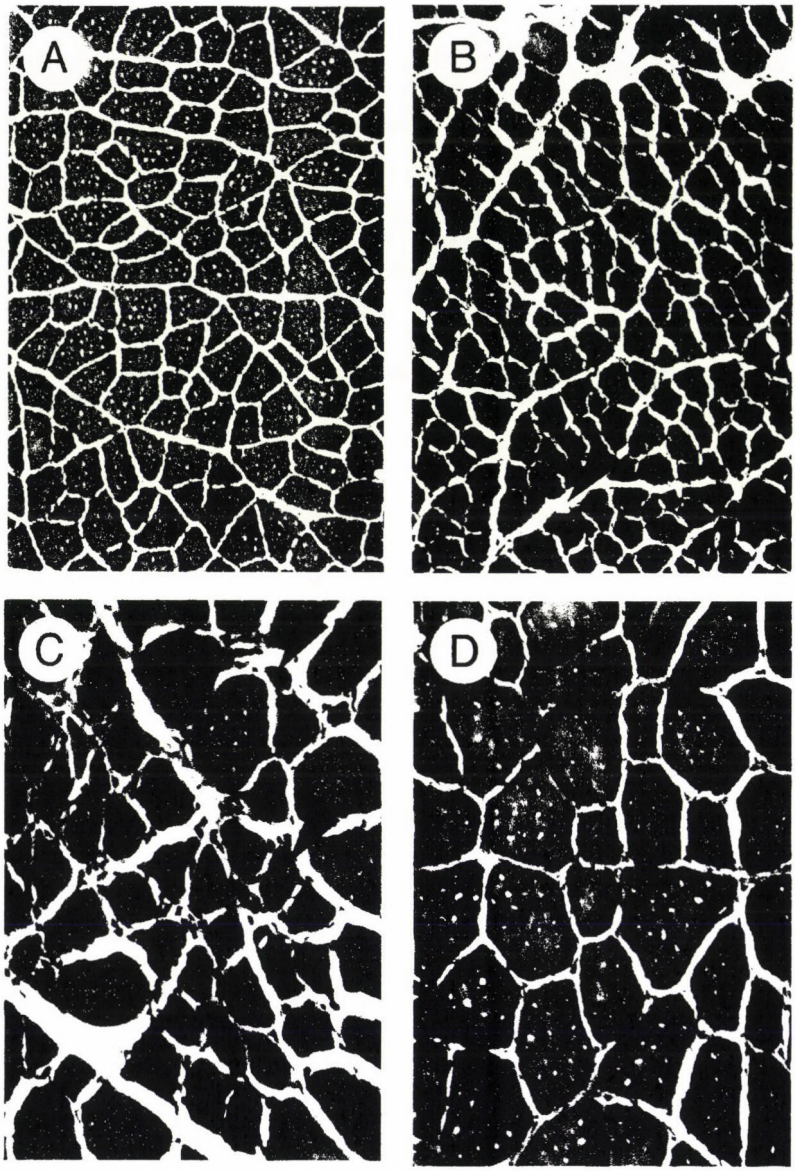

Fig. 4. Muscle cross-sections stained with hematoxylin and eosin. A: 2 day CP presented a normal histological appearance. B: 2 day OP was characterized by several indices of focal damage including the presence of widened interstitial spaces between the muscle fibres and apparent mononuclear cell infiltration into the interstitial spaces (see arrowheads). C: 7 day OP exhibited several small fibres characterized by central nuclei (see arrowheads). D: 30 day OP once again demonstrated normal histological appearance. Magnification – A and B ×125; C and D ×500

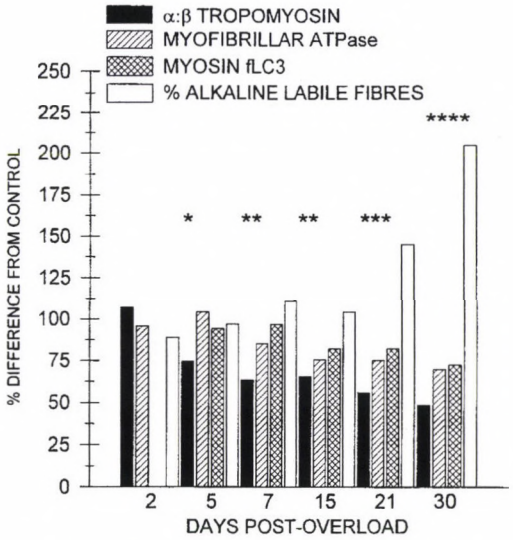

Fig. 5. Fibre type transformation in mature skeletal muscle occurs as a sequence of alterations in various components of the muscle fibre. * represents a significant difference from control ($P<0.05$)

Our results (Fig. 5) showed that a reduction in the α:β TM ratio occurred within 5 days of mycetomy and myofibrillar ATPase activity was decreased by 7 days. In contrast, increases in slow myosin content as assessed by SLC_1 and the percentage of alkaline labile fibres did not occur until 21 to 30 days postmyectomy. Although the time course of these changes supports the hypothesis that fibre type transformation in mature skeletal muscle occurs as a sequence of alterations in isoform expression, the functional dynamics involved in these changes are not understood completely.

The present study demonstrates that the adaptive response of skeletal muscle to the compensatory overload model is complex and cannot simply be ascribed to long-term changes in muscle phenotype. This is emphasized by histological evidence of damage to muscle fibres, the time-course of changes in the α:β TM ratio, the apparent dissociation of the myofibrillar ATPase activity from measures of myosin phenotype and the transient decrease in normalized maximum tetanic isometric force. A scheme which could explain the response to overload following ablation of synergists is presented in Fig. 6. The interesting aspect of this scheme is that muscle injury, and fibre degeneration and regeneration play an integral role in the observed changes during the early phase of adaptation to compensatory overload.

During the first two weeks of overload, we observed evidence of extensive muscle damage, phagocytosis and regeneration in OP muscles similar to that reported

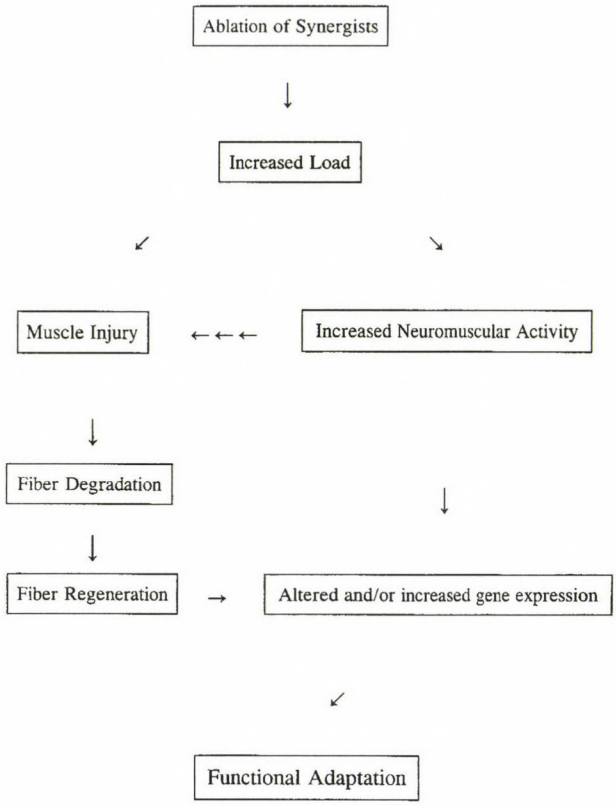

Fig. 6. Proposed mechanism for functional adaptation to compensatory overload. Note that some of the early changes may be a consequence of muscle injury resulting from increased load

by others [1]. Small muscle fibres with central nuclei, presumably at the myotube stage of development, were observed very early following the initiation of overload. These observations are consistent with the report that during the first week after ablation of synergistic muscles, up to 60% of the fibres in soleus muscles of rats showed evidence of ultrastructural damage which appeared to be associated with subsequent satellite cell incorporation and muscle fibre repair [33]. Whether the injury is produced as a consequence of increased neuromuscular activity, or increased loading due to the ablation of synergists, or a combination of both is not known, but there is little evidence to suggest that increased neuromuscular activity, in and of itself, produces substantial damage to skeletal muscle [16, 21]. In contrast, previous studies have shown that repeated stretches of contracting skeletal muscles (pliometric contractions), over relatively small ranges of displacement, can cause

considerable damage to muscle fibres [2, 23, 24]. During the early stages of overload, it seems likely that a large number of fibres in OP muscle are exposed to repeated pliometric contractions when the animal attempts to support its own body weight.

Three additional factors suggest that muscle injury is involved in the adaptive response to compensatory overload and that it is not simply a sequential transition of muscle phenotype. First, the histological time course of the injury and recovery following the initiation of overload is similar to that reported following protocols of pliometric contractions [2, 23]. In addition, during the first weeks of compensatory overload there is a parallel decrease in maximum specific force (N/cm^2) which generally recovers over a period of time [17]. We observed decreases in normalized maximum isometric force (N/g) of a similar magnitude to that of Kandarian and White [17], but showed a more complete recovery of normalized maximum isometric force. This latter observation may be due to the less precise method we used to normalize maximum force. Second, Roy et al. [31] have suggested that the β isoform of tropomyosin is preferentially expressed in muscle undergoing rapid myofibrillogenesis. This may be because the β isoform may polymerize better than the α isoform [35]. Hence, the early changes in the β isoform composition of OP muscle may be more closely associated with muscle degeneration and subsequent fibre regeneration and growth, than with the fast-to-slow transformation of the OP muscles. In this context, the reversion of the α:β TM ratio at 77 days post-myectomy suggests that α and β TM are not part of the long term adaptive response produced by compensatory overload. Finally, the rapid decease in myofibrillar ATPase activity is inconsistent with the observation that myofibrillar ATPase activity in mature skeletal muscle is associated closely with myosin composition [10]. Compared with non-operated controls, we observed a decreased myofibrillar ATPase activity in CP and OP muscles 2 days post-myectomy and significant differences between CP and OP muscles were present by 7 days. In contrast, our data and that of others [26, 34] demonstrate that an increase in the proportion of slow myosin does not occur until 21–30 days postmyectomy. The initial decrease in myofibrillar ATPase activity of CP and OP muscles without an associated increase in indicators of slow myosin is more likely a reflection of surgical trauma and/or mechanical strain on both myosin cross-bridges and myofibril integrity than a result of rapid changes in the expression myosin phenotypes. The inability of ourselves and others [34] to clearly distinguish the myosin light chain composition of either CP or OP muscles early after surgery supports this latter suggestion.

In summary, compensatory muscle overload initiates a sequence of morphological and functional changes in the rat plantaris muscle. The early transition in TM isoform ratio and later changes in the myosin light chain and histochemical profile of the overloaded muscle are consistent with a sequential fast to slow fibre transformation.

However, indices of fibre damage and regeneration, a simultaneous depression in myofibrillar ATPase activity in both CP and OP and the eventual recovery of a normal α:β TM ratio, suggest that the adaptive process is complex and that muscle damage and subsequent recovery may play a role. Of interest in future studies will be identification of the molecular signals responsible for the transduction of muscle injury and/or increased neuromuscular activity to functional adaptation.

Acknowledgement

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Changes in the activity of some lysosomal enzymes and in the fine structure of submandibular gland due to experimental diabetes

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The aim of this study was to establish and quantify changes in the activities of the some lysosomal enzymes and to determine the type of changes in the ultrastructure of the submandibular gland in rabbits caused during progression of diabetes. The experiment was conducted on 89 New Zealand rabbit males. Diabetes was induced by the intravenous administration of 10% alloxan solution at a dose of 10-mg/kg-body weight. On the seventh day after alloxan administration, the level of glucose in blood was determined. Rabbits were divided into five groups: intact (n=18), 21-day diabetes (n=18), 42-day diabetes (n=17), 90-day diabetes (n=19) and 180-day diabetes (n=17). From killed animals in each group, the submandibular glands were removed and fixed or stored. Enzyme activities were assayed by spectrophotometric methods using substrates (Sigma) which release 4-metyloumbeliferol when they react with the proteases. Fixation procedure was done according to standard methods. Semi-thin and ultra-thin specimens were prepared by use of clearly visible after 42 days of diabetes. Mitochondria were damaged, accumulation of large amounts of lipids in the intracellular spaces was observed. After 90 days the presence of vacuoli and swollen lysosomes were observed, some cells also contained myelin figures. After 180 days the greatest changes were observed in the blood vessels, which had thickened walls and were often occluded. We concluded that the total activity of acid phosphatase and β-N-acetyl-glucosaminidase in the submandibular gland was correlated with the level of glucose but there was no correlation between total β-galactosidase activity and the serum concentration level of glucose has been detected during course of diabetes. The activities of the free fractions of acid phosphatase, β-galactosidase and β-N-acetyl-glucosaminidase in the submandibular gland were higher than the bound fractions in all groups of rabbits. The changes in the ultrastructure of the submandibular gland were correlated with changes in serum glucose level and with lysosomal enzymes activities during progression of experimental diabetes in rabbits.

Keywords: diabetes mellitus, submandibular gland

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Diabetes has become one of the most important health problems of the world. This disease is associated with mostly absolute insulin deficiency (type I) or mostly relative insulin deficiency (type II), which causes disorders in the metabolism of carbohydrates, proteins, and fats, as well as morphological changes in many organs [25]. This disease affects two to four per cent of global population. During the recent 20 years, a twofold increase has been observed in the incidence of insulin-dependent diabetes among people over the age of 65 [7, 26]. Diabetes is a metabolic disease with symptoms in many organs including the oral cavity. In fact, many of these symptoms that appear in aged people in the oral cavity which were thought to be connected with ageing are – according to the recent clinical research conducted in past decade – the result of either parotid gland dysfunction in general syndromes and the effects of their treatment [6, 7].

Disorders in mechanisms regulating excretion of parotid glands that means nervous and hormonal system can evoke many changes in metabolism [3, 13, 15, 19]. It is widely known that parotid excretion is controlled by the autonomic nervous system. Vegetative neuropathy and disorders of the direct effect of insulin on cells are found in diabetes. Secretion of the parotid glands is controlled by sympathetic and parasympathetic nervous system. Stimulation of the sympathetic system provokes excretion of a small amount of rich in protein saliva followed by degranulation in cells and ducts of the gland. Stimulation of the parasympathetic system induces excretion of large amount of poor in protein saliva. Little or no degranulation follows [2, 3]. Many researchers often investigate changes in the pancreas concomitantly with those in the parotid gland; however, the abnormalities in the submandibular gland in the course of diabetes are considerably less examined [1, 2, 3, 12, 19, 23, 28].

The aim of this study was to establish and quantify changes in the activities of the some lysosomal enzymes and to determine the type of changes in the ultrastructure of the submandibular gland in rabbits caused during progression of diabetes.

Materials and Methods

Eighty-nine male rabbits, New Zealand breed (Experimental Animals Laboratory, Chorzelow n/Warsaw, Poland), weighing 3030±150 g. were used in experiment. Animals were housed one per cage under 12 h/12 h light/dark cycle at 21±2 °C temperature and 50% relative humidity with standard granulated food (Motycz, Poland) and water available. Body weight was measured before induction of diabetes and before decapitation. Bioethical Committee of Medical University School of Lublin approved the experimental protocol. Diabetes mellitus was induced by a single injection of alloxan (Sigma Chemical Company, St. Louis, MO, USA) at a dose of 10 mg/kg into

the auricular vein [18]. On day 7 the glucose level in the whole blood was measured by a glucometer (Boehringer, Germany) to confirm the presence of diabetes. From this, the daytime of disease was counted.

The rabbits were divided into the following groups: Group 1 – intact (n=18), Group 2 – 21 days diabetes mellitus (n=18), Group 3 – 42 days diabetes mellitus (n=17), Group 4 – 90 days diabetes mellitus (n=19), Group 5 – 180 days diabetes mellitus (n=17). After above-mentioned periods, blood samples were taken and the rabbits were killed by decapitation. The final level of glucose in the sera was determined spectrophotometrically by enzymatic method using ready kit GS-120L (Cormay, Lublin, Poland) at the wavelength 500 nm. The right submandibular gland was removed and stored at –20 °C.

The samples were defrosted in 0.9% solution of NaCl at 4 °C. One gram of the submandibular gland was taken for the biochemical examination. The samples were dissolved and placed into a 0.3 M sucrose solution at 4 °C in proportions of 1.0 *g* of gland tissue to 5.0 ml of sucrose and homogenised. The obtained homogenate was centrifuged for 10 minutes at 2.200×g at 4 °C.

The supernatant was decanted and centrifuged for 20 minutes at 35,000×*g*. The obtained sample containing the free fraction of enzyme was assigned as supernatant 1 [24, 31]. The precipitate was placed into 5.0 ml of 0.3 M sucrose containing 0.1% Triton X-100 and stored for 24 hours at 4 °C. Triton was used to rupture the lysosomal membrane. The precipitate was then centrifuged for 20 minutes at 35,000×*g*. The supernatant, containing the fraction of bound enzyme, was decanted and assigned as supernatant 2.

Enzyme activities were assayed by the spectrophotometric methods using substrates (Sigma) which release 4-metyloumbeliferol when they react with the proteases [4, 27].

The rule of acid phosphatase [EC 3.1.3.2] activity designation is based on degradation it by 45 mg of sodium phosphate 4-metyloumbeliferol dissolved into 100 ml 0.1 M acetate buffer (pH 5.0). The rule of β-galactosidase [EC 3.2.1.23] activity designation is based on degradation it by 51 mg of 4-metyloumbeliferylo-β-D-galactopiranoside dissolved into 100 ml 0.1 M citrate buffer (pH 3.6). The rule of β-N-acetyl-glucosaminidase [EC 2.3.1.3] activity designation is based on degradation it by 57.2 mg of 4-metyloumbeliferylo-β-N-acetyl-glucosaminidine dissolved into 100 ml 0.1 M citrate buffer (pH 4.3) with 0.3 M NaCl.

One hundred µl of 1st and 2nd supernatants were incubated with 500 µl of above mentioned substratum by 18 hours at 37 °C. Next 600 µl alkaline buffer were added because of breaking reaction and after 5 min of extinction had been read by waves of 360 nm on the spectrophotometer. Quantitative of designation protein had been carried

out by Lowry's method [22]. The values were expressed as nmol/1mg of protein/1 hour of incubation. The total activity was counted as the sum of free and bound fractions.

The statistical analysis was done using the SAS system v. 6.11 (SAS Institute Inc., SAS Campus Drive, Carry, NC 27513, USA). Results are expressed as mean ±SD. Differences between groups were analysed by analysis of variance (ANOVA). The correlation coefficients between analysed characters were counted. If $P<0.05$, differences between the mean values were considered statistically significant.

From three killed animals in each group, the left submandibular gland was removed and fixed. Fixation procedure was done according to standard methods [11]. The samples were fixed for 4 hours in 4% glutar aldehyde, in 0.1 M cacodylate buffer of pH 7.4, at a temperature +4 °C. The specimens were then washed twice for 10 minutes with 0.1 cacodylate buffer and refixed for 2 hours in 1% osmium tetroxide in the same buffer at a temperature of +4 °C. After dehydration in a series of ethanol solutions and propylene oxide, the specimens were saturated with SPURR Low-Viscosity Polysciences resin. Semi-thin and ultra-thin specimens were prepared by the use of Richert Om-U3 ultramicrotome, and subsequently observed and documented by Tesla BS-500 microscope.

Results

The initial serum glucose concentration had an average value of 6.36± 2.03 mmol/l. Twenty-one days after injection of alloxan, the concentration had increased to 21.79±9.49 mmol/l. On the day 42, the level was 32.02±19.11 mmol/l, which decreased, thereafter in all groups at 23.15±10.83 mmol/l on day 180 (Group 5). Statistical analyses of the serum glucose concentration are presented in Table I. In all the diabetic groups the P value was lower than 0.01, and so the differences between the control and diabetic groups were statistically highly significant (Table I).

The highest decrease in body weight occurred in 180-day diabetes group, slightly lower in 21-day diabetes group and the lowest in 90- and 42-day diabetes groups (data not shown). There were no significant changes in animals body weight during experiment ($P>0.05$).

Statistical characterisations of the activity of acid phosphatase, β-galactosidase and β-N-acetyl-glucosaminidase are presented in Table II.

During the course of disease the total activity of acid phosphatase increased and β-N-acetyl-glucosaminidase decreased while the activity of β-galactosidase was fairly constant.

Table I

The serum glucose concentration (nmol/l) of the control and alloxan-diabetic rabbits

		Diabetic groups			
	Group 1 Control	Group 2 day 21	Group 3 day 42	Group 4 day 90	Group 5 day 180
No. of animals	18	18	17	19	17
Glucose level	6.36 ± 2.03	21.79 ± 9.49*	32.02 ± 19.11*	31.33 ± 19.21*	23.15 ± 10.83*

*$p<0.01$

The mean total activity of acid phosphatase in the intact group was 117.11 nmol/mg of protein/h. This value increased by 26% in Group 2, by 50% in Group 3 and by 62% in Group 4. On the day 180 this activity was somewhat less than on day 90. The analysis of variance showed, that there were significant differences between the mean values of acid phosphatase activity of intact (Group 1) and all diabetic groups (Groups 2, 3, 4, 5) ($p<0.05$).

Table II

Mean activity of acid phosphatase (ACP), β-galactosidase (β-GAL) and β-N-acetyl-glucosaminidase (NAGL) in the rabbit's submandibular gland during the course of experimental diabetes

		Diabetic groups			
Enzyme	Group 1 Control	Group 2 day 21	Group 3 day 42	Group 4 day 90	Group 5 day 180
ACP free	59.77 ± 15.69	75.68 ± 15.92	89.74 ± 15.72	98.58 ± 10.46	84.10 ± 15.86
ACP bound	57.34 ± 14.25	72.32 ± 25.19	85.34 ± 13.25	90.49 ± 33.18*	77.00 ± 17.56*
ACP total	117.11 ± 22.84	148.00 ± 33.16*	175.08 ± 22.54*	189.07 ± 38.41*	161.10 ± 25.49*
NAGL free	167.52 ± 33.54	122.21 ± 31.40	90.57 ± 43.21	118.79 ± 23.40	133.43 ± 21.71
NAGL bound	40.31 ± 13.93	35.41 ± 8.45	22.76 ± 12.14	33.36 ± 10.11*	45.53 ± 15.95*
NAGL total	207.83 ± 40.51	157.62 ± 35.82	113.33 ± 49.28	152.15 ± 28.56	178.96 ± 29.89
β–GAL free	12.90 ± 6.75	19.00 ± 7.96*	14.90 ± 6.71*	16.69 ± 6.73*	17.41 ± 8.31
β–GAL bound	7.71 ± 1.89	5.01 ± 2.44	5.65 ± 2.62	12.15 ± 3.82*	7.05 ± 1.31
β–GAL total	20.61 ± 7.68	24.01 ± 9.28	20.55 ± 8.62	28.84 ± 8.66	24.46 ± 8.96

* $p<0.01$

The values are expressed as nmol/1 mg of protein/1 hour of incubation. The values of standard deviation are given in parentheses.

The mean total activity of β-N-acetyl-glucosaminidase in the intact group was 207.83 nmol/mg of protein/h. This value decreased by 24% in Group 2 and by 45% in Group 3. Over the course of the next periods the percentage of decrement tended to diminish increased to 152.15 (in Group 4) and to 178.96 nmol (in Group 5). The statistical analysis showed that there were significant differences between the mean values of β-N-acetyl-glucosaminidase activity of Group 1 and Groups 2, 3 and 4 ($p<0.05$).

The mean total activity of β-galactosidase ranged from 20.55 to 28.84 nmol. ANOVA showed that there were no significant differences between controls and the different diabetic groups.

In the glandular cells of the intact group, an enlarged secretory system was observed in the form of large secretory granules, which were mainly electron loose. Other cell organelles were normal in structure and maintained their typical position. Relatively numerous, intact blood vessels were present. A large nucleus and a relatively poor cytoplasm of the dark cells resembled lymphocytes, whereas long digitiform processes were typical of Langerhans cell.

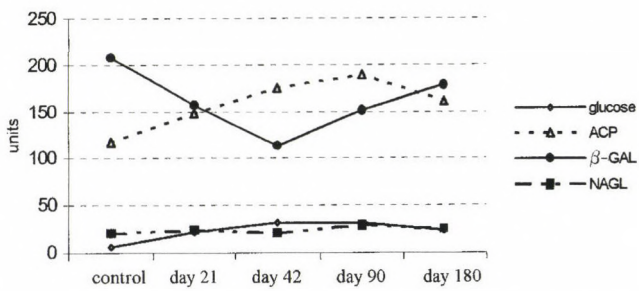

Fig. 1. Changes of serum glucose concentration and activities of ACP, β–GAL and NAGL with the course of diabetes

In the group inducted with 21 days of diabetes, the changes observed in rabbit submandibular glands were relatively slight. It was demonstrated either a reduced density of nuclei and cytoplasm, or contrarily, a high density of nuclei. The merging of secretory granules was noted. The cells of excretory ducts remained intact.

After 42 days of manifest diabetes, the changes in the glandular ultrastructure were clearly visible. The intercellular space was considerably widened in some places, and contained lipids and relatively numerous collagen fibbers (Fig. 2). Secretory granules merged in most cells. Rough endoplasm reticulum of some cells was frequently observed but the epithelium of excretory ducts remained intact.

On day 90 of diabetes entailed an intensification of the changes observed in the previous group. There was the widening of the intracellular space, and fibrosis and widening of the endoplasmic reticulum. The merging of secretory granules was common and resulted in cell organelles being pushed to the peripheral parts of the cell. The mitochondria were swollen and tended to create vacuoli. Changes of occlusive character were noticed in blood vessels (Fig. 3). The epithelial cells of ducts remained intact.

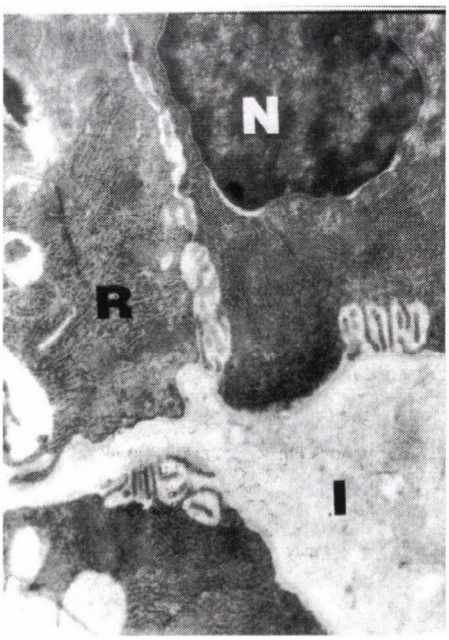

Fig. 2. Submandibular gland in rabbit, 42-day diabetes. Considerably widened intercellular space (I) and extended endoplasmic reticulum (R). Cell nucleus (N). ×10,000

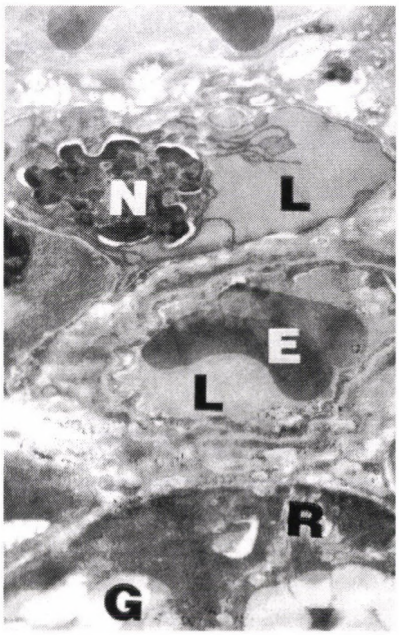

Fig. 3. Submandibular gland in rabbit, 90-day diabetes. Altered blood vessels with lumen (L) which contain erythrocytes (E). Secretory cells with granules (G) and cellular interdigitations of the enlarged reticulum (R). Plasma cell with dilated reticulum and nucleus (N). ×4,000

In the Group 5, the greatest changes were observed in the blood vessels, which had thickened walls and were often occluded (Fig. 4). Endothelial cells showed degenerative changes in the form of irregular shapes; they became thinner, and occasionally created cytoplasmic processes in the direction of the lumen of the vessel. Moreover, a greater widening of the endoplasmic reticulum of the gland cell was noted,

with the creation of large vacuoli. Widening and fibrosis of intracellular space was also observed (Fig. 5).

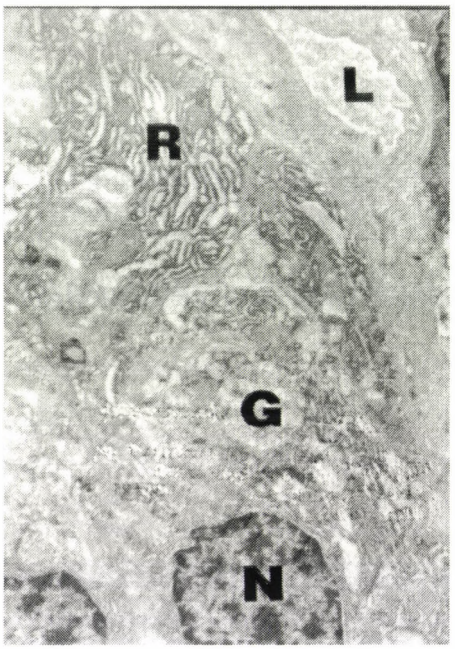

Fig. 4. Submandibular gland in rabbit, 180-day diabetes. Considerably enlarged reticulum (R) and secretory granules (G) with blurred outlines. Section of the vessel (L), cell nucleus (N). ×4,000

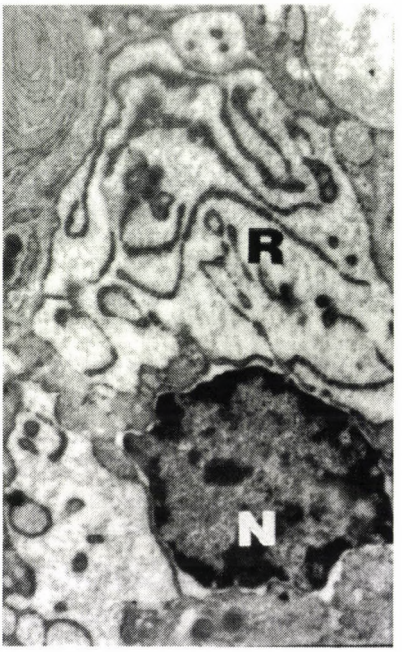

Fig. 5. Submandibular gland in rabbit, 180-day diabetes. Massive oedema of endoplasm reticulum (R) with creation of large vacuoli. Cell nucleus (N). ×6,000

The mean level of glucose and the mean activity of acid phosphatase, β-galactosidase and β-N-acetyl-glucosaminidase were presented in Fig. 1 as the curve of changes. On the axis Y the units (mmol/l×10 for glucose concentration and nmol/mg/h for enzymes activity) were presented commonly for comparative reasons.

The statistical analysis of correlation coefficients between the data characters showed that there was positive correlation between glucose concentration and activity of acid phosphatase ($p=0.05$) and that was negative correlation between glucose concentration and β-N-acetyl-glucosaminidase activity ($p<0.05$). There was no correlation between glucose concentration and β-galactosidase activity ($p>0.05$).

Discussion

The submandibular gland of a rabbit is of heterocrine type, and the secretory terminal sections consist of seromucous alveoli and tubuli [11, 14, 29, 30]. The changes in ultrastructure of the submandibular gland appeared by the 21st day of the alloxan-induced diabetes, and their intensification was observed during the course of the disease. By the 21st day, only the increased or decreased density of the nuclei and cytoplasm were noted, whereas more severe changes occurred on the 42nd day. A widening of the intracellular spaces and the endoplasm reticulum manifested these changes. Mitochondria were also damaged. The accumulation of large amounts of lipids in the intracellular spaces was also observed after the 42nd day of disease, which is partially inconsistent with reports by some authors [1]. In 90- and 180-day diabetes, the presence of vacuoli and swollen lysosomes were observed. Secretory granules merged already 21 days after the induction of diabetes; some cells contained myelin figures. Apart from slight differences, the changes described above were generally consistent with the literature data [28]. The lack of apparent changes in the cells of striated ducts was also characteristic, and their mitochondria were not damaged until after 90 days of diabetes, which is reported by other authors [21]. Since secretory ducts participate in the creation of saliva through the resorption of sodium ions and secretion of potassium ions, the lack of disturbances in the concentration of these ions in the saliva is evidence that the ducts remain intact [28].

Model of alloxan-induced diabetes in rabbits is interesting in particular, due to the fact that considering lipid accumulation, a rabbit model is more alike to Human than rats [8, 9]. Dixit et al. [10] reported in 1962 that insulin content in beta cells decreased to 5% of normal value 48 hours after alloxan administration. In our experiment it was observed a significant increase of blood glucose level reaching its maximum value on 42nd day. It remained on a high level until 90th day, and slightly decreased on 180th day. Even at this time it was three times higher than the control. Disorders related to impaired blood glucose levels are usually accompanied by other biochemical changes [16, 17]. Opposite to our results, a significant body weight loss has been reported by numerous authors [16, 17], and it is often regarded as characteristic feature of diabetes.

We have found only a few papers devoted to the issue of lysosomal enzyme activities and ultrastructural changes in salivary glands due to experimental diabetes [5, 20, 23]. We can conclude that the activities of acid phosphatase and β-N-acetyl-glucosaminidase in the rabbit's submandibular gland changed over the course of alloxan-induced diabetes, and changes are comparable either with the changes in the ultrastructure of the gland or with changes in serum glucose level. Secondly, we

established that the activity of the free fraction of acid phosphatase, β-galactosidase and β-N-acetyl-glucosaminidase in the submandibular gland was higher then the bound fraction in all groups of rabbits.

Conclusions

1. The total activity of acid phosphatase and β-N-acetyl-glucosaminidase in the submandibular gland was correlated with the level of glucose.
2. There was no correlation between total β-galactosidase activity in the submandibular gland and the level of glucose in the course of diabetes.
3. The activity of the free fraction of acid phosphatase, β-galactosidase and β-N-acetyl-glucosaminidase in the submandibular gland was higher then the bound fraction in all groups of rabbits.
4. The changes in the ultrastructure of the submandibular gland are correlated with changes in serum glucose level and with lysosomal enzyme activity during progression of experimental diabetes in rabbits.

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Blood flow of the right and left submandibular gland during unilateral carotid artery occlusion in rat: Role of nitric oxide*

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The aim of the present study was to investigate the effect of unilateral carotid artery occlusion on the blood flow of submandibular gland in anesthetized rats and identify the role of nitric oxide (NO) in blood flow changes after the artery occlusion. L-NAME (Nω-nitro-L-arginine-methyl-ester; 10 mg/kg/day, per os) dissolved in tap water was used to block nitric oxide synthase. Glandular blood flow was measured using Sapirstein's indicator (^{86}Rb) distribution technique.

In the control animals the blood flow of left (ligated side) submandibular gland was lower than in the right (unligated side) one (right: 76.4±15.4 ml/min/100 g, 64.1±13.4 ml/min/100 g, $p<0.01$). The blood flow of submandibular glands decreased in NOS blocked group versus control. The vascular resistance after L-NAME treatment was elevated (control: 11±2.3 R/kg, L-NAME: 17.5±4.1 R/kg, $p<0.001$). In L-NAME group the difference between blood flow value of the left and right submandibular gland was significantly lower than in the control group (control: –16%, NAME: –8%, $p<0.01$).

Conclusion: The maintenance of the blood flow in the left submandibular gland during ligation of the left common carotid artery could be due to the good vascular anastomotic system at these regions and adaptation of the submandibular vessels to the decreased perfusion pressure. Nitric oxide may have a role in the regulation of blood flow under this condition.

Keywords: submandibular gland blood flow, carotid artery occlusion, nitric oxide

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One of the reason of the ischaemic problem of the oro-facial tissue could be the carotid artery stenosis or occlusion. It is important to know what circulatory changes occur in organs peripherial to the point of vascular occlusion. To obtain information about changes in the circulation of submandibular gland in the present study the blood flow of the submandibular gland at the two sides was compared during unilateral common carotid artery occlusion.

Some investigators measure the local perfusion pressure at the oro-facial regions during carotid artery occlusion and found it significantly less than systemic arterial blood pressure [2, 13]. During decreased perfusion pressure constant blood flow in the organs was maintained by autoregulatory mechanisms. Several studies have shown that basal release of nitric oxide (NO) influences the vascular resistance and blood flow in the submandibular gland in physiological conditions [4, 8, 14]. The possible activation of NO-mediated mechanisms in controlling blood flow during common carotid artery occlusion was also studied under such conditions.

Material and Methods

Female Wistar rats weighing between 160 and 220 g were used. The animals were kept in a 12-hour light/dark cycle.

Group 1 rats (control, n=10): were maintained on standard rat chow and tap water *ad libitum*.

Group 2 rats (L-NAME, n=13): received the same diet with drinking water completed with L-NAME (Nω-nitro-L-arginine-methyl-ester, $C_7H_{15}N_5O_4 \cdot HCL$, Sigma Chemical Company, St. Louis, Missouri USA; 10 mg/kg/day) dissolved in tap water (0.1 mg/ml) for four days to block nitric oxide synthase.

Surgical preparation

The animals were anesthetized with sodium pentobarbital (CEVA Sanofi santé animale s.a., 37 avenue George V. 75008 Paris, France; 60 mg/kg b.w., i.p.) and placed on a heated table. Rectal body temperature was kept at around 37 °C controlled by thermometer (Experimetria Ltd.). A catheter was inserted into the right atrium via the right external jugular vein, and the left common carotid artery was cannulated. Tracheostomy was performed and animals were allowed to breathe spontaneously through the tracheal cannula. After completion of the surgery, heparin (Richter Gedeon Rt., Budapest) (500 IU/kg iv.) was administered. The measurements were made after the systemic arterial pressure remained constant.

Experimental protocol

Systemic blood pressure was continuously monitored through the carotid artery canal using Statham pressure transducer connected to electromanometer. Glandular blood flow was measured using Sapirstein's [10] indicator accumulation technique. According to Hársing and Pelley [6] the ^{86}Rb isotope injected into the systemic circulation is suitable for the determination of blood flow (F) to any organ or tissue if tissue or organ radioactivity (q) per total injected radioactivity (Q) is multiplied by cardiac output (CO), i.e.,

$$F = \frac{q}{Q} \times CO .$$

Cardiac output was measured by ^{86}Rb indicator according to Stewart-Hamilton's formula [12].

$$CO = \frac{60 \times Q}{\int_0^\infty Ca(t) \times dt}$$

where: $\int_0^\infty Ca(t) \times dt$ denotes the area under the arterial dilution curve of the indicator activity during the first circulation.

^{86}RbCl (0.5 MBq) dissolved in 0.15 ml physiologic saline was injected as a bolus into the right ventricle via the right external jugular vein. Arterial blood samples were taken from the left carotid artery at 0.8 s intervals with the aim of plotting the isotope dilution curve. Sampling of arterial blood was started at the time of ^{86}RbCl injection and continued for 10 s. Animals with a drop of blood pressure more than 10 mm Hg during this procedure were discarded from the study. Ninety seconds after the administration of the isotope rats were sacrificed, and wet weight of the submandibular glands was determined. After alkaline boiling, the radioactivity of the blood and tissue samples was measured in Beckmann gamma counter.

Total peripheral resistance (TPR) was calculated as quotient of the mean arterial blood pressure and the cardiac output. The vascular resistance was calculated as quotient of the mean arterial blood pressure and the blood flow, since the left carotid artery was occluded, the vascular resistance for the left submandibular gland was not calculated. Percent changes of blood flow, vascular resistance in the submandibular gland were calculated as the difference of mean values of L-NAME treated and control animals divided by the control value.

Statistics

All values are presented as mean ±SD. Student's paired *t*-test was used to test the difference between the blood flow of two sides. Student's unpaired *t*-test was used to evaluate the effect of the L-NAME treatments. To determine that higher systemic blood pressure measured in an animal means higher blood flow in the submandibular gland regression analysis was made between blood pressure values and blood flow values. The acceptable level of significance was $p<0.05$.

Results

The blood pressure, the TPR were significantly elevated, the cardiac output was significantly decreased in L-NAME group (Table I). The blood flow of right (unligated side) submandibular gland was lower, the vascular resistance was higher in NOS blocked group than in controls (Table II).

Table I

Effect of L-NAME treatment on haemodynamics parameters

	Control	NAME
	$\bar{x}$±SD, n=10	$\bar{x}$±SD, n=13
Arterial blood pressure (mm Hg)	135 ± 10	156 ± 16*
Cardiac output (ml/min/100g)	31.7 ± 5.9	22.0 ± 5.3*
TPR (mm Hg×ml^{-1}×s×kg^{-1})	26.2 ± 4.4	44.8 ± 10.6*

* NAME vs. Control, $p<0.001$

In the control group the blood flow of left submandibular gland was lower by 16% ($p<0.01$) than that of the right one. In the L-NAME group the difference between blood flow value of the left (ligated side) and right submandibular gland was –8% ($p<0.01$), which was significantly ($p<0.01$) lower than that in the control group.

No correlation was found (Fig. 1) between the blood pressure and blood flow in the right submandibular gland in controls either ($r=0.32$, $n=10$, $p>0.36$) or in L-NAME treated group ($r=0.006$, $n=13$, $p>0.98$).

Table II

Effect of L-NAME treatment on haemodynamics of the submandibular gland

		Control	NAME
		$\bar{x}$±SD, n=10	$\bar{x}$±SD, n=13
Blood flow (ml/min/100g)	right	76.4 ± 15.4	56 ± 11.6**
	left	64.1 ± 13.4	51.3 ± 11.4*
Left vs. right		$p<0.01$	$p<0.01$
Vascular resistance (mm Hg×ml^{-1}×s×kg^{-1})	right	11 ± 2.3	17.5 ± 4.1**

NAME vs. Control in right submandibular gland, * $p<0.05$, ** $p<0.001$

Discussion

The glandular branches of the facial artery furnish the blood supply to the submandibular gland [1]. The facial artery is originated from the external carotid artery which is one of the main branches of the common carotid artery. It is unexpected that after the ligation of the left common carotid artery the blood flow of the ipsilateral (left) submandibular gland is lower only by 16% comparing to the contralateral side. This could be explained by the significance of the vascular anastomotic system in the oro-facial region, which can supply blood during carotid artery occlusion.

The local arterial pressure measured in cervical stump of ligated common carotid artery is only 30% of the systemic blood pressure in anesthetized rats [2] and the local arterial pressure measured in the small arteries at the facial region is 57% of the systemic blood pressure after bilateral common carotid occlusion in dogs [13]. It seems that lower perfusion pressure only slightly influence blood flow, which is due to the good autoregulatory mechanism in the submandibular gland. This is illustrated by the lack of correlation between blood pressure and blood flow values.

The blood pressure was significantly higher (16%) in the L-NAME group in spite of the decrease of cardiac output, because the TPR elevation (71%) was more pronounced than the attenuation of the cardiac output (–30%).

The glandular blood flow decreased with L-NAME due to the pronounced vasoconstriction (vascular resistance increased by 59% in the right gland). These results are in good agreement with our earlier findings [14] and the observations of other authors [3, 4, 8] and support the possible role of NO in maintaining basal blood flow rate in the submandibular gland. In case of artery occlusion the increase in NO production could compensate the falling in blood flow due to the decrease in perfusion

pressure via vasodilatation. If the NO had an important role in the autoregulation the abolish of NO synthesis could have resulted more pronounced decrease in blood flow after artery occlusion. In our experiment the difference of blood flow between the right and left gland was significantly less in L-NAME treated rats than in control ones and NOS inhibition did not alter the pressure-flow relationship (Fig. 1). These data indicate that nitric oxide does not play significant role in controlling autoregulation in the submandibular gland of rat.

On the basis of other studies NO mediates the flow-induced dilatation *in vitro* and *in vivo* [5, 7, 11]. Increase in blood flow velocity elicited a significant increase in arterial wall shear stress followed by increase in diameter of arterial vessels, which consequently resulted in decrease in wall shear stress [9]. Based on this mechanism in our experiment the blood flow velocity and wall shear stress could decrease after artery occlusion due to the attenuation of local perfusion pressure. After artery occlusion the blood flow in the left (ligated side) submandibular gland is controlled by interaction between the autoregulatory mechanism (induced vasodilatation) and decreased wall shear stress induced vasoconstriction. This theory could explain our results: lack of NO in submandibular gland vessels attenuates the effect of carotid artery occlusion on lowering the blood flow.

The maintenance of the blood flow in the left submandibular gland during ligation of the left common carotid artery could be due to the good vascular anastomotic system at this regions and adaptation of the submandibular vessels to the decreased perfusion pressure. Basal release of nitric oxide may decrease during carotid artery occlusion, this way NO plays an inhibitor role in the maintenance of blood flow during occlusion.

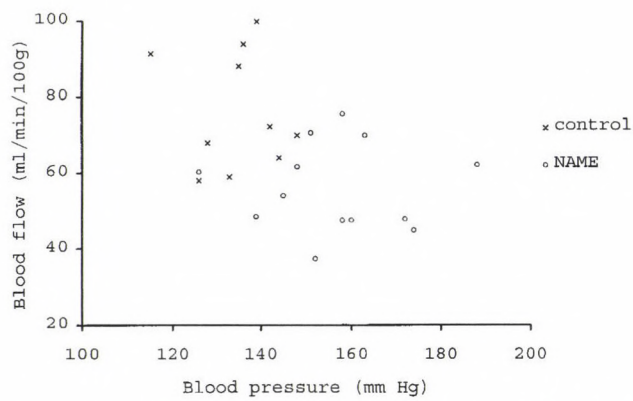

Fig. 1. Relationship between blood pressure and blood flow of right (unligated side) submandibular gland in anesthetized rat. Control: r=0.32, n=10, p>0.36; L-NAME treated group: r=0.006, n=13, p>0.98

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Identification of acute intermittent porphyria carriers by molecular biologic methods

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Acute intermittent porphyria (AIP) is an autosomal dominant disease caused by mutations of the gene coding for hidroxymethylbilane synthase. Acute attack of AIP is a potentially life-threatening condition precipitated by certain drugs, alcohol, fasting and stress. Biochemical diagnosis before the manifestation of the symptoms is problematic, and genetic screening is required to identify asymptotic carriers. The aim of this study was to establish a fast, reproducible and reliable genetic method to detect mutations causing AIP. Exon 10 of one healthy individual and 12 AIP patients was studied using a recently developed method, temporal temperature gradient electrophoresis (TTGE). Mutation of exon 10 was detected using TTGE in one patient. DNA sequence analysis confirmed the presence of a heterozygous point mutation causing substitution of the arginine in position 173 of the gene with glutamine. These results were also confirmed using restriction enzyme analysis, and this method and TTGE identified a child of this patient as an asymptotic carrier of AIP.

Keywords: acute intermittent porphyria, hidroxymethylbilane synthase, DNA sequence analysis, mutation, restriction enzyme analysis, asymptotic carrier of acute intermittent porphyria

Acute intermittent porphyria is an autosomal dominant disease caused by mutations of the gene coding for hidroxymethylbilane synthase (HMBS) (EC 4.3.1.8.)

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the third enzyme in the human heme biosynthetic pathway. AIP is clinically characterized by neurovisceral attacks often precipitated by certain drugs, alcohol, starvation and stress. The acute attack is a life-threatening condition therefore the detection of gene carriers is very important in the prevention of attacks. Symptomatic heterozygotes who have increased levels of the porphyrin precursors δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) are easy to identify. However, the diagnosis of asymptotic heterozygotes (ca. 80% of the patients), who usually have normal levels of urinary ALA and PBG is problematic due to the significant overlap between high heterozygote and low normal values of HMBS activity in erythrocytes [1]. In this group of patients genetic analysis of HMBS is the only reliable diagnostic method [2].

Previous studies have identified over 130 mutations in the HMBS gene using several different mutation detection methods including heteroduplex analysis, single-stranded conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE). However, genetic analysis of the HMBS gene is particularly difficult, because its coding region is located on 15 exons [3]. In order to minimize this problem we tried to find a method, which is fast, predictable, reproducible and reliable to provide the proper diagnosis. Temporal temperature gradient electrophoresis (TTGE) is a recently developed method, which appeared to be highly suitable to satisfy these criteria. The running conditions of TTGE can be determined from the melting profile of the DNA fragment calculated by a computer software. The aim of the present study was to establish this genetic method for detection of mutations causing AIP.

Methods

Genomic DNA from samples of one healthy control individual and 12 previously diagnosed unrelated AIP patients were analyzed. The diagnosis was established according to clinical and biochemical abnormalities and the measurement of HMBS activity in red blood cells.

Genomic DNA was prepared from EDTA-anticoagulated blood samples according to E.S. Kawasaki [4]. Previous analyses of AIP patients have shown that exon 10 of the HMBS gene is a frequent location of mutations causing AIP [5]. For this reason exon 10 was analyzed in the present study. The sequence of exon 10 was amplified in a polymerase chain reaction (PCR) using sense and antisense primers located in intron 9 and intron 10, respectively. The antisense primer contained a 30-base GC-rich sequence (GC-clamp) to prevent full denaturation of the PCR product during the TTGE. Thirty-six cycles of DNA amplification were performed in 50 μl reaction mixture containing 200–400 ng of genomic DNA, 25 pmoles of each primer

(sense primer: 5'-CCGACACTGTGGTCCTTAGCAA-3', antisense primer: 5'-cgcccggcggccctcccgcgggcggcgggcTGGGGATGACTGTAAGGCAGAA-3'), 200 μmol/l of each dNTP and 2.5 unit of RedTaq polymerase (Sigma). During the first cycle the DNA samples were denatured for 3 min at 95 °C and in all additional cycles for 60 sec at 95 °C. Annealing and primer extension were performed for 45 sec at 56 °C and 60 sec at 72 °C, respectively. After the completion of the last cycle of the PCR reaction the samples were heated at 95 °C for 5 min and incubated for 5 min at 72 °C to facilitate the formation of heteroduplexes. Aliquots of 5 μl of each amplified DNA were submitted to TTGE.

TTGE involves electrophoresis of double-stranded DNA fragments through a polyacrylamide gel containing a constant concentration of denaturant in the gel. During electrophoresis, the temperature of the buffer is increased gradually and uniformly providing a linear temperature gradient. In the present experiments these conditions were achieved using the DCode universal mutation detection system (Bio-Rad). Electrophoretic mobility is decreased as DNA denaturation begins. Heteroduplex and mutant or wild-type homoduplex molecules have different melting behavior and thus begin to denature at different points in time resulting in a separation on the gel. The addition of a GC-rich sequence to the one end of the DNA fragments prevents full denaturation of the DNA molecules and results in a single melting domain allowing the detection of sequence variation across the entire fragment.

Conditions for the TTGE were designed using the WinMelt software (provided with DCode), which calculates and graphs the theoretical melting profile of DNA sequences. The temperature range for the gradient can be calculated from the melting profile graph by first determining the lowest and the highest non-GC clamped melting temperature of the DNA sequence. The theoretical melting temperatures can be lowered by adding urea to the gel. We used 8% acrylamide/bis-acrylamide (37.5:1) gel containing 8M urea. The gel was run on the DCode system at 130 volts, 1.5 × TAE buffer, temperature range of 59–66 °C, and a ramp rate of 1.8 °C/h. After electrophoresis, the gel was stained with ethidium bromide and visualized by a Fluor-S MultiImager (Bio-Rad).

Automated DNA sequencing was performed using ABI PRISM BigDye Terminator Cycle Sequencing kit (Perkin-Elmer). The PCR product with altered mobility and the control PCR product were sequenced in both directions using the primers described above without adding GC-clamp.

For the incubation with restriction enzyme MspI (Fermentas) we used 2 units of the enzyme, 1.5 μl of the 10x buffer Y and 7 μl amplified DNA fragment in final volume of 15 μl. After incubating at 37 °C for 2 hours 10 μl of sample was run on 4% NuSieve 3:1 agarose gel, stained with ethidium bromide and visualized by Fluor-S MultiImager.

Results and Discussion

Genomic DNA samples of one healthy individual and 12 patients diagnosed with AIP using biochemical methods were analyzed with TTGE. Oligonucleotides were designed as described in Methods to amplify the sequence of exon 10 of the HMBS gene. As expected the sample of the healthy control individual shows a single band on the TTGE gel (Fig. 1, lane 1). This band corresponds to the homoduplex wild-type PCR product. Samples of 11 patients (Fig. 1, lanes 2–4 and 6–13) gave single bands in a similar position suggesting that the mutations causing AIP in these patients are not located on exon 10. The sample of one patient had a different migration pattern on the TTGE gel (Fig. 1, lane 5). In this patient four bands were observed. The fastest migrating band corresponded to the wild-type homoduplex PCR product, and 3 additional bands were detected, which correspond to the mutant homoduplex and the two heteroduplexes. As a consequence of the destabilization of the heteroduplex DNA by the mismatch, it is likely that the two slowest migrating bands are the heteroduplexes (Fig. 1, arrows).

The DNA sequence of the PCR product of the latter patient was determined by sequencing and compared to those of the healthy control individual. The DNA sequence of the patient had a heterozygous G→A point mutation at position 518 of the HMBS gene, which caused the substitution of the arginine (CGG) in position 173 with glutamine (CAG). This mutation (R173Q) of exon 10 was first identified as a genetic alteration causing AIP by Delfau et al. [6].

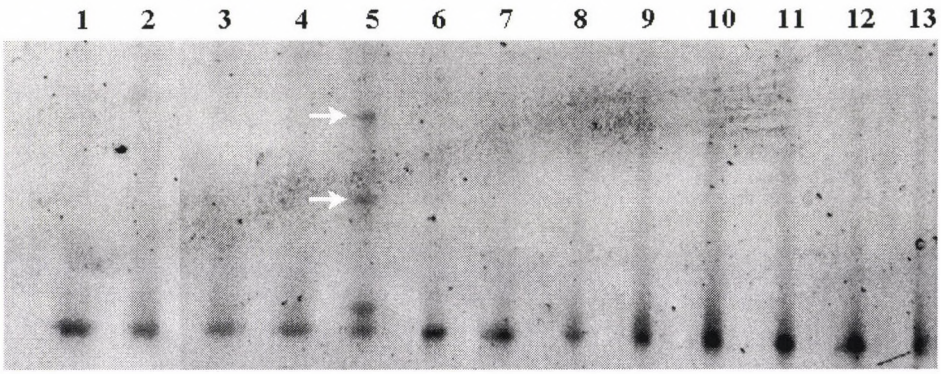

Fig. 1. Analysis of exon 10 of the HMBS gene by TTGE. Ethidium-bromide-stained gels after TTGE of PCR-amplified fragments from 1 healthy individual (lane 1) and 12 AIP patients are shown. The white arrows point at the location of heteroduplex DNAs in lane 5

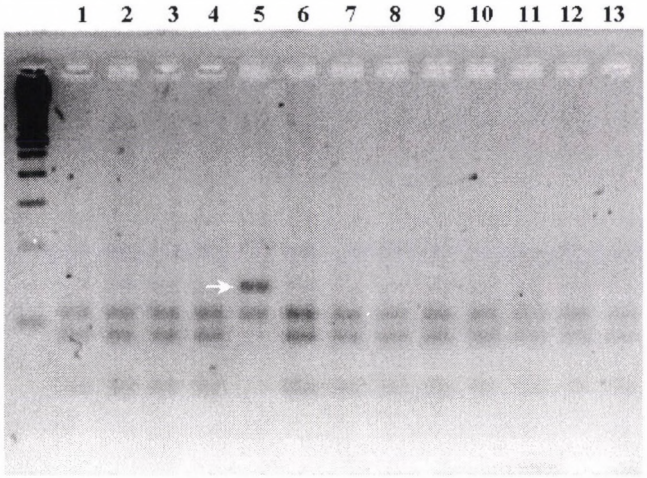

Fig. 2. Restriction enzyme patterns of exon 10. DNA fragments created by MspI treatment of the PCR products from 1 healthy individual (lane 1) and 12 AIP patients are shown. The white arrow points at the location of the 134-basepair fragment, which was eliminated in all samples except for that of lane 5

If the mutation causing AIP creates or eliminates a restriction enzyme recognition site simple restriction enzyme screening can be used to identify asymptotic carriers among the family members of the patient. The mutation identified in the present study eliminates a recognition site of the MspI restriction enzyme (CCGG). To verify the presence of this mutation the PCR products of all tested individuals were digested with MspI and run on a gel containing 4% agarose. A 134 basepair fragment of the patient carrying the G518A mutation resisted the MspI treatment (Fig. 2, lane 5). In the PCR products of all other patients this fragment was cleaved by MspI creating 2 smaller bands.

Genetic DNA was prepared from all three children of the patient with the G518A mutation, and the sequence of exon 10 was amplified by PCR as described above. The PCR products were treated with MspI. The 134 basepair fragments of the PCR products of the patient with the G518A mutation (Fig. 3, lane 1) and that of one child (Fig. 3, lane 4) were resistant of MspI treatment, but in two other children the fragments were cleaved (Fig. 3, lanes 2 and 3). These data demonstrate that one out of 3 children (Fig. 3, lane 4) is an asymptotic carrier of AIP. However, in many cases the exact

mutation is not known or there is no convenient alteration in the restriction enzyme pattern of the studied DNA. In these cases mutation detection methods, such as TTGE, are the most convenient way to screen the family members. Screening of the family members with TTGE was performed, and the gel of the child, identified with restriction enzyme screening as a carrier of the mutation, showed the heterozygous pattern of 4 bands, while the 2 siblings showed a single PCR product, which corresponded to the wild-type homoduplex (data not shown).

These data demonstrate that TTGE is a fast and reliable mutation detection method, and it is suitable to detect unidentified mutations of the HMBS gene. The major advantages of this method are its simplicity and the possible ability to detect mutations, which have not been identified. After locating the mutant exon of a proband, it is enough to perform TTGE on the affected exon of the family members, furthermore in some cases elimination or creation of a restriction enzyme by the mutation can further accelerate the screening for AIP carriers.

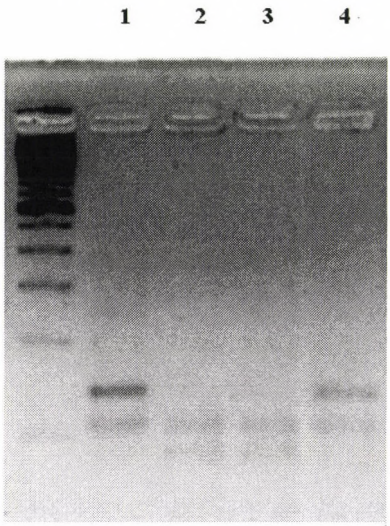

Fig. 3. Restriction enzyme patterns of exon 10 of the patient with the G518A mutation and those of 3 children. The PCR products treated with MspI restriction enzyme are shown. The 134-basepair fragment resisted Msp I treatment in the patient (lane 1) and in 1 child (lane 4)

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Effects of bradykinin in the cerebral circulation*

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All components of an intracerebral kallikrein-kinin system have been described. Thus, bradykinin (BK) acting from the parenchymal side as well as from the blood side may influence cerebral microcirculation.

BK is a potent dilator of extra- and intraparenchymal cerebral arteries when acting from the perivascular side. The vasomotor effect of BK is mediated by B_2 receptors which appear to be located at the abluminal membrane of the endothelial cell. Signal transmission from the endothelial to the smooth muscle cell is mediated by NO, prostanoids, free radicals or H_2O_2 depending on the animal species and on the location of the artery.

Selective opening of the blood-brain barrier for small tracers (Na^+-fluorescein; MW, 376) has been found in cats during cortical superfusion or intraarterial application of BK. This leakage is mediated by B_2 receptors located at the luminal and abluminal membrane of the endothelial cells and probably mediated by an opening of tight junctions.

Formation of brain edema has been found after ventriculo-cisternal perfusion or interstitial infusion of BK. This can be explained by increase of vascular permeability and cerebral blood flow due to arterial dilatation thus enhancing driving forces for the extravasation.

An increase of the BK concentration in the interstitial space of the brain up to concentrations which induce extravasation, dilatation and edema formation has been found under several pathological conditions. Thus, BK may be involved in edema and necrosis formation after cold lesion, concussive brain injury, traumatic spinal cord and ischemic brain injury.

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The mediator role of BK in brain edema is further supported by therapeutic results. Brain swelling and the area of necrosis due to cold lesion or ischemia could be diminished by treatment with kallikrein-inhibitors or B_2 receptor blockers. Similarly, dilatation of cerebral arterioles after concussive brain injury was reduced by blockade of B_2 receptors.

In conclusion, all criteria discussed above favour BK as one mediator of vasogenic edema.

Keywords: intracerebral kallikrein-kinin system, cerebral microcirculation, bradykinin, endothelial cell, blood-brain barrier

Whereas the effects of bradykinin (BK) in the peripheral circulation have been studied in detail its influence upon the cerebral microcirculation has been neglected for a long time. All components of an intraparenchymal kallikrein-kinin system have been described for the brain [12, 13]. Thus BK acting from the parenchymal side as well as from the blood side may influence cerebral microcirculation [11–13].

Vasomotor effects of bradykinin

The vasomotor effect of BK in isolated arteries is dependent on the species, the cerebral region and the experimental design. After pharmacological precontraction BK (10^{-9}–10^{-5} M) elicited relaxation in all isolated extraparenchymal arteries tested, including human vessels [2, 8, 9, 14, 15, 17, 18], except the nonresponding feline basilar artery (BA) [14, 15].

In situ, BK is a potent dilator of extraparenchymal cerebral arteries. When applied by perivascular microapplication the BK induced dilatations of feline pial arteries (PA) were concentration dependent with an EC_{50} of 4.4×10^{-7} M and a maximal response of 45% [8, 9, 14]. Cortical superfusion revealed similar results but tachyphylaxis appears to develop after 30 min [3, 6, 10]. Thus, BK appears to be an important autacoid for the local adjustment of cerebral microcirculation [12].

Endogenous BK released from brain kininogen by cortical superfusion of kallikrein elicited dilatation which was inhibited by the kallikrein-inhibitor aprotinin [3].

In contrast, intracarotid infusion of BK in cats revealing concentrations of 10^{-6} to 10^{-5} M in the sagittal sinus blood did not alter the diameter of pial vessels [6] or CBF [12]. These findings clearly indicate that the kinin receptors inducing vasomotor responses can only be reached by BK from the extravascular side.

Effects of kininase II inhibitors on the vasomotor action of bradykinin

Kininase-like activity has been found in the brain. Part of the total cerebral kininase activity is due to kininase II (K II). Two peptidylpeptidases are considered to belong to the family of KII. One, K II-ACE appears to be identical with the angiotensin-

converting enzyme (ACE). K II-ACE can be inhibited by several compounds such as BK-potentiating-peptides 5a (BPP_{5a}) and 9a (BPP_{9a}), BK-potentiator C, and captopril.

Studies employing cerebral arteries *in vitro* [9, 15, 16] and *in situ* [9] failed to detect an influence of these inhibitors on the dilating effect of BK. These findings do not indicate the absence of KII-ACE in the vessel wall or the surrounding of the arteries was confirmed by testing the vasomotor effect of angiotensin I (AI). To induce contraction AI has to be converted to AII by KII-ACE. This could be inhibited by the blocking agents *in vitro* [15, 18] and *in situ* [20].

These data demonstrate that K II-ACE is present in the vessel wall, the perivascular space or the adjacent tissue. K II-ACE is necessary for the conversion of AI to AII but unimportant for the degradation of BK. Thus, other enzymes with kininase like activity may be involved in the degradation of BK [9, 12].

Effects of bradykinin on blood-brain barrier (BBB) permeability

Cortical superfusion with BK induced extravasation of Na^+-fluorescein (MW, 367) at 4×10^{-7} to 4×10^{-6} M [6, 10]. Leakage started as single fluorescent spots around small veins. Extravasation of fluorescein isothiocyanate (FITC) labelled albumin (MW, 67,000) or FITC dextran (MW, 19,400–62,000) could not be induced in the cat even at BK concentrations up to 4×10^{-3} M [6]. These data demonstrate that BK selectively opens BBB only for small compounds in the cat. From the hydrodynamic radius of Na^+-fluorescein one can estimate that BK increased BBB permeability by opening of functional pores with a diameter of at least 1.1 nm [6]. An opening of tight junctions may be the mechanism [5]. Thus, BK reduced the transmural electrical resistance in frog cerebral venules [12, 13] which would be compatible with an opening of tight junctions.

Contradictory results were obtained during intravascular administration of BK. Leakage of horseradish peroxidase (MW, 40,000) has been found after intracarotid infusion of 10^{-5} M BK in the rat whereas no extravasation of the tracers was detected by others [12]. However, an increased ionic conductance was detected in frog pial venules during intravascular administration of BK [12]. Similarly, intracarotid infusion of BK leading to concentrations of 10^{-6} to 10^{-5} M in sagittal sinus blood evoked leakage of Na^+-fluorescein but not of FITC dextran (MW, 62,000) from cat pial vessels [6]. Even lower intracarotid concentration of BK (3×10^{-8} M) induced an unspecific tracer extravasation from rat glioma vessels [13].

Kinin receptors in cerebral arteries

Kinin receptors of cerebral arteries were characterized by studying the effects of agonists and antagonists of various affinity to the B_1 and B_2 receptors. Employing the agonists BK, methionyl-lysyl-BK, and des-Arg^9-BK it was demonstrated that the relaxation of large arteries *in vitro* [14, 16, 19] and of small PA *in situ* [6, 14] was mediated by B_2-receptors. Correspondingly, *in vitro* and *in situ* the dilating effects of kinin agonists could be inhibited by the B_2-receptor antagonists Hoe 140 and Thi^{5,8} D-Phe^7-BK [2, 19] but not by the B_1-receptor antagonist des-Arg^9-Leu^8-BK [6, 14]. Since the dilating effect of BK is endothelium dependent [2, 18] and can only be elicited by extravascular but not by intravascular BK [6] this B_2 receptor has to be localized to the antiluminal side of the endothelial cell.

Since the opening of the BBB could not be induced by a B_1-receptor agonist and the extravasation due to BK was not inhibited by a B_1-receptor antagonist [6] the effect of BK is obviously mediated by B_2 receptors which have to be localized to the luminal and antiluminal side of the endothelium.

Mechanisms mediating the bradykinin induced effects

Since opening of BBB by BK was prevented by pretreatment with inhibitors of cyclooxygenase a coupling between the kallikrein-kinin and prostanoid systems was suggested [11–13]. An interaction between the kallikrein-kinin system, arachidonic acid and free radicals has been also proposed by Ellis et al. [1] to occur after brain injury. These authors concluded that the effects of BK were finally mediated by free radicals. However, we [11–13] found divergent vasomotor and permeability effects of BK, arachidonic acid, and free radicals in our experimental model.

The dilating effect of BK is endothelium dependent [2, 18] and can be mediated by several endothelial factors depending on animal species and/or location of the vessel. The dilating effect of BK appears to be mediated by NO in some cerebral arteries like BA of dog and rat or middle cerebral artery (MCA) of the rat [2, 13]. However, NO is not involved in the dilating effect of BK in PA of rat and cat where H_2O_2 and hydroxyl radicals appear to be involved as mediators, respectively [12–13]. Prostacyclin appears to mediate the dilating effect of BK in rabbit PA *in situ* [3]. However, the involvement of dilating prostanoids in the response to BK could not be demonstrated in isolated MCA or BA of cats and humans [8, 9, 14, 15, 18].

Bradykinin as a mediator of brain edema

Evidence has been accumulated that kinins are involved in the formation of vasogenic brain edema since ventriculo-cisternal perfusion with artificial CSF containing 2.5×10^{-6} M BK significantly increased brain tissue water content [11–13]. Formation of kinins, in particular in perifocal edematous tissue, was increased by additional cerebral ischemia due to marked rise of intracranial pressure [4]. For this state, BK-concentrations of 10^{-7} to 10^{-6} M in the interstitial fluid can be estimated which is effective to open BBB permeability and to induce vasodilatation. Similarly, a significant increase of brain kininogen content which was accompanied by protein leakage and increase of cerebral tissue water content was detected in the rat after fluid percussion injury [12, 13]. Furthermore, tissue kininogen and kinin content were increased after traumatic spinal cord injury [12, 13].

The mediator role of BK in brain edema was further supported by therapeutical studies in rabbits with cold-induced brain swelling [7]. Inhibition of kinin formation by treatment with aprotinin reduced significantly the hemisphere swelling. Similarly, activation of the cerebral kallikrein-kinin system and formation of brain edema due to ischemia induced by bilateral carotid occlusion could be reduced by treatment with aprotinin [12, 13]. Furthermore, the arterial dilatation following fluid percussion neural trauma could be inhibited by pretreatment with a B_2-receptor antagonist [1]. In addition the B_2 antagonist, CP-012, a B_2 antagonist produced a significant inhibition of edema in a rat cold lesion model (Whalley, unpublished observations). Finally, recent observations in our laboratory revealed that the necrosis induced in the parieto-temporal cortex of rats by a cold lesion (–78 °C, ∅ 3 mm, 6") could be reduced significantly by 19% with pre- and posttreatment of Hoe 140 (300 ng/kg/min), a B_2 antagonist, in comparison to vehicle treated controls.

In conclusion, the vasomotor responses to BK that can enhance driving forces for bulk flow, the increase in cerebral vascular permeability, the edema formation, the change of BK concentration in tissue or interstitial fluid during pathological event and the therapeutic results favour BK as one factor mediating secondary brain damage [11–13].

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Selective inhibition of neuronal nitric oxide synthase fails to alter the resting tension and the relaxant effect of bradykinin in isolated rat middle cerebral arteries

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The role of the neuronal isoform of the nitric oxide (NO) synthase (nNOS) in the regulation of the cerebrovascular tone was studied *in vitro*. Selective inhibition of nNOS by 7-nitro indazole monosodium salt (7-NINA) failed to alter the resting tension and the relaxant effect of bradykinin in isolated rat middle cerebral arteries. These results indicate that 1./ 7-NINA is selective for nNOS and 2./ cerebrovascular nNOS is involved neither in the resting NO production nor in the mediation of the relaxant effect of bradykinin. Therefore, nNOS- derived NO that contributes to the maintenance of the resting cerebral blood flow *in vivo* appears to be released from neurons and/or glial cells.

Keywords: neuronal nitric oxide synthase, 7-nitro indazole monosodium salt, bradykinin, rat middle cerebral artery, endothelium

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Nitric oxide (NO) production by constitutive and inducible NO synthase (NOS) enzymes plays an important role in the regulation of the cerebral blood flow (CBF) [for review see: 4]. Inhibition of the L-arginine – NO pathway results in cerebral vasoconstriction and reduction of the CBF, indicating that the basal release of NO provides a resting relaxant tone in the cerebrovascular bed [for review see: 8]. Selective inhibition of the neuronal isoform of the constitutive NOS (nNOS) also reduces blood flow and increases vascular resistance in the brain, indicating that basal NO release by nNOS contributes to the maintenance of the resting CBF [7, 9, 10, 12, 15]. Since nNOS is present both in the cerebrovascular endothelium and in cerebral perivascular nerves [3, 5, 11], in the present study we investigated the possible physiological role of this cerebrovascular nNOS system. Specifically, in isolated rat middle cerebral arteries we studied whether selective inhibition of nNOS by 7-nitro indazole monosodium salt (7-NINA) [13] may influence the resting tone or the relaxant effect of bradykinin.

Materials and Methods

Adult male Wistar rats were exsanguinated rapidly under deep ether anaesthesia. Ring segments of the MCA were prepared for measurement of isometric force as described previously [6]. Special care was taken to preserve the endothelium during preparation of the artery. The segments were transferred into 5-ml organ baths filled with a modified Krebs solution of the following composition (mM): NaCl, 119; KCl, 4.6; NaH_2PO_4, 1.2; $CaCl_2 \cdot 2H_2O$, 1.5; $MgCl_2 \cdot 6H_2O$, 1.2; $NaHCO_3$, 15; glucose, 10. The bath solution was bubbled continuously with a humidified gas mixture (90% O_2/10% CO_2). The MCA segments were mounted on two L-shaped tungsten wires (50 μm diameter), one of which was fixed to the bath and the other connected to a force transducer for measuring isometric force. The vessels were allowed a 90-min equilibration period, during which the resting tension was adjusted to 1.5–2 mN. Thereafter each segment was exposed to a 124-mM K^+ Krebs solution to elicit a reference contraction. Following a 60-min resting period the segments were precontracted with 100 μM UTP (Serva) and the relaxant effect of bradykinin (0.01–10 μM, Calbiochem) was determined. According to our previous study [6] segments that did not exhibit at least 15% bradykinin-induced relaxation of the precontraction were regarded as endothelium-negative and excluded from the study. Thereafter 7-NINA (100 μM, Calbiochem) was applied and the relaxant effect of bradykinin was determined after inhibition of nNOS.

Results are presented as mean ±SEM, the number of MCA segments studied was 9. The bradykinin-induced relaxation was calculated as percent decrease of precontraction due to UTP while the effect of 7-NINA on the resting vascular tone was

expressed as percentage of the reference contraction induced by 124 mM K^+ Krebs solution. Results were analysed statistically using Student's *t*-test and a p value less than 0.05 was considered significant.

Results and Discussion

7-NINA induced a weak increase of the resting vascular tone (2.6±0.7%). However, this effect is negligible compared to the contractile effect of the nonisoform-selective NOS inhibitor nitro-L-arginine (40.6±2.9%) applied in the same concentration in our previous study in isolated rat MCAs [2].

In vivo inhibition of the nNOS with 7-nitro indazole (7-NI) results in a significant reduction of the CBF, indicating that nNOS-derived NO is an important factor in the maintenance of the resting perfusion of the brain [7, 9, 10, 12, 15]. Since 7-NINA failed to induce a significant vasoconstriction in the present study cerebrovascular nNOS does not appear to be involved in the process of NO generation under resting conditions. Therefore, inhibition of the NO production by neurons and/or glial cells appears to be responsible for the CBF-decreasing effect of 7-NI *in vivo*.

7-NINA also failed to influence the vasodilator effect of bradykinin in the present study (Fig. 1). This observation, together with our previous results demonstrating a significant reduction of the bradykinin-induced relaxation in rat MCA after NOS

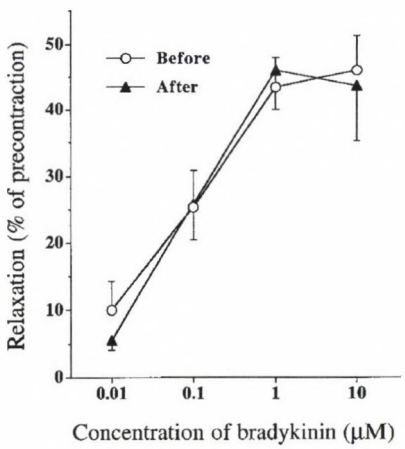

Fig. 1. The relaxant effect of bradykinin (0.01–10 μM) in 9 isolated rat middle cerebral artery segments before and after the administration of 100 μM 7-nitro indazole monosodium salt (7-NINA). The values (mean ± SEM) are expressed as percentage of the precontraction induced by 100 μM UTP. There was no significant difference between the corresponding values before and after 7-NINA

blockade by nitro-L-arginine [6, 14], indicates that bradykinin stimulates only the endothelial isoform of NOS in the rat MCA. Our recent studies, however, indicate that cerebrovascular nNOS may have an important physiological role in the mediation of the acetylcholine-induced vasorelaxation [1].

Zagvazdin and colleagues [16] have reported some nonisoform-selective effects of 7-NI *in vivo*. Our present study clearly demonstrates that 7-NINA has no such side-effects *in vitro*, because in a relatively high concentration it did not induce similar effects to those of nitro-L-arginine: vasoconstriction and attenuation of the relaxant effect of bradykinin [2, 6].

In conclusion, our results indicate that cerebrovascular nNOS is involved neither in the process of NO generation under resting conditions nor in the mediation of the relaxant effect of bradykinin. The nNOS-derived NO that contributes to the maintenance of the resting CBF *in vivo* must therefore be released from neurons and/or glial cells.

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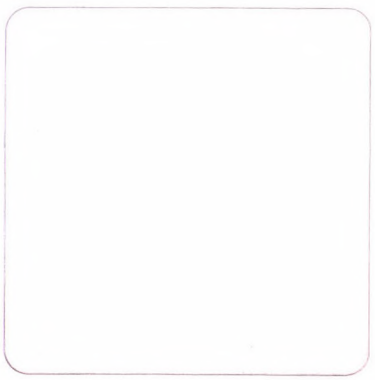

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64th Annual Meeting of the
Hungarian Physiological Society

July 5–8, 1999, Budapest, Hungary

SHORT COMMUNICATIONS

Section A

Interactions between physiological sciences and primary prevention – scientific research and teaching

Acta Physiologica Hungarica, Volume 86 (3–4), pp. 169–170 (1999)

Section A

Interactions between physiological sciences and primary prevention – scientific research and teaching

Introductory remarks

It was a pioneering initiative by the organizers of the *64th Annual Meeting of the Hungarian Physiological Society* (July 5–8, 1999, Budapest) to put together an "experimental" Session entitled **"Interactions between physiological sciences and primary prevention – scientific research and teaching".**

As a result of industrial, scientific, informational, and economical "revolutions", large masses of people rush rather blindly towards promising ultramodern ages – labelled frequently as "Global", "Knowledge", "Informational", "Network", "Postindustrial", or "Risk Society". This process, however, can bear serious risks for the health of the individual involved. A consequence of this overaccelerated man made "evolution" is that the "natural" physiological balance between physical and psychological strains of man has broken up, and this instability has reached a dangerous extent. Typically enough, we individuals – in a dense information-cascade – do less and less physical exercise, but eat more, we hope to find a company in mass entertainments, and in the meantime become more and more lonely. We get distressed, our stress absorbing ability diminishes, and we easily put our health at stake, seek primitive pleasures. Consequently, our body and soul may become victimes of second-hand activities. At the same time, our knowledge lacks sufficiently detailed, scientifically founded, up-to-date information on the normal functions of our own organism and on the conditions of protecting its healthy functioning. We are not even motivated appropriately in keeping our own physical and mental health. Unfortunately, this applies to a great part of the medical community, too, although it would primarily be the task of professionals in medicine to make the society aware of the miracle of how the human organism functions and how its health could be protected.

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New efforts must be made for supporting primary prevention, i.e. protection, maintenance, and improvement of health, also on behalf of physiologists. We should integrate, develope further, and distribute our knowledge for providing firm scientific foundation of preventive actions in medicine, and to build up a scientifically supported health culture in the society. By definition, physiological science can be regarded as one of the most reliable allied force of those who are ready to act for these purposes. These tasks provide new challenges and promising perspectives for physiology.

In an attempt to stimulate further activities in this field, including international dialogues and new original publications, six presentations of the above successful Session are published on the following pages as short communications. We belive that there are good reasons for fostering among physiologists such action that has not been brought up before.

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Vegan diet in physiological health promotion*

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We have performed a number of studies including dietary interventions and cross-sectional studies on subjects consuming uncooked vegan food called living food (LF) and clarified the changes in several parameters related to health risk factors. LF consists of germinated seeds, cereals, sprouts, vegetables, fruits, berries and nuts. Some items are fermented and contain a lot of lactobacilli. The diet is rich in fiber. It has very little sodium, and it contains no cholesterol. Food items like berries and wheat grass juice are rich in antioxidants such as carotenoids and flavonoids. The subjects eating living food show increased levels of carotenoids and vitamins C and E and lowered cholesterol concentration in their sera. Urinary excretion of sodium is only a fraction of the omnivorous controls. Also urinary output of phenol and p-cresol is lowered as are several fecal enzyme levels which are considered harmful.

The rheumatoid arthritis patients eating the LF diet reported amelioration of their pain, swelling of joints and morning stiffness which all got worse after finishing LF diet. The composite indices of objective measures showed also improvement of the rheumatoid arthritis patients during the intervention. The fibromyalgic subjects eating LF lost weight compared to their omnivorous controls. The results on their joint stiffness and pain (visual analogue scale), on their quality of sleep, on health assessment questionnaire and on general health questionnaire all improved.

It appears that the adoption of vegan diet exemplified by the living food leads to a lessening of several health risk factors to cardiovascular diseases and cancer. Rheumatoid patients subjectively benefited from the vegan diet which was also seen in serum parameters and fecal analyses.

Keywords: vegan diet, plant materials, physiological health promotion

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* Vegan diet contains only plant material. The plants are rich natural sources of antioxidants in addition to other nutrients. The subjects eating mostly plants appear to have less cardiovascular diseases and some tumors.

Well-balanced diet with moderate physical activity is one of the basic components in the physiological approach in health promotion. Plants form the main component of our food. There are populations which live exclusively on plant materials (vegans) [8] and vegetarians who consume also some animal products (as milk (lacto), eggs (ovo) or fish (pesce) and semivegetarians). In India there is a strong vegetarian tradition because of the Hindu culture, which has now also followers in other countries [45]. Most Seventh Day Adventists prefer a lacto-vegetarian diet [25]. We have been studying an extreme kind of vegan diet. It is called living food (LF), because none of the components have been cooked.

Meat and other animal foods are often considered best to meet the human needs. This can be claimed not healthy as are also the vegan diets by some authorities. It is possible, however, to combine different plant items to provide balanced diets. At present various vegetarian diets are gaining more interest in western societies on ethical and/or nutritional basis.

Epidemiological studies have shown that the consumption of several plant foods has statistically significant association with lessened coronary heart disease risk [9]. In addition, the populations which eat mostly vegetarian diets may also have a lower risk for constipation, diverticular disease, gallstones and appendicitis [17]. Also the risk to die in some forms of cancer may be lowered [e.g. 47], although in some studies no difference has been found [17]. High meat diets seem to increase breast, bowel and prostate cancers [4]. Vegetarian men have lower incidence of prostate cancer than omnivorous males [6].

Most carbohydrates in the omnivorous diets come from the plant items. Lipid component varies a lot between the different plants, but usually their lipid content is low and the fatty acids are unsaturated. Protein content of plants is also low. On the other hand, the animal foods are rich in protein, but they can also contain high amounts of lipid with usually saturated fatty acids. Vegetable rich diets contain much fiber. Fiber promotes the gut function, but it may also affect the absorption of some nutrients [10, 13, 22, 26].

Plants are natural rich sources of antioxidants. Plants face heavy load of light. UV light generates radicals in their tissues. Furthermore during photosynthesis oxygen in statu nascendi is generated in their chloroplasts. When the plants are oxidizing nutrients, their mitochondria are releasing oxygen derived radicals as in the animal cells. The plants must also defend themselves against the attacks of microorganisms and animals. They use in this process radicals, too. All this means that plants must be very well prepared to meet the challenges of the oxygen radical stress. They contain therefore a broad variety of antioxidant chemicals in addition to the enzymes catalysing their interaction [42].

Metabolic radicals are either the cause or outcome of several disease processes. The plants in diet thus provide many kinds of compounds to help the antioxidant defence in man, although the enzymes of the plant tissues are destroyed during the digestion by the high concentration of hydrochloric acid in stomach and the proteolytic enzymes in our gastrointestinal tract.

We have studied the users of the living food in Finland. This diet has not earlier been scientifically studied although several books have been published for lay users. The diet has been developed by Wigmore [50] by personal experience.

The idea has been that by studying the extreme we learn something also about the basic physiology. The subjects have been either volunteers having interest to test the diet rheumatoid patients. Some rheumatoid patients had spontaneously adopted the living food diet, and they had provided anecdotal evidence on the benefits of that in their problems. Rheumatoid disorders disable significant numbers of people by pain and other symptoms. The pathophysiology of rheumatoid disorders is poorly understood. Inflammation is, however, related to the symptoms.

The aim of this presentation has been to give an overview on the effectiveness of the living food and some other vegetarian diets on the common risk factors of cardiovascular diseases and cancer as well as on the symptoms of rheumatoid arthritis and fibromyalgia patients.

Composition of living food

Living food is an extreme uncooked vegan diet developed by Wigmore [50] in United States. The diet consists of germinated seeds, cereals, sprouts, vegetables, fruits, berries and nuts. Some items are fermented and contain a lot of lactobacilli [23]. The diet contains no coffee, tea, alcohol and table salt. The diet provides a lot of fiber [15]. Diet has very low Na-content. It contains no cholesterol, and its lipids are rich in unsaturated fatty acids [15, 39, 40, 51].

Food items like berries and wheat grass juice are rich in antioxidants such as carotenoids and flavonoids. For example cranberry, bog whortleberry, lingonberry, black currant and crowberry have total contents of flavonols quercetin, kaempherol and myricetin (100–263 mg/kg) higher than the common fruits and vegetables, except onion and broccoli [14, 48].

Living food lowers risk factors of cardiovascular diseases

Obesity is one of the problems related to cardiovascular diseases. It hampers the general metabolism and means a stress to the pancreatic insulin secretion. Even if the living food diet provides equal amounts of energy with the omnivorous diet, the users lose weight under supervised conditions [15]. The fibromyalgic subjects who adopted LF diet and were eating it *ad libitum* at home lost also weight ($p=0.0001$) [16].

The living food diet contains no added sodium chloride. This was reflected to very low daily urinary exretion of sodium, when the subjects were on the living food diet [15, 27, 28]. Subjects eating LF had also lower blood pressure than their omnivorous controls [15]. This is in accordance with that the vegetarian diets are consistently associated with reduced blood pressure [49] and that diet rich in fruits, vegetables, nuts and low-fat dairy products (i.e. low in saturated fat and cholesterol), sugar and refined carbohydrate lowers blood pressure [44]. The intracellular sodium level in red cells as well as their sodium potassium cotransport activity, which increase in patients with hypertension, were lower in subjects having been eating LF for several years than in the omnivorous controls. They had also lower systolic and diastolic blood pressures than the controls [31].

As expected the serum cholesterol levels dropped, when the subjects were shifted to the living food diet, because it contains no cholesterol. The subjects having normal Finnish serum cholesterol levels showed about one millimole lower serum total cholesterol levels already within a week on the diet than before or their controls [15, 21].

The living food contains only plant lipids. Therefore the saturation degree of the fatty acids is much lower than the fatty acids in the normal Finnish diet [15, 51]. In the lipids of the red cell membranes of the long-term living food users the proportion n-3 fatty acids, eicosapentaenoic and docosahexenoic especially, radically diminished, although chloroplasts in plants in their food are rich in n-3 fatty acids. The ratio of n-3 to n-6 lowered, too [51]. It has been reported that the vegetarians also have significantly lower proportion of eicosapentaenoic and docosahexenoic acids in their plasma lipids, significantly shortened bleeding time and increased blood platelet count and *in vitro* platelet function. Plasma levels of all coagulation or fibrinolytic factors and natural inhibitors synthesized in the liver were lower in vegetarians than in controls [24].

All the plants contain high amounts of several vitamins. The subjects adopting living food diet obtain significantly higher amounts of carotenes, vitamins E and C. Their blood levels were also higher than in the omnivorous controls. The vegans got significantly less selenium than the omnivorous controls. The erythrocyte dismutase levels were higher in the blood of the vegans than their omnivorous controls [39, 40]. The high levels of both water and lipid phase antioxidants diminish the lipid peroxidation. The oxidized lipids increase the risk of cardiovascular diseases.

Vegetarians have significantly less oxidized LDL as wells as an increased total antioxidant status and molar ratio vitamin E/cholesterol which all indicate a more effective protection of lipoproteins against oxidation [27].

Fecal bacteria and health

Fermented living food items contain great numbers of lactobacilli [23]. As one could expect the number of lactobacilli increased also in the fecal samples, when the people started to eat the living food diet [43].

The LF diet caused also other changes in the fecal microflora. The cultivation of the fecal bacteria is very time consuming and difficult, if at all possible [33]. The easiest way to follow the shifts in flora is to analyse the lipid profiles of the fecal flora. These measurements indicated that the flora was very different after the subjects started to eat living food diet. The flora returned back again, when the subjects returned again to their previous omnivorous diet [34].

The intestinal microflora has a great capability to transform the substrates available in the gut. When the diet changes, also microflora changes as also the metabolism in the gut lumen.

When the subjects started to eat living food diet, we observed a radical change in the fecal urease level, which dropped to about one-third and returned back to the previous level after the readoption of the omnivorous diet. Urease hydrolyzes urea to ammonia, which is quite toxic. Also the fecal beta-glucuronidase activity fell. This enzyme hydrolyzes various glucuronides. For instance bilirubin diglucuronide is split and free bilirubin can again be reabsorbed and increase the chemical load of the liver. Bilirubin metabolism can also proceed after the removal of glucuronic acid recidues in the gut contents, and these products also can be absorbed. Glycocholic acid hydrolase activity lowered also after the adoption of the living food diet and returned back to the earlier level, when the subjects readopted their omnivorous diet [19]. When the bile component glycocholic acid is hydrolyzed by the microbial enzyme in the gut lumen, further metabolism of this steroid derivative by the gut bacteria is possible. The changes in the harmful bacterial enzymes may be due to the increase of lactobacilli in the gut contents, because their supplementation affects these enzyme levels [18, 20]. If less substrate is available, less metabolites are possibly produced. Some of the steroid metabolites are related to the carcinogens or compounds which are somehow related to the process leading to malignancy. The toxic ammonia can contribute to the lessened defence.

Gut bacteria convert aromatic amino acids of both exogenous and endogenous proteins to phenols. These phenols are toxic. The adoption of the living food diet caused a drop of both phenol and para-cresol levels in the blood as well as in the daily urinary output [15].

The rich fiber content of the living food increases the fecal volume and speed up the gut function [3, 13, 15]. Both of these probably also lessen the absorption of harmful metabolites from the gut contents. Delayed glucose absorption has been shown to be related to the dietary fiber [5].

Plant fibers can be converted to lignans and isoflavonic phytoestrogens by bacteria e.g. in the gut. These compounds have been suggested to lower the risk of breast, colon, and prostate cancers as well as coronary disease. Substates for their synthesis are available in unrefined grain products, various seeds, beans and probably also in pulses, peas and berries. Some types of fiber seem to influence sex hormone and bile acid metabolism mainly by partial interruption of the enterohepatic circulation, by alteration of intestinal metabolism and by increasing fecal excretion of these compounds [1, 2].

Symptoms of rheumatoid patients

The rheumatoid patients eating the LF diet reported significant amelioration of their pain ($p=0.03$), swelling of joints ($p=0.03$) and morning stiffness ($p=0.0008$) which all got worse after finishing FL diet. The composite indices (Disease Activity Score, DAS) [46] and Relative Activity Index, RAI [28] of objective measures showed also improvement of the rheumatoid arthritis patients during the intervention ($p=0.02$) [28, 29]. The disease activity in rheumatoid arthritis appeared to lessen in relation to the change in fecal microbial flora, when the subjects were the eating LF diet [29, 35].

When the fibromyalgic subjects adopted the living food, a number of positive results were recorded. The results on their joint stiffness ($p=0.001$) and pain (visual analogue scale) ($p=0.003$), on their quality of sleep ($p=0.0001$), on health assessment questionnaire ($p=0.03$) and on general health questionnaire ($p=0.021$) all improved [16].

Possible risks of vegan diets

It is well documented that vegan diets pose especially infants and young children to vitamin B-12 deficiency. This risk is relevant also for adult vegans. Manifestation of symptoms depends on several personal factors including time on vegan diet, the size of vitamin B-12 stores and the efficiency of the enterohepatic circulation [12].

In our studies the subjects having eaten living food for several years showed quite variable levels of serum vitamin B-12 [39, 40]. None of the subjects had any clinically detectable problems of vitamin B-12 deficiency even, when the measured serum level was low. Some subjects who had been on this diet for years showed normal vitamin B-12 levels [39, 40]. In spite of this LF eaters should not rely on their food as a sole source of vitamin B-12, but supplement their diet with this vitamin.

It has been found that there is a correlation between the level of vitamin B-12 and total plasma homocysteine. This and the increased platelet function may counteract the known cardiovascular health benefits of vegetarian diets [24].

Another big nutritional problem in vegan diet is the scanty supply of calcium combined with the low dietary intake of vitamin D [32]. This is especially harmful for growing children but also for vegans of all ages. Hence, careful individual dietary planning combined to calcium and vitamin D supplementation is recommended.

When studying all the common components of the living food we observed that the sea weeds of the diet can contain very variable and sometimes also low or high amounts of iodine. Some of the LF eaters had thus quite low and some high daily urinary output of iodine. None of them showed, however, any signs of thyroid dysfunction. The high intake of iodine from the sea weeds can mean a risk as well as the low intake from other components of the diet [38]. The increased risk of iodine deficiency with vegetarian nutrition has received also recently attention [41].

Discussion

Food is one of the basic starting points in the physiological health promotion. The different vegetarian diets are getting popular among people who are becoming more aware of their potential health advantages. Some adopt the vegetarian diets also from ethical reasons, because in the meat production the conversion of feed to product is usually poor and deterioration of nature is of general concern. Plant foods are often much richer in taste. Although there are a number of studies on vegetarian diets, much more information is needed. For the optimal diet the plants should be known by their components.

Wild herbivores rely in nature on plants only. It would be interesting to carefully study what they actually eat to learn more about the physiology of diets in general and to help the derivation of vegan or vegetarian diets for human populations in different geographic and climatic areas providing different plants.

The diet of people in affluent countries is not healthy as seen from the statistics of cardiovascular diseases and the occurrence of several forms of cancer. A deterioration

of health has also been observed in Japan and some other countries where the traditional diets have been abandoned and replaced by the western style eating habits.

Our studies show that one can elaborate vegan diets in such a way that no obvious symptoms of nutrient deficiency can clinically be observed. Furthermore even the vitamin B-12 levels can be quite normal. Because those having low vitamin B-12 levels in our studies had no signs of deficiency, it is possible that other components of the diet can compensate the low intake of vitamin B-12. It is known that some fermented foods may contain minute amounts of biologically active vitamin B-12 [11].

The adaptation to the low intake of some other food components can also be speculated.

The rather low intake of calcium in vegetarian diet can be compensated by the low intake of sodium chloride, because the sodium excretion in urine is related to the calcium output ie the lower sodium output can save the calcium stores in the body [7, 30].

One can conclude from our studies that the vegan diet can be composed so that the level of several risk factors of cardiovascular diseases as well carcinoma are significantly lowered. The patients of rheumatoid diseases appear to benefit both subjectively and objectively from the vegan diet rich in fiber, various antioxidants and lactobacilli.

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Vitality diagnostics and preventive medicine for the well-being of people at advanced age

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This paper emphasises the importance of vitality diagnostics in relation to healthy ageing and prevention of age-associated diseases. Ageing, reducing the reserve capacity, decreases the adaptability of various systems and increases the risk of functional disorders. The early recognition and treatment of functional disorders in vitality diagnostics laboratories provides an opportunity to prevent or delay the onset of degenerative diseases related to advanced age.

Keywords: vitality diagnostics, preventive medicine, well-being of people, advanced age, reserve capacity, functional disorders

This paper emphasises the importance of vitality diagnostics laboratories in relation to healthy ageing and prevention of age-associated diseases. What is the difference between a department of geriatrics and another one which deals with patients suffering from chronic illnesses? A department cannot be considered as a department of geriatrics just because of the advanced age and chronic illnesses of the patients. Nowadays the activity of a department of geriatrics includes not only the adequate and special treatment of elderly patients, but also the determination of their biological age,

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general biological status and vitality [1, 2]. These departments take into consideration the relationship between vitality and

- actual diseases,
- the risk of additional diseases,
- the possible efficiency of rehabilitation.

The chronological and biological age of elderly people are not necessarily coincident with each other. Sometimes the difference between them is very significant. The determination of biological age gives valuable information to basic health care and special treatment of elderly people.

Vitality diagnostics [3] includes, under laboratory conditions, the assessment of

- circulatory (blood pressure, cardiac index) and respiratory (vital capacity, PO_2) system,
- general metabolic parameters (blood glucose, uric acid, cholesterol, triglyceride, homocysteine etc.),
- sensory impairments (hearing, vision),
- osteoporosis,
- sleep disorders,
- affective functions,
- cognitive functions.

Vitality diagnostics does not mean a number of mechanical or static measurements, but it means a dynamic and functional assessment, in other words, the assessment of adaptability of various organs and the whole organism to standardised stimuli [5]. It is well known that ageing, reducing the reserve capacity, decreases the adaptability of various systems and finally the whole organism [4]. Remarkable individual differences have been found in this diminution. Individuals with a better adaptability than the average value of their age group have higher vitality and can be considered younger in biological sense. On the other hand, in individuals with decreased adaptability as compared to the average value of their age group, the early detection and treatment of functional disorders can prevent the clinical manifestation.

Functional disorders, to a certain extent, can be treated without any medical or pharmacological interventions, with a modification in the life style [6]. In elderly people with definitive diseases the assessment of vitality and adaptability may have an additional advantage as well. It can be helpful in estimating the perspectives and planing the strategy of rehabilitation. The up-to-date gerontology has a preventive view. Vitality diagnostics can be advisable even from the age of 35 years. In many cases the early recognition of functional disorders provides an opportunity to prevent or delay the onset of degenerative diseases related to advanced age.

Geriatric centres play a very important role not only in the therapy but also in the prevention of age-associated diseases and rehabilitation of elderly patients. These geriatric centres have three basic functions: medical care, research and education. In this multidimensional structure of geriatric centres, vitality diagnostics laboratories play an integrating role (Fig. 1).

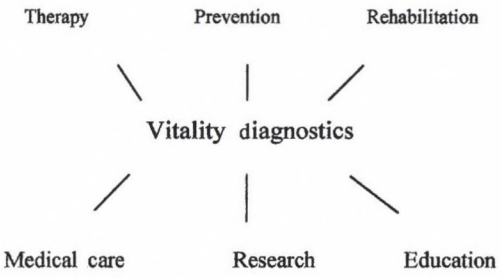

Fig. 1. Vitality diagnostics play an integrating role in the research and education of ageing as well as in the medical care of elderly people

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Atherosclerosis risk factors in children of high risk families

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The **Objective** of this study was to determine the frequency of care reactive atherosclerosis risk factors in children of parents with premature coronary heart disease observed before their 45 years of age for the promotion of the effectivity of the preventive work started in childhood and adolescent ages. **Methods.** Height and weight was measured. Body mass index (BMI) was calculated. Fat analysis was performed in children with overweight. Blood pressure was measured and both 24 hour monitoring and fundoscopy were performed in cases with a blood pressure higher than 90 centile values. Fasting blood sugar (BS) level was measured. Oral glucose tolerance test was made in cases with a fasting BS level higher than 5 mmol/l. Serum total cholesterol (TC), HDL-cholesterol (HDLC) and total triglyceride (TT) levels were measured and LDL-cholesterol (LDLC) level was calculated. The plasma thiobarbituric acid reactive system (TBARS) was investigated. Statistical analyses were performed by χ^2 and Student *t*-probes. Data of 1140 offsprings and 457 referents without any high atherosclerotic risk family history were analyzed. **Results.** BMI of 87 offsprings was higher than the 90 centile value. The fat percent of the body of these children was higher than 40. The blood pressure of 311 children and adolescents was higher than the 90 centile value. Fasting BS level was higher than 5 mmol/l in 47 cases 17 of them showed a pathologic oral glucose tolerance test. High serum TC level was observed in 67 cases, high serum TT level was found in 8 cases. 245 offsprings had a low serum HDLC level.

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The plasma TBARS level was high in 241 cases. Data of referents differed significantly from those of offsprings. Their serum TC, LDLC levels and plasma TBARS level were lower, serum HDLC level was higher than that of children and adolescents with high risk atherosclerotic family history. **Conclusion.** Risk factors of atherosclerosis are detectable in children and adolescents of high risk families. The measurement of these factors may help the effectivity of the preventive work.

Keywords: atherosclerosis, prevention, childhood, adolescent ages

Atherosclerosis starts in childhood [5]. Some risk factors as the high serum total cholesterol (TC) and/or low serum high density lipoprotein cholesterol (HDLC), high serum total triglyceride (TT), high blood pressure (BP) levels and/or overweight were detected in childhood and adolescent ages [2]. Earlier we found high plasma thiobarbituric acid reactive system (TBARS) levels as a marker of the higher lipid peroxidation in children, too [9]. The early detection followed by an useful intervention may prevent the later development of the atherosclerosis [3]. The risk factor screening of all children and adolescents (mass approach) is very expansive why the investigation of offsprings of parents with premature coronary heart disease (CHD) for the effective prevention was advised [6]. The detection of the frequency of care reactive risk factors (risk factors expected to change after an effective intervention) in these populations may promote the effectivity of the paediatric preventive work.

Methods

Data of 1140, 3–18 year-old children and adolescents whose parents' CHD was detected by ECG and enzymatic methods before their 45 years of age were analyzed and compared to data of 457 age matched children and adolescents without any high risk family history. Height and weight were measured and body mass index (BMI) was calculated. Fat analysis was performed by a BT 905 Skylark analyzer in cases with a BMI higher than 90 centile. Blood pressure was measured. Children and adolescents with a blood pressure higher than the 90 centile value were investigated by both methods, they were monitored during 24 hours by a Meditech 002 ambulatory blood pressure monitor (ABPM) and their fundus of the eye was investigated by a non-midriatic Topcon fundus camera connected with a computer with a version 1.53 software. After an overnight fast blood was collected from the cubital vein of children and adolescents. Blood sugar (BS), serum TC, HDLC, TT and plasma TBARS levels were measured by enzymatic methods and fluorimetric method described by Yagi [10], respectively. The serum low-density lipoprotein cholesterol (LDLC) level was calculated by Friedewald [4]. Glucose tolerance test was performed in cases with a BS level higher than 5 mmol/l.

The frequency of high BS (above 5 mmol/l), high blood pressure (above 90 centile), high BMI (above 90 centile), high serum TC (above 5.2 mmol/l), TT (above 1.4 mmol/l), LDLC (above 3.5 mmol/l), low HDLC (below 0.9 mmol/l), high plasma TBARS (above 2.5 nmol/ml), high fat percent (above 40%) levels and pathologic glucose tolerance test or fundus was measured and the offsprings' frequency was compared to that of referents by χ^2 probe.

BS, serum TC, TT, HDLC, LDLC, plasma TBARS mean levels of offsprings and referents were compared by Student *t*-probe.

Results

Frequency of the high BMI and fasting BS values above 5 mmol/l was similar in both the offsprings and referents. The fat percentage of children and adolescents with high BMI was more than 40. The ratio of children and adolescents with pathologic glucose tolerance tests was similar in both group, too (Table I). High serum TT level was found very rarely and the frequency was nearly the same in both groups (Table II).

High serum TC, LDLC and low HDLC levels were more frequent in offsprings than in referents. The high plasma TBARS levels were found more frequently in offsprings, too (Table II).

Table I

Frequency of the high BMI and BS levels of children

Determined values	Offsprings number of patients	% of inv. pop.	Referents number of patients	% of inv. pop.	Statistical probability
BS > 5 mmol/l	47	3	21	4	$p > 0.5$
Pathologic glucose tol. test	17	35*	8	35*	$p > 0.5$
BMI > 90 centile	87	8	47	10	$p > 0.5$

* % of the patients with high BS

Table II

Frequency of the high serum TC, TT, LDLC and plasma TBARS and low HDLC levels

Determined values	Offsprings number of patients	% of inv. pop.	Referents number of patients	% of inv. pop.	Statistical probability
Serum TC >5.2 mmol/l	67	6	4	1	$0.05 > p > 0.02$
Serum TT >1.4 mmol/l	8	1	11	1	$p > 0.5$
Serum LDLC >3.5 mmol/l	63	6	5	1	$0.05 > p > 0.02$
Serum HDLC <0.9 mmol/l	245	21	12	3	$0.01 > p$
Plasma TBARS >2.5 nmol/ml	241	21	9	2	$0.01 > p$

Elevated blood pressure levels detected during 24 hours were found more frequently in offsprings than in referents. The fundus of the eye showed pathologic signs in about 1/3 of these cases (Table III).

The mean serum TC, LDLC and plasma TBARS levels were significantly higher, and the serum HDLC levels were significantly lower in offsprings than that in referents (Table IV).

Table III

Frequency of the high blood pressure and pathologic fundus of the eye

Determined values	Offsprings number of patients	% of inv. pop.	Referents number of patients	% of inv. pop.	Statistical probability
Blood pressure > 90 centile value	311	27	36	8	$0.05 > p > 0.02$
Continuously high blood pressure (as a result of the 24 hour monitoring)	121	11	9	2	$0.02 > p > 0.01$
Pathologic signs on the fundus of the eye	42	35*	2	22*	$p > 0.5$

* % of patients with continuously high blood pressure

Table IV

Mean serum lipid and plasma TBARS levels

Investigated parameters	Offsprings	Referents	Statistical probability
Serum TC (mmol/l)	5.1 ± 0.7	3.9 ± 0.5	$0.05 > p > 0.02$
Serum TT (mmol/l)	0.8 ± 0.2	0.9 ± 0.2	$p > 0.5$
Serum LDLC (mmol/l)	3.7 ± 0.5	2.7 ± 0.4	$0.05 > p > 0.02$
Serum HDLC (mmol/l)	1.1 ± 0.3	1.4 ± 0.2	$0.02 > p > 0.01$
Plasma TBARS (nmol/ml)	3.2 ± 0.4	1.9 ± 0.2	$0.001 > p$

Discussion and conclusion

The higher frequency of the high blood pressure, serum TC, LDLC and plasma TBARS levels and low serum HDLC levels in offsprings of parents with premature CHD reinforces the presence of the atherosclerosis risk factors in childhood and adolescence and underlines the importance of the screening of these children for the early detection and the preventive care as soon as possible similarly to other countries [7] in Hungary, too. The high plasma TBARS levels suggest the supplementary effect of antioxidant vitamins and trace elements to the cholesterol lowering dietotherapy [8]. The low serum HDLC levels suggest the important role of the physical excercise in the preventive care [1].

The similar frequency of the higher fasting BS levels, pathologic glucose tolerance tests and BMI levels in both groups show a wider importance of these factors in children and adolescents with or without high atherosclerotic risk family history.

The ABPM seems to be very useful to differ children with real higher blood pressure from that with so-called "white coat" hypertension [10].

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Behavioural medicine in health promotion

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Behavioural medicine is a rapidly developing interdisciplinary field that integrates the physiological and psychosocial aspects of human behaviour and applies them to prevention. In the early stage of chronic non-infectious illnesses of great epidemiological significance the most important risk factors are the reversible psychophysiological regulation disturbances. According to the behavioural medicine model depressive symptomatology, hopelessness, anxiety, non-adaptive ways of coping, dysfunctional attitudes are common risk factors in the background of self-destructive behavioural disturbances, such as smoking, alcohol and drug abuse and suicidal behaviour. The basic link between physiological and psychological phenomena is the decision making process, the cognitive appraisal, evaluation of the given situation, which is very subjective and depends on the socialization process. The modern civilised way of life continuously creates situations in which we experience loss of control, and therefore the psychological and physiological balance can only be obtained with great difficulty. Especially under conditions of sudden cultural and socioeconomic transition strengthening adaptive ways of coping and preventing emotional disturbances are fundamental in health promotion.

Keywords: psychophysiology, behavioural medicine, health promotion, prevention, risk factors, hopelessness, anxiety, behavioural disturbances

Over the last two centuries, although technology and civilisation have achieved unbelievable successes, little of attention has been given to what effect these absolutely new historical "experiments" have had on man. Modern society has broken up the foundations of those relationships and processes, which are of vital importance from the

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point of view of human personality development. The process of sociocultural identification, the passing of values, the learning process of adaptive ways of coping became highly disturbed.

Game theory model

The individual can be considered as one of the players, in a game theory model, where the other player is the natural and social environment. The environment tries to impose its own demands and conditions on the individual, while he is an active being who shapes his surroundings and tries to achieve his aims and values.

For the human being attitudes, values, perceived self-efficacy and self-esteem, which were developed during the socialization, dominate in controlling the actual behaviour with the same or greater force as conservation of the physiological balance. The modern, civilised way of life continuously creates situations in which the psychological and physiological balance can only be maintained with great difficulty. This interdependent control, especially under conditions of sudden cultural and socioeconomic transition might result in the sense of loss of control, not only in individuals, but in masses of people [2].

Decision-making process

Figure 1 shows the schematic of the decision making process controlling behaviour in the system of man and his environment. In every waking moment of our life we have to decide whether we are capable of accomplishing the given environmental expectations, whether we are capable of solving given situations on the basis of information from our previous experiences stored in our memory. In actual fact we do not examine the harmony between the objective expectations and our real capacities, but only that which we realize from it.

The basic characteristic of a healthy personality is the ability to judge his own capacities and the expectations of the environment. From the point of view of emotional and behavioural disturbances the degree of reality of the subjective evaluation of the given life situation is fundamental.

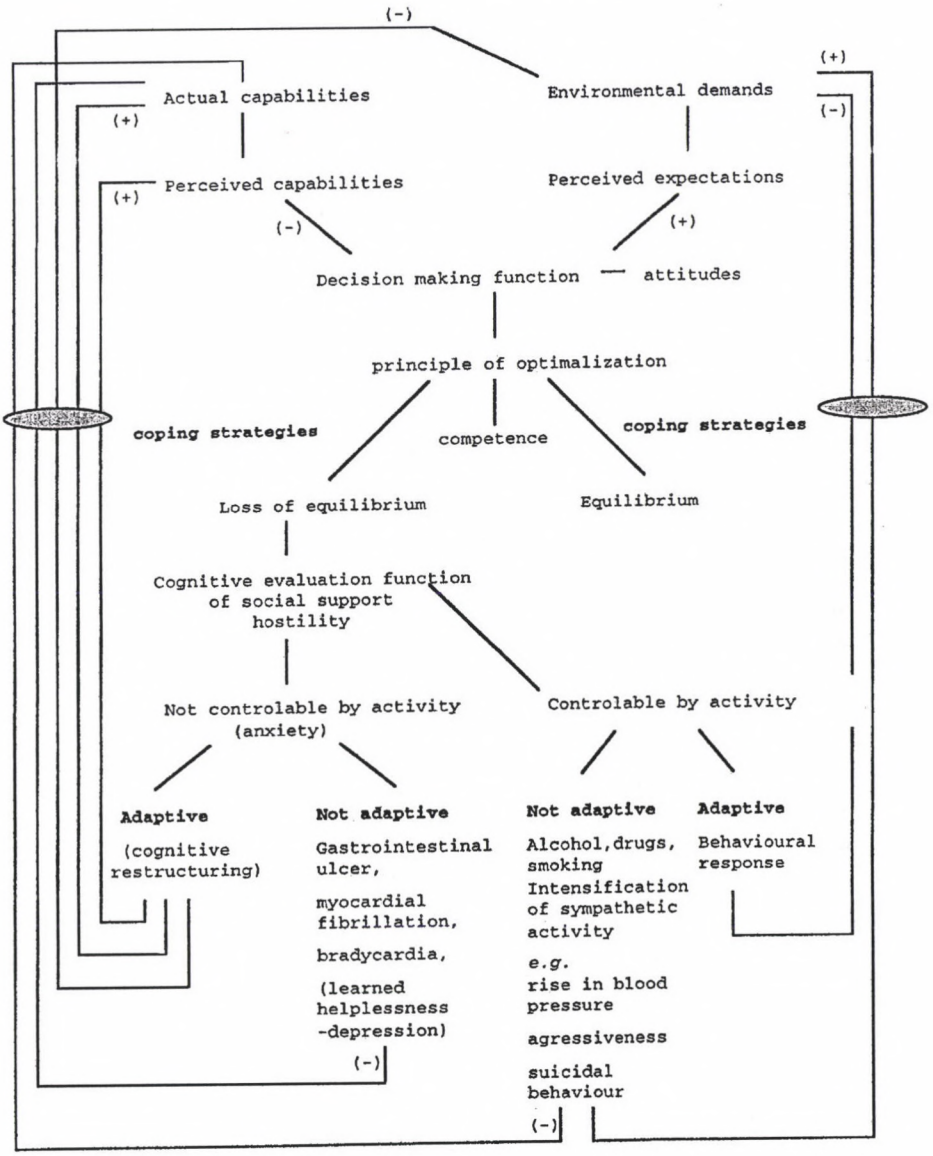
(-)
(+)
(-)
Actual capabilities
Environmental demands
(+)
(+)
Perceived capabilities
Perceived expectations
(-)
(+)
Decision making function — attitudes
principle of optimalization
coping strategies
competence
coping strategies
Loss of equilibrium
Equilibrium
Cognitive evaluation function
of social support
hostility
Not controlable by activity
(anxiety)
Controlable by activity
Adaptive
(cognitive
restructuring)
Not adaptive
Gastrointestinal
ulcer,
myocardial
fibrillation,
bradycardia,
(learned
helplessness
-depression)
(-)
Not adaptive
Alcohol,drugs,
smoking
Intensification
of sympathetic
activity
e.g.
rise in blood
pressure
agressiveness
suicidal
behaviour
(-)
Adaptive
Behavioural
response

Fig. 1.

Cognitive appraisal and psychophysiology

The cognitive evaluation, that is the appraisal of the situation means, that we compare our perceived capabilities which are available to solve the situation with the perceived environmental expectations, and decide on the basis of our previous experiences whether we are capable of solving the problem.

This appraisal process is the basic connecting point of psychological and physiological phenomena, psychophysiology, since the environmental effects can cause physiological symptoms only through cognitive appraisal. If we interpret a situation as dangerous, if we react with an alarm reaction – independent of the degree of danger present in a situation – the sequence of vegetative reactions develops. Our image of our actual self is formed continuously by the influence of environmental feedback, we classify ourselves according to this feedback. During socialization, the complex learning process forms what we would like to become, what our self-ideal is, what values we accept and with which values we identify ourselves. In each life situation, we compare it with the self-ideal, whether we can come up to the expectations demanded of us. If somebody makes unrealistic demands of himself, then he can find himself facing unaccomplishable tasks and hence always classifies himself negatively. If somebody expects some kind of ideal behaviour from his environment the reality rarely matches these expectations.

Self-esteem, self-efficacy and competency as optimalization principles

The experience and demand for successful and purposeful behaviour and the ability to control our own situations is called self-efficacy or competence – this is the most generally formulated optimalisation basic principle, towards which man is striving with his behavioural decisions. Naturally personal elements of perceived self-efficacy or competence optimalisation are very different. For an Indian fakir competence can mean the highest level of meditation, for an American businessman business success and for a person who from childhood grew up in "video-company", without real relationships, the optimalisation of consumption. So the same situation, in which one person classifies himself negatively, can be a goal for another. Hence, for example, a man from a consumer society wants to avoid suffering by all means, whilst on the contrary the humanistic culture considers the voluntary willingness to sacrifice oneself for others a basic value.

Coping strategies (3)

Figure 1 shows that both the behavioural patterns and the cognitive feedback are characteristic of the person's conflict solving ability, the so-called coping strategy. These conflict solving or coping strategies develop through socialization. It is obvious from the model that the non-adaptive conflict solving strategies play an important role in causing, maintaining and recrudescing physical symptoms and illnesses of psychological origin. During sudden cultural and socioeconomic transition there is a need for change in attitudes, ways of coping, therefore in such a situation only people with flexible coping resources can adapt successfully.

Pathological alarm reactions – anxiety, depression

The mean feature of anxiety is the subjective experience of loss of control in an emotionally negative situation. If we consider the ability to control our situation as the basic principle or optimalisation principle of behavioural regulation, it is evident that the conditions of loss of control or anxiety are the basic background factors of behavioural disorders. In animal experiments the consequences of prolonged loss of control are gastro-intestinal ulcerations, cardiac arrythmias, bradycardia and lastly the condition of "learned helplessness", which is considered to be the best animal model of depression [5].

In such a situation the cardiovascular and metabolic response might dissociate, as Cannon [1] had already described: "The organism prepares for action and, should the increased preparedness endure without the development of activity, this can be followed by catastrophic consequences."

The depressive condition affects perceived state of health and can lead to disability even without organic illness. Depression has a very close relationship with self-destructive behaviours, such as smoking and alcohol consumption, and suicidal behaviour is especially common among depressive people. Those suffering from permanent mood disorder and depression are more vulnerable to various diseases and are less able to improve their social situation, so that they easily fall into a sustained vicious cycle. In recent decades, depression, "vital exhaustion" and hopelessness has been identified as important independent risk factor to coronary disease. Learned helplessness or learned hopelessness, which can be regarded as the most appropriate model for depression, is associated with decreased immunological activity and affects tumour growth, and vulnerability to various infections [6].

Pathological anxiety

The interpretation of a given situation is very subjective. It depends on inherited abilities, previous life events and the current condition. There are some situations, which cause anxiety for everybody, in which case a realistic judgement of the situation would classify it as unsolvable or uncontrollable by action. Such a situation could be our own or a close relative's grave illness, or the situation in which the inhabitants of Sarajevo found themselves during the civil war.

Anxiety becomes pathological if certain situations unrealistically produce an alarm reaction, if we classify our situation as unsolvable or uncontrollable by action even when, in reality, it is not from an objective judgement threatening, or could be controllable or solvable by action. The various different schools of psychotherapy endeavour to treat such unrealistic alarm reactions in many different ways. Some of them, such as psychoanalysis, attempt it by a radical restructuring of the personality, whilst others, such as the cognitive approach, attempt it by correction of the unrealistic appraisal or interpretation.

Non-adaptive active control strategies, alcohol, drug, self-destructive behaviour

According to Figure 1, if a standard is set too high by the self-ideal this can cause non-adaptive behaviour patterns. The essence of this is that by sacrificing the physiological balance of the organism for a long time, we create the psychological balance. The most common non-adaptive coping strategy is using drugs or alcohol to re-establish the cognitive balance. Negative emotional states, the avoidance of anxiety and achieving control over our situation are very powerful motivational factors. All those responses, which intensify this condition, or the feeling of control over our situation, can remain and get permanently fixed as conditioned responses. Other non-adaptive active behavioural reactions are those which assure the person's cognitive evaluation balance with an abnormal increase of sympathetic activity. In this case the psychological expectations of ourselves, as an inner compulsion, assert themselves with greater efficacy in shaping our way of living than does preserving the physiological balance.

On the basis of the above behavioural medicine model of human behavioural regulation, preventing depression and strengthening the adaptive ways of coping are fundamental in effective health promotion.

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Is it reasonable to involve kinesiology in medical education?

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The purpose of this study is to argue for teaching kinesiology for medical students. To become familiar with the basics of the science of human motions is essential for the medical practice. There is substantial evidence that regular physical exercise is beneficial to improve health and prevent disease, and has a key place among the strategies in therapy and rehabilitation. However, before prescription of exercise programmes the physician must take into consideration all the benefits and risks of that exercise. The present curriculum offers insufficient information about the physiological effects and physiologically essential limits of physical exercise. Therefore, a regular study of human motions is required by the medical practice. The need for such education is also shown by the everlasting interest of medical students in credit courses of kinesiology and sports physiology.

Keywords: kinesiology, medical education, physical exercise, therapy and rehabilitation, medical practice, sports physiology

Machines have taken so "comfortable" our life that we are the most physically inactive generation that ever lived; we spend most of our lives immobile. We sit while we work, we even sit during our recreational hours (for example driving our car), we punch the TV remote control instead of standing up and walking a few steps to change channels. However, it turned out that this lifestyle is risky for our physical and psychological well-being. The increase in the different diseases related to the reduced physical activity arises new sort of tasks also for the physician. Nowadays our medical

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education does not give sufficient assistance to manage these problems. Certain basic and clinical disciplines direct the student's attention toward the effects of regular physical exercise. However, these annotations do not create a synthesised knowledge of kinesiology that should help the preventive-therapeutic work of the physician. To improve the factual knowledge is probably easier than to establish an attitude towards the utility of physical exercise in medical practice. The physician's way of thinking is based primarily on medical education. A topic that is not included in the medical education is not able to influence conspicuously the physician's attitude. Therefore, a well-defined subject of the science of human motions is essential in the medical curriculum.

The place of physical exercise in medical practice

The regular physical exercise plays a key role in prevention, treatment and rehabilitation of various diseases [3, 5, 8, 10].

Nowadays most, if not all physicians regard regular physical exercise as effective tool in maintaining general physical and psychological health. The regular physical activity reduces the morbidity and mortality for conditions such as coronary heart disease, hypertension, obesity, diabetes, osteoporosis and mental problems (depression, anxiety, etc.). However the benefits of physical activity must be weighed against its potential adverse effects, which include injury, osteoarthritis, myocardial infarction, and, rarely, sudden death.

Besides its preventive role, the physical activity is essential part of therapeutic exercises to improve motion, strength, motor control and function. Physical activity is the most effective physiological stimulant. Therefore it is used successfully to treat also psychological problems (e.g. depression).

Physical exercises are involved in special programs of rehabilitation.

It is important to emphasize that any physical exercise prescription is advantageous only if it is based on its physiological effects and limitations.

The physician's concern for kinesiology

In spite of the importance of physical exercise in prevention, treatment and rehabilitation of certain diseases, the prescription of exercise programmes, and the control of their effects on the participant is often related to other specialists (physical education instructors, kinesiologists, physical therapists, etc.). Evidently, the work of these specialists is essential but it does not reduce the physician's responsibility for the

patient's health. The physician who says to his patient that "you need more physical activity", but does not explain the expected benefits and risks of exercising, makes as if he says to the patient that "you need some medicaments" without giving any specification.

The first important obligation of the physician is the counseling (Table I).

Many people need information about the benefits of exercise to them and personal advice about safe and suitable programmes. According to an investigation among adults living in London [9] only 11% men and 6% of women had ever received any such advice. Guidelines or recommendations have been established to help to increase the efficacy and reduce the risks of the physical activity [1, 6]. An emphasis on regular, moderate-intensity physical activity rather than on rigorous exercise is reasonable in sedentary persons.

Before beginning any exercise program the person should have a complete medical evaluation. The physician should determine the patient's psychophysical condition, and provide information on the expected effects of physical activity. It is advantageous to define the individual's goal of exercising. The physician may then assist the person in selecting appropriate types of physical activity. The exercise prescription involves the mode or type of exercise and the limits (frequency, duration and intensity of the exercise bout). The changes in person's health status should be controlled by periodic medical evaluations.

The credit course

Sports Physiology courses for medical students have been held at Medical University of Pécs since 1978, and Kinesiology courses since 1997 (Table II). The number of participants in both courses was 10±2% of the total second year class (15–30 students) in each academic year. At the end of the course they have to pass a written exam to receive credit points. The school achievement of the participants was 3.5±0.5 after the second year. It shows that the students having good-average results were interested in these courses rather than the excellents or the weaks.

Table I

Questions to be answered during medical counseling

1.	Diagnosis: What is the health status of the patient?
2.	Is beneficial any regular physical exercise in his/her present condition?
3.	What effects of the regular physical exercise are expected to improve the patient's health?
4.	What type of exercise is the most effective?
5.	What conditions and limits of exercise (intensity, frequency, duration) are advantageous?

The motives for participation were:

- to accumulate credit points,
- to pass more easily the final exam of physiology,
- to prepare the clinical studies,
- to use this knowledge later in practice as a medical doctor,
- being an active athlete, to improve his or her sport performance.

The students were satisfied with this theoretical program, but they wanted to do also manual practices. Therefore we prepare appropriate practices, e.g. special measurements and examinations to corroborate the theoretical lectures. Data in the literature also show the increased interest in practical work both among medical students and residents [2, 4, 7]. It indicates that even the students feel the need for the basics in the science of human motion.

Table II

The topics of the sports physiology and kinesiology credit courses

Sports physiology	Kinesiology
What is sports physiology?	Kinesiology, the science of human movements. Basic kinesiology, applied and clinical kinesiology.
Neuromuscular aspects of exercise. Neuro-humoral regulation.	Structural basis of the human movements.
Exercise metabolism.	Kinematics of the human movements.
Physical activity and the circulation.	Kinetics of the human movements.
Physical activity and the respiration.	Elementary motor patterns. Posture and locomotion.
Sports and stress.	Motor control. Motor learning.
Goals and physiological effects. of special types of training.	Integration of movement: sensation, action and performance.
Factors influencing human performance.	Motor behaviour.
Health, physical activity and sports.	Health benefits of exercise.
Principles of measurement in sports physiology.	Motor disturbances. The essentials of clinical kinesiology.

Conclusions

Both the developments of medical sciences and the health condition of the population suggest newer and newer topics for the medical education. It is a high responsibility of the university professors to improve continuously the medical education. However, it is very difficult to decide what is essential and what is unnecessary for the graduate medical curriculum having limited number of hours. However, some new teaching techniques and organisations may help the judgement about the recommended subjects. Useful data might be obtained from the optional credit

courses. The selection of credit courses by the students is a good test for the new subject. Our experiences, especially the continuous interests of the students, support the idea that medical education should involve the basics of the science of human motions.

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The way teaching human physiology can support primary prevention

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In this article, a rationale is provided to prove that teaching human physiology – according to its definition and characteristics – could effectively support also the cause of health culture and prevention by establishing an appropriate, high level scientific background knowledge in the medical schools. The concept of a reform curriculum of integrative physiology developed in order to serve this task is outlined. An international cooperation is suggested to develope further this concept.

Keywords: teaching, human physiology, primary prevention, health culture, medical schools, integrative physiology, international cooperation

Definition of human (medical) physiology

Human physiology studies the functions of healthy living organism at rest and the changes that occur during activity. The word "physiology" is derived from the Greek words "physis" and "logos" which mean "inquiry into nature". It is also called the Logic of Life.

Traditionally, teaching physiology in medical schools is expected to provide an integrated, firm scientific base for studying later, in preclinical and clinical disciplines, the pathological functions of the human organism, their diagnosis, as well as their

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therapy. In addition – as we suggest in this article – physiology should be recognized as an obligate background knowledge *also for health promotion and protection, which is called primary prevention.*

Integrative and quantitative characteristics of physiology

Physiology applies an *integrative approach* to analyze and understand the processes, relationships, and regulatory mechanisms that reflect the functions of the organism at the hierarchical and also at the heterarchical organisation levels. Hence, physiology, a basically integrative life science, comprises knowledge acquired at a number of levels: submolecular, molecular, subcellular, cellular, tissue, organ, organ system, and organismic ones.

From such an approach, this discipline raises a number of important questions and searches for the right answers:

- How does the human organism function as a whole and in its subsystems?
- What are the mechanisms underlying the normal somatomotor, visceromotor, and psychomotor functions of the organism?
- How can the organism maintain its normal functions against internal and external "disturbances" of various extent, and how does it remain healthy?
- What is the nature of processes and parameters that characterize normal healthy functions?
- What are the short-, intermediate-, and long-term regulatory mechanisms of normal bodily processes?
- At which magnitude of displacement, velocity, rate, incidence, frequency, amplitude, concentration, do the above processes normally occur in the body? etc.

The nature of these questions imply that integrative knowledge of physiology also requires a *quantitative approach.* Since all human beings are different, and individual physiological values are statistical variables, the so-called "normal values" we teach generally hold true only for a hypothetical, actually non-existing "standard" subject. Thus, while creating a quantitative scientific outlook or generalizing details about the human species, individual features should also be considered. This dialectical integrity of the general laws and the individual properties is one of the exciting phylosophical beauties of physiology.

Experimental nature of physiology

Basically, human physiology is an *experimental science.* However, apart from carefully planned laboratory experiments performed with sophisticated equipment and data processing, the role of direct observations in acquisition of knowledge is still of value. Although animal models play a crucial role in the development of physiological knowledge and in the education of medical students, the dramatically fast development of non-invasive methods has enabled scientists to perform more and more reliable studies on healthy human "experimental subjects". In addition, computer simulation and experimental animation also play an increasing part in physiological research and teaching.

Relationship of physiology to life sciences

Physiological science covers a wide field of life sciences, including human, clinical, sport, pathological, veterinary, botanical, and comparative branches of physiology, in which human physiology occupies only a relatively small part. Following the principle of the entity of structure and function, physiological science is closely related to morphology, as well as to behavioral and to population sciences (Fig. 1). Through its analytical research methods, physiology is related to biochemistry, molecular biology, and biophysics, whereas the integrative (system) nature links this science with general biology, biomedical engineering, control theory and informatics.

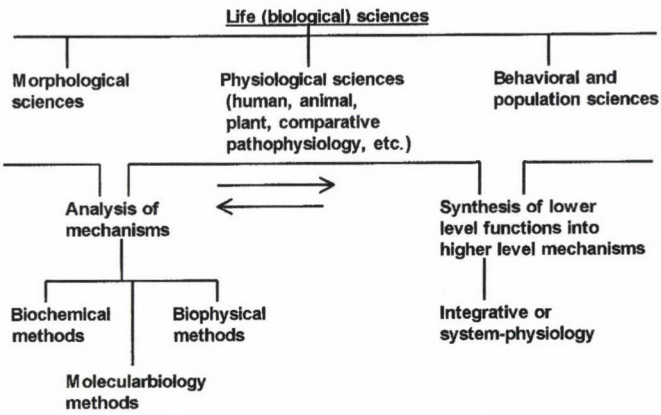

Fig. 1. Relationship of physiology to other branches of life sciences

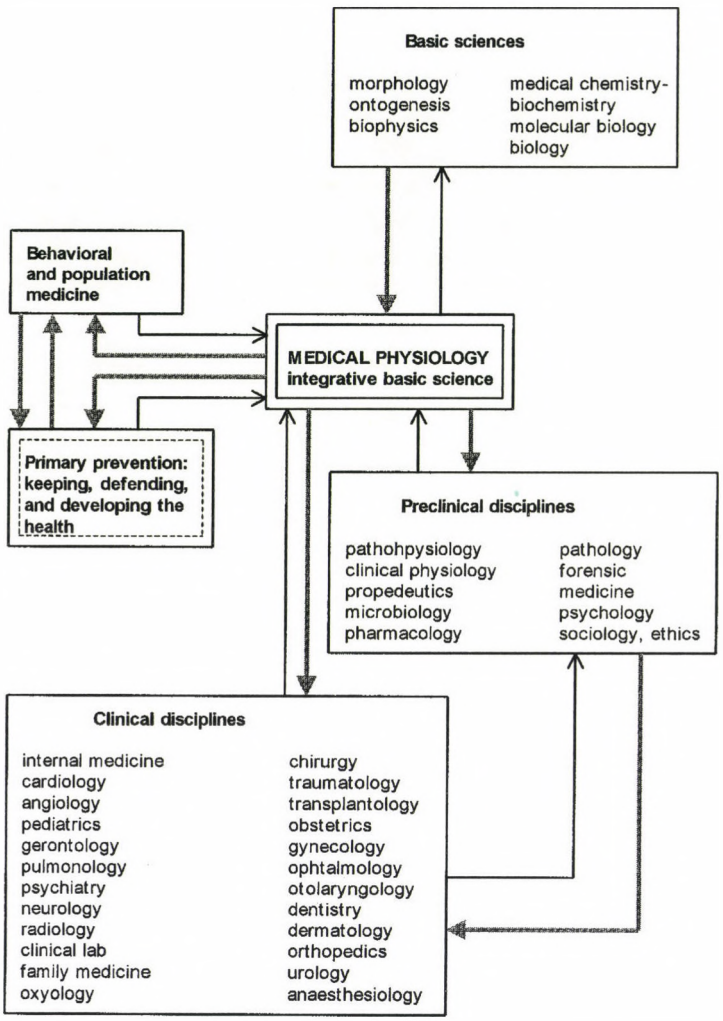

Fig. 2. The integrative interaction of medical physiology with other medical disciplines in the medical curriculum

The integrative role of physiology in medical education

As an integrative link between different basic and clinical disciplines, physiology plays an outstanding key role in the medical curriculum. (For some details see the block diagram in Fig. 2.). While physiology education is substantially based on the methods

and data of morphology, embryology, biophysics, biochemistry, and molecular biology, medical physiology itself remains an important integrative subject, that introduces and supports the teaching of preclinical and clinical disciplines. These disciplines, of course, feed back positively to the developement of physiological knowledge. Lately, a positive interaction started to develope between teaching physiology and such behavioral and population sciences as hygiene, public health, health management, behavioral physiology, and medical psychology.

Teaching physiology and primary prevention

In contrast to the multiple interactions of the above disciplines, scarcely any effort has been made worldwide to combine teaching physiology with the theoretical basis of health promotion, prevention, and health culture (=primary prevention) during the preparatory phase of medical education. The main lines of basic and clinical professional trainings in medical schools are focussed primarily on diseases (that is they are *disease-oriented*); and in addition, the existing controversial health financing systems prompt physicians themselves to become dominantly *disease-interested* all over the world.

Recently, in our Department, we decided to develope and introduce a reform curriculum of physiology in order to convince young medical students, the future doctors in their "imprinting age", that working for primary prevention is just as important moral obligation of the medical practice as treating diseased patients. In addition, such efforts may have also important economic implications, because early investments in health promotion and prevention will be returned to the society with substantial profit in the long run. We believe that physiology – in accordance with the definition we gave in the first paragraph – can be regarded as the most reliable *allied force* of those who are ready to fight for health protection and prevention.

Major features of our reform curriculum involve, chapter by chapter of the integrative physiology, the explicit identification and explanation of those scientific evidences (e.g. data, mechanisms, interactions, etc.) which may highlight their close relation to some important aspects of primary prevention. Special emphasis is placed on the evidences which closely relate to the following components of health protection and promotion:

- Regular physical exercise of adequate intensity;
- Hygiene of the body and bodily functions;
- Psychological (mental) hygiene;
- Healthy nutrition including both quantitative and qualitative aspects;
- Adequate rhythm of life and sleep;

- Morality in sexual relations;
- A healthy attitude to stress situations, tolerance in conflicts;
- Healthy relationship with the psychological, social, and natural environments;
- Regular participation in disease-prevention activities (e.g. in medical check-ups);
- Avoiding smoking and alcohol consumption;
- Refusing drugs;
- Establishing adequate conditions for habitation, clothing, and work;
- Cultured general conduct of life;
- Self-awarness, self-education ("nosce te ipsum").

Of course, we do not teach the detailes of practical knowledge and methods of the above listed aspects of primary prevention. This task belongs to other disciplines such as hygiene, mental hygiene, and public health. We intend to elucidate convincingly the basic scientific roots which show the high values of the healthy human organism, and the major risk factors.

In parallel with the curriculum reform, a new generation of computerized laboratory instrumentation is also introduced to our physiology teaching program. The computer aided lab experiments are combined with multimedia based educational softwares. These animation labs are expected to make physiology education even more colorful and convincing, and at the same time they link experimental observations obtained in normal healthy organs or tissues directly with aspects of health promotion and prevention.

The mid-term, semi-final, and final examination test materials of medical physiology will be modified according to the changes we introduce in the curriculum and lab programs.

In order to open an international discussion and cooperation about further development of our new teaching program, we plan to expose it to the Internet in the near future.

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An approach to primary prevention from the aspect of applied physiology

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The main reason for our decreasing population number – a most remarkable indicator of the inadequacy of our health culture – is the high rate of overall mortality. In its background one finds a number of risk factors of high prevalence, such as hypertension disease, addiction pathology, reduced stress tolerance as well as physical and psychic inactivity. Patterns of life that are positive are scarce and as yet not attractive or efficient. The spirit of primary prevention is yet far from permeating medicine; the most the clinical side did realize has been a recognition of the population's need for regular medical screenings.

A completely new approach that involves prevention programs embracing the whole of society, and an elaboration of new strategies are badly needed to achieve a desirable change in the present set of values. One of the already available remedies is to give full and science-based support to the positive life patterns in our culture, for instance by demonstrating how p h ysiology can be applied to human life, by putting the latter within a broader scope, namely that of psychophysiology and social psychology. In this framework the elements to be discussed are such aspects of culture as dietary habits, physical exercise, and mental and sexual hygiene. Placing greater emphasis on sports and intense habitual physical exercise can promote a healthier lifestyle, above all in our youth.

Keywords: applied physiology, health culture, mortality, risk factors, stress tolerance

The first point we have to admit is that primary prevention, its importance and the pressing need for it, is still not a fully recognized notion in the eyes of physicians.

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Its actual implications still escape the interest of medicine, several of its basic constituents have not been realized in their entirety. And this state of affairs seems to persist despite that we have witnessed more than a decade of real efforts to overcome the menace of risk factors that afflict a large part of our society. Most of these efforts were doubtless technically well-founded and valuable, yet the negative trends – as manifest in the unfavourable rates of morbidity and mortality – have proved to be much stronger than the strategies devised to counteract them.

Further points worth considering are a distortion of common sense, of our priorities in the set of values, and a disorder in the general norms of mental health. These failures have a not negligible part both in the development of the factors that endanger somatic health, and in their growing severity. The obvious issues and interrelations have become conspicuously manifest in the apparently unstoppable decrease of our population.

The first part of this paper provides evidence to support the statements outlined above. The second part attempts to disclose the mechanisms at work and to suggest means that may help reversing this decline of health.

Evidence for the need of urgent action

Table I relies on the publications of the Central Office of Statistics and refers to some basic indices of demography that demonstrate the tendencies observable in the past decades and in the nineties. As shown, the number of marriages has halved in the past ten years. The figures were 96,612 in 1970 and 45,000 in 1998. The rate of decrease has grown with every decade and keeps progressing. In the first quarter of this year it was 92.2% when compared to the respective period of 1998 [3].

The dramatic drop in the number of births has started later. All of us remember the so-called demographical wave of the mid-seventies. In reality, this period was the last one when the numbers of the fresh cohorts could be still considered normal, and they were not at all extremely high. We are right in attributing importance to the measures then taken by the social and family policy. However, negative influences have soon prevailed, and they are a strong indication of the fact that the main concern has its roots in a change of priorities and cannot be reduced simply to one of living standards. Although the balance was very modest, the birth rate in 1980 made natural reproduction figures still positive. Then, in the course of the eighties the number of live births decreased steadily, and it kept decreasing also in the nineties, but at a faster rate. The fact that this trend still continues is obvious in the figure of the current first quarter.

Table I

Demographic data (source: Central Office of Statistics)

Year	Marriages (1000)	Live-born (1000)	Still-born (1000)	Natural increase or decrease (1000)
1970	97	152	120	32
1980	80	149	145	3
1990	67	127	146	–20
1998	46	98	148	–44
1999 I–II	92.2%	94.5%	146.7%	–46%
% of prev. yr.				
1999	51.23	22.75	39.38	–16.62
1998	53.58	24.07	35.40	–11.33

The process of decrease in the number of the population has become a standing trait since the eighties, because mortality kept being high in addition to the smaller birth frequencies. It is noted that while in 1960 the recorded number of deaths was only 101,515, the figures characteristic since 1980 are around 145,000. The presently largest death figure of 150,244 was recorded in 1993, the smallest of 139,434 in 1997. No one ascribed excessive importanceto the latter, but there was some hope that – arising from the increased efforts of health preservation – a sort of improvement may have started. Although one should not rule out this possibility entirely as the years before and after this date were also better than the previous ones, the data for the current first quarter point to a continuation of the untoward tendency.

Most of the experts in demography and sociology are of the opinion that the main cause in this now apparently lasting annual population loss of about 40,000 is to be found in the permanently high rate of mortality. This implies that the low birth rate characteristic of the developed countries would not be too great a concern if it were coupled with their also characteristic low rate of mortality. Our mortality is now very close, however, to that of the third world.

We cannot but agree in regarding mortality as the greatest contributor to the nation's disease, namely, progressive population loss, and we share also the view that a further reduction of mortality among premature babies and an improved quality of life for all live borns should be first priority rather than an increase in birth rates. Nevertheless, it is our conviction that these goals do not preclude the objective of promoting stable marriages, complete and sound family life and more sensible child care by influencing the set of values upheld now by our society.

Anyway, efforts to reduce mortality and to broaden survival chances have to become a focus of our attention. It is a generally acknowledged fact that health status depends on genetic endowment, life style and environmental factors alike, and the level

of health care is secondary to all these. That, however, should not mean that we may slacken in our attempts to improve the latter. This is of very great importance for the better and more efficient medicine is, the larger the number of death cases that become avoidable, so at least the negative side of medicine can be reduced. We want to draw attention to the fact that while the part of curative clinical medicine in the population's health status is around 10% in the most developed countries, its role in Hungary is 20%. Let us refer to another rather characteristic fact: when in the early nineties the palette of therapy included the best medical drugs for the first time, the basic tendencies failed to improve despite that both the amount and the expenses of drug prescription rose.

All what has been said points toward the need for developing strategies of primary prevention.

Mechanisms in the background and means suggested to counteract them.

Most of the measures taken until now against the risk factors have – reasonably and approvably – been materialized in medical screening. For instance, monitoring blood pressure and studies of blood cholesterol. However, morbidity and mortality data have revealed an important contribution of such pathological conditions in the development of which addictive states, a narrowing stress tolerance, somatic and psychic inactivity and/or depression – several times, in combination and mutually deteriorating each other – have a primary role. This state of affairs prescribes for us that by inventing new strategies we should do our best also in propagating the positive patterns of life style towards every stratum of our society. The focus of these new strategies should centre around such diverse facets of our culture as recommendations concerning diet, physical activity, sexual behaviour and mental hygiene [1, 2].

In order to become efficient, these strategies need a firm basis of applied physiology. To take a trivial example, any campaign the target of which is the man of the street, in particular the young one, when it can provide impressive facts based on the pathophysiology of chronic alcohol abuse, is bound to have a more lasting impact than broadcasting flat general statements or everyday witticisms. There is often a need for shocking the public mind, and when there is sound science behind the intended shock, the message hits with much greater efficiency. The American campaign of increasing habitual physical activity started with the presentation of the shockingly poor health status of the nation.

An important aspect is that the various layers of society have to be approached separately, taking specific steps toward the two sexes, the respective age groups, but also in gaining the interest of the dissimilar levels of education. One may rightly ask whether any probability exists for breaking the vicious circles of a deranged social set of values or of a wrong style of general life, and if so, what that may be. Our answer is affirmative: we have seen fine examples of it in young people that became engaged in one or other event of sports. We admit that a lot depends on the coach, because an

optimum combination of the somatic issues of regular physical training and the effects of sport moulding the mind and the lifestyle is by no means a spontaneous development that does not require sincere efforts.

In what follows we wish to demonstrate some effects of regular physical conditioning, effects that may have a part in preventing the development of cardiovascular diseases. Concerning these echocardiographic studies [4] it is important to note that such effects do not depend on a talent in sport performance, but on the regularity and adequate intensity of physical training so one can expect them to develop even at an older age although at a slower rate. Regular physical training induces left ventricular hypertrophy at almost all ages. In contrast to senile or pathological hypertrophy, this exercise-induced one is not associated with an impairment of diastolic functions, but – as shown by left ventricular compliance expressed by the E/A quotient – with an improvement of the same, in particular in the most sensitive older ages.

Table II

Echocardiographic parameters of male and female non-athletic and athletic subjects
(mean ± s.d)

Parameter	Age	Males Non-athletic	Males Athletic	Females Non-athletic	Females Athletic
Rel. LVMM	<15	75.7 ± 12.2	**82.2 ± 14.1 o**	67.6 ± 5.2 +	**80.9 ± 13.8** +
	15–18	66.0 ± 13.3	**85.4 ± 17.5***	62.8 ± 6.6	***74.9*** ± ***10.2****
	19–30	73.1 ± 9.4	**94.6 ± 17.0***	*60.2* ± *7.5**	***76.2*** ± ***11.4****
	31–45	75.9 ± 11.3	**91.3 ± 12.7***	70.5 ± 15.5	**84.9 ± 9.3 o**
	45<	86.3 ± 23.1	84.5 ± 18.3	85.6 ± 16.0	82.1 ± 11.2
E/A	<15	2.00 ± 0.38	2.04 ± 0.36	2.20 ± 0.32	2.04 ± 0.38
	15–18	1.81 ± 0.37	**2.23 ± 0.59 o**	2.05 ± 0.62	2.45 ± 0.87 +
	19–30	1.91 ± 0.38	**2.08 ± 0.51 o**	*2.26* ± *0.43**	*2.27* ± *0.70 o*
	31–45	1.43 ± 0.27	**1.73 ± 0.35 !**	*1.71* ± *0.43 o*	1.87 ± 0.32
	45<	1.24 ± 0.36	1.56 ± 0.40 +	1.14 ± 0.30	**1.52 ± 0.37 !**

Rel. LVMM: relative left ventricular muscle mass, E/A: ratio of the peak velocity during early and late phase of the transmitral flow. Bolded numbers: significant difference between non-athletic and athletic subjects, italics: significant difference between males and females. +: $p < 0.1$, o: $p < 0.05$, ! : $p < 0.01$, * : $p < 0.001$

We are quite certain that our strategy of primary prevention can be renewed provided that it relies on knowledge based on applied physiology. We have to gain above all the true interest of medical people in the ways and means by which the common public is to be reached. Our hope is that laying a physiological foundation for health policy is one of the best recommendations in the sight of physicians and other experts in health care so they shall back such efforts more willingly and understanding better the goals to be achieved.

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Central thermoregulatory effects of neuropeptide Y and orexin A in rats

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Orexin A and neuropeptide Y that are known to induce a feeding response when applied centrally, in the present studies also caused hypothermia. Neuropeptide Y elicited hypothermia by depressing metabolic rate (without affecting heat loss mechanisms), while orexin A acted through enhancing peripheral heat loss (without affecting metabolic rate). Neither peptide induced co-ordinated thermoregulatory changes, both of them appeared to influence thermoregulation via different effector mechanisms.

Keywords: NPY, orexin, hypothermia, thermoregulation, heat-loss index

Energy homeostasis and thermal balance are closely interrelated. Fasting animals maintain lower body temperature than well-fed controls [4], and pyrogenic substances usually also cause anorexia [5]. Investigation of common central mediators may yield more information on the ways the two regulatory systems may co-operate. Both neuropeptide Y (NPY) and orexin A (OxA) are such possible common central mediators. Neuropeptide Y, that is produced in the lateral hypothalamus, has been shown to increase food-intake and to lower body temperature when administered centrally [1, 2, 3, 8]. A newly discovered hypothalamic neuropeptide, OxA, was demonstrated to increase food-intake upon central administration [3, 6, 7], and might have some thermoregulatory effects as well.

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In the present study the possible central thermoregulatory effects of Orexin A were investigated and compared to those of NPY.

Materials and methods

Adult, female, cold-adapted Wistars rats served as subjects (n=7 and 8 in the groups receiving NPY or OxA, respectively). At least three weeks before the experiments the animals were moved into a cold chamber of 3–5 °C, with a 12/12-h light-dark cycle and *ad libitum* food and water supply. At least one week before the tests a stainless steel guide-cannula was implanted into the right cerebral ventricle of the rats through which NPY, OxA, or saline was administered in a volume of 5 µl. The semi-restrained animals were equipped with thermocouples measuring colon and tail skin temperatures (T_c and T_s, respectively) and were placed into an open circuit metabolic chamber the temperature of which was 15 °C, i.e. moderately cool for cold-adapted animals. During the experimental sessions T_c, T_s and CO_2 production, indicating metabolic rate (MR) were continuously recorded. On the basis of peripheral vasomotor response a heat-loss index (HLI) was calculated according to the following formula: $(T_s-T_a)/(T_c-T_a)$. In case of a maximal vasoconstriction the value of HLI is close to 0, in case of a maximal vasodilation the HLI approaches 1.

NPY was administered intracerebroventricularly (ICV) in a dose of 10 µg, the OxA dose was 2 µg.

The results are presented as mean±S.E.M. of individual results. Student's *t*-tests were used for statistical analysis of the data.

Results and discussion

Central injections of physiological saline failed to have any influence on the regulation of body temperature. There were no significant differences in the initial values of MR or those of T_c between the NPY and OxA groups. Initial MR was 12.39± 0.53 $W \cdot kg^{-1}$ and 14.38±0.38 $W \cdot kg^{-1}$ in the NPY and the OxA group, respectively. Initial core temperatures were 37.93±0.13 °C and 37.67±0.06 °C, respectively, in the groups receiving NPY or OxA injections.

Central administration of NPY was followed by an acute decrease in T_c, due to decreased metabolic rate. Maximal fall of MR was observed 30 min after NPY administration, reaching the value of –2.77±0.52 $W \cdot kg^{-1}$. In moderately cool environment the animals maintained maximal vasoconstriction, that is, HLI did not rise above zero (Fig. 1).

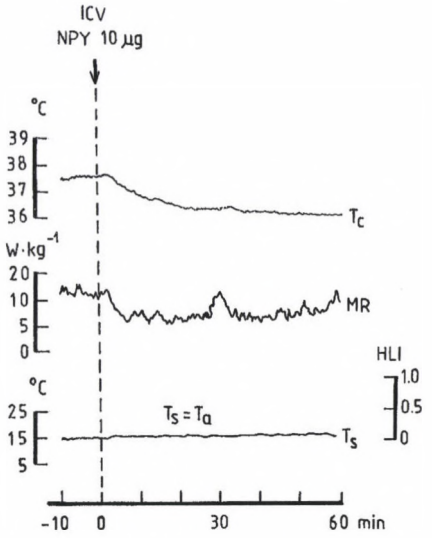

Fig. 1. Individual recording of the thermoregulatory effects of 10 µg NPY, injected ICV at an environmental temperature (T_a) of 15 °C. The upper, middle and lower panels show the NPY-induced decrease in core temperature (T_c), the NPY-induced decrease in metabolic rate (MR), and the continuously low tail skin temperature (T_s), respectively. Heat-loss index (HLI) remained zero throughout the experiment

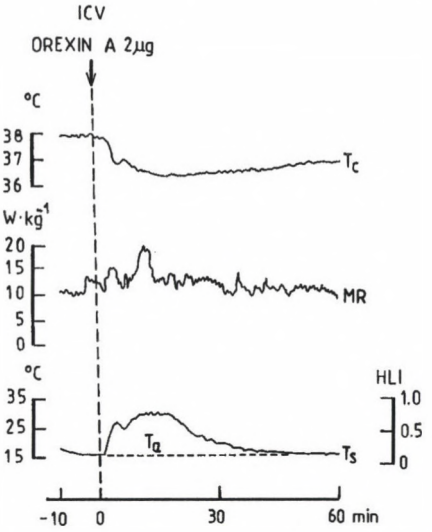

Fig. 2. Individual recording of the thermoregulatory effects of 2 µg OxA, injected ICV at an environmental temperature of 15 °C. The upper and middle panels show the OxA-induced acute drop in T_c and the course of MR, respectively. The OxA-induced increase in peripheral heat-loss is demonstrated in the lower panel, presented as an increase in T_s (on the left axis) or as a change in HLI (on the right axis). For abbreviations see legend to Fig. 1

OxA, upon ICV administration, elicited an acute decrease in T_c, due to vasodilation and consequently increased peripheral heat-loss. The HLI was 0.58±0.12 with a maximum around 10–20 min after OxA. There was no significant change in MR. Maximal change of MR was observed 30 min after OxA administration reaching a value of +1.23±0.55 W·kg^{-1} (Fig. 2).

Apparently, both neuropeptides have some central thermoregulatory effects in addition to their contribution to food-intake regulation, both of them causing hypothermia. However, neither of them caused hypothermia through co-ordinated changes that would indicate an important role in the regulation of body temperature (i.e. there was no simultaneous fall in heat production with a rise in heat-loss). These results suggest that the two neuropeptides influence separate effector functions in thermoregulation.

Acknowledgements

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The effect of detraining on echocardiographic parameters due to injury

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Regular exercise training modifies the morphological and functional properties as well as the autonomous regulation of the heart. Such changes constitute what is termed an athletic heart, and were found to be reversible so after a discontinuation of regular exercise cardiac parameters gradually return to the non-athletic values.

As yet, however, it has not been fully settled
1) how long a period of detraining is necessary to elicit such reversion, and
2) whether the various characteristics of an athletic heart would move concurrently or the time course of their change is different.

In order to get more insight, the echocardiographic correlates of these problems were studied in 22 female and 23 male physical education students forced by an injury to discontinue their exercise for some time. A sex and age matched group of non-athletic subjects and data published about a group of elite athletes served as contrast. The studied resting parameters were the wall thickness and internal diameter of the left ventricle, left ventricular relative muscle mass, relative stroke volume and cardiac output, and heart rate.

It was inferred that – in respect of the echographical parameters – the time taken until the first signs of detraining depended on the athlete's previously attained level of conditioning, and that there was a definite order of sequence in that functional changes preceded morphological ones.

Keywords: detraining, echocardiographic parameters, injury, exercise training, regulation of the heart, athletic heart, cardiac parameters

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A previous echocardiographic study in road cyclists [3] revealed that a detraining period of 14–60 days did not cause observable changes in left ventricular wall thickness and internal diameter, but elicited some functional modifications. Cardiac output at rest (which in endurance athletes is usually slightly smaller) was found to have increased and even exceeded the level of non-athletes, while resting heart rate remained low. However, other authors did report on changes in heart morphology already after some weeks of inactivity [1, 2]. This raised a question of the possible causes of dissimilar responses given to detraining. The second point of interest was the timing, intensity and manner of the changes.

In the present study an attempt was made, therefore, to get further data concerning the problem of an abrupt cessation of regular exercise training in athletes. To this end cardiac functions were examined by echocardiography during detraining, and compared to data obtained active period.

Materials and methods

The test group consisted of 22 female (weight: 58.62±4.88 kg; height: 167.766± 5.39 cm; age: 22.15±3.2 yrs.) and 23 male (weight: 73.84±7.3 kg; height: 182.66± 6.36 cm; age: 21.4±3.65 yrs.) physical education students who had to stop their physical training because of injury and volunteered to take part as subjects in the study. In one subject, measurements were made twices, when they were active before passive period as a control examination or when they became active again, but not before a minimum of 3 months of retraining, and once during their involuntary detraining period, at least 2 and at most 12 weeks after their injury.

For orientation, data of a control group of 22 sedentary females (weight: 57.83± 5.05 kg; height: 165.91±4.98 cm; age: 21.8±2.37 yrs.) and 23 sedentary males (weight: 75.4±6.85 kg; height: 181.37±7.46 cm; age: 21.76±3.11 yrs.) was also demonstrated in the Table I.

A Dornier AI 4800 echocardiograph was used with a 2.5 MHz transducer. Two-dimensionally guided M-mode measurements [5] were made in the parasternal view for estimating the morphological and functional parameters of the heart muscle. In every instance several cardiac cycles were recorded, and the average of these cycles was used as an entry in the calculations.

End-diastolic (EDV) and end-systolic volumes (ESV), and left ventricular muscle mass (LVMM) were calculated by cubing the respective diameters [5]:

$$LVMM = \{(IVST + PWT + EDD)^3 - EDV\} \cdot 1.05,$$

where IVST is interventricular septal thickness, PWT is posterior wall thickness, and EDD is the end-diastolic diameter of the left ventricle, all in mm. EDV was taken to equal EDD^3, while 1.05 is the density of the cardiac wall.

In order to obtain size-independent measures for comparison, thicknesses and diameters were related to the square root of body surface area (BSA), while LVMM was related to the cube of the square root of BSA, as done already previously [4]. Stroke volume was calculated as EDV–ESV.

By the valuation we compared the dates of the active and passive periods by t-test for dependent samples.

Results

Table I demonstrates the average echocardiographic values of our subjects during detraining and in their retraining, newly active period. For further information, the means for a healthy, sedentary control group are also indicated in the 3rd row.

After cessation of regular exercise training relative left ventricular wall thickness (rel.LVWT) decreased slightly but significantly, while there was no change in relative left ventricular internal diameter (rel.LVID). A very slight, not significant decrease was seen in relative left ventricular muscle mass (rel.LVMM). On the other hand, a significant increase was seen in resting heart rate and in cardiac output (CO), but none of these reached the level of the non-athletic subjects.

Table I

Some echocardiographic data during and after a detraining period in athletic subjects (Means ± SD)

Parameter	Left ventricular			Heart rate	Relative	
	wall thickness [mm/$\sqrt{m^2}$]	internal diameter [mm/$\sqrt{m^2}$]	muscle mass [g/$(\sqrt{m^2})^3$]	[beat/min]	stroke volume [ml/min/$(\sqrt{m^2})^3$]	cardiac output [L//min/$(\sqrt{m^2})^3$]
Active	13.75±1.46	35.91±2.37	81.16±16.33	59.07±8.11	33.83±1.27	1.99±1.76
Detraining	**13.39±1.32**	36.16±2.49	79.00±16.86	**62.83±8.67**	34.97±0.97	**2.19±0.152**
Untrained	12.06±1.51	35.31±2.56	67.28±16.87	74.86±7.14	32.31±0.83	2.40±0.097

(Sedentary data are shown in the last row)

Note: bold figures denote significant differences between active and detraining states

Discussion

Table II is a symbolic comparison between the studied test group during detraining and data reported on similarly detraining Austrian road cyclists [3]. The two groups differed mainly in the response of relative LVWT and cardiac output. In the morphological parameters our subjects displayed a small decrease in rel.LVWT, but not any in rel.LVID and rel.LVMM. There was, however, a difference between the two groups in the parameters of cardiac regulation. In contrast to the cyclists' data, heart rate showed a slight, significant increase in our subjects. Cardiac output also increased significantly, but the extent of this increase was not so great as in the road cyclists, in that it did not exceed the normal, untrained level.

Table II

Symbolic comparison of the test group with elite, but detraining Austrian road cyclists in respect of the changes occurring in the echocardiographic parameters during detraining

	Wall thickness	Internal diameter	Ventricular muscle mass	Heart rate	Cardiac output
Road cyclists	=	=	=	=	++
P.E. students	–	=	=	+	+

Symbols: =: comparable; +: became greater; ++: much greater; –: smaller during detraining.

The results of both the present investigation and those of previous studies [1, 2, 3, 6] indicate that during detraining the studied echocardiographic parameters do not change simultaneously. The earliest modifications are likely to appear in the autonomous regulation of the heart, first in sympathetic activity, then in parasympathetic tone. The change of the morphological parameters, if any appear markedly late. This course of events needs little explanation, because the nervous component of regulation responds fastest.

The intensity and manner of the various changes, however, as they actually appeared, only became explicable when detraining athletes of a dissimilar previous degree of conditioning were compared. It could be shown only by this comparison that the way in which such changes occur depends on an athlete's physical condition before detraining. In athletes of excellent condition even several weeks of detraining only led to an increase of resting sympathetic autonomous activity, as shown by the Austrian cyclists [3]. As the ventricular myocardium has only sympathetic innervation, stroke volume would increase during detraining and, owing to the still marked hypertrophy, cardiac output at rest might exceed the normal, sedentary level.

When the level of physical conditioning is lower, e.g. in athletes of a lower qualification or in our material, the change in these and other components of cardiac function occurs sooner and in a faster sequence: an elevation of resting sympathetic activity becomes manifest in an increase of resting cardiac output, a drop in resting parasympathetic tone in a faster heart rate, and also some of the morphological parameters may show modifications. This must have been the case in our present study and in the available relevant publications [1, 2]. Our present results seem therefore slightly more similar to those of Ehsani et al. [1] and Maron et al. [2] than to the results obtained in Austrian road cyclists [3].

As a conclusion, results indicate that detraining elicits modification in the echocardiographic parameters, but these changes appear in different time of detraining period.

Characteristics of the autonomous nervous system change the soonest, morphological modifications appear later. After how much longer these changes happen, depends on several other factors. The most important is on the actual level of trainedness of the athlete.

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Characteristics influencing changes in aerobic performance of children aged 7–9

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Interpretation of the actual level of aerobic endurance in a growing child is difficult. Endurance capacity *per se* is influenced by a number of factors, e.g. by genetic endowment, developmental rate, body composition and habitual physical activity. The respective effects of these factors cannot be distinctly separated from one another, so their particular role is not clearly definable in the various age periods.

The aim of our investigation was to define the actual level of aerobic endurance performance in children aged 7–9, and to analyse the relevant factors that may affect this kind of performance according to the different level of sport activities.

Two primary school classes (N=42) were selected for the experiment. Both groups had physical education classes every day (five times a week). One group (AG, N=31) consisted of children taking part in regular sport courses at least twices a week (e.g. basket ball, karate, triathlon swimming and gymnastics), as well, the other group (NAG, N=11) served as comparison group.

Aerobic performance was estimated by measuring cardiorespiratory response during a Jaeger treadmill run using a "vita maxima" (all-out) testing protocol. The subjects were measured in the Laboratory for Spiroergometry of the Hungarian University of Physical Education twice, in 1997 and 1998. Body composition was assessed by the Drinkwater–Ross [4] body mass fractionation technique. Robustness of the body was described by using the plastic index (PLX) of Conrad's growth type [2], morphological age (MORF AGE) was estimated by using the method

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of Mészáros and Mohácsi [5]. Exercise performance was studied in the laboratory by using a Jaeger 6000 LE model treadmill and a Jaeger μ-DATASPIR model gas analyser. The functional status of the subjects' cardiopulmonary system was estimated by spiroergometric parameters and total mechanical work (WORK).

The AG group had a better endurance performance in 1998 than that of non-athletic group. A factor analytic study (principal component method) of the employed variables revealed that the higher level of endurance fitness in the athletic group was not only affected by growth in size, but also by an increased level of cardiorespiratory performance capacity.

Keywords: aerobic performance, genetic endowment, developmental rate, body composition, habitual physical activity, sport activity

Fitness level, as the performance ability of the cardiovascular system can be characterised well by measuring maximal oxygen consumption (VO_2max) in exercise tests [10]. It is difficult to tell us the required level of endurance fitness in prepubertal children, because spontaneous growth itself (changes in body dimensions and cardiopulmonary function) brings about an improvement of aerobic and anaerobic performance. It is almost impossible to separate the effect of growth from that of habitual physical activity in the measured performance parameters.

Burdukiewicz and Janus [1] followed 7–15 year-old children's progress with attention to their somatic development and physical activity. Test results of physically active children proved to be better, and the effect of sexual difference in performance values was smaller in the active group, than that of non-active ones. Sall and Viru [10] found that sportactivities 2–3 times a week lasting twenty minutes occasionally were not enough for increasing aerobic performance of children aged 4–10.

Our aim was to assess endurance performance and body composition of children aged 7–9, and to analyse the role of some relevant characteristics in a system of relationships of the variables. The elementary school where we have made our investigation has extremely good circumstances for sport activities than other schools. The children have physical education classes five times a week. The school has an own sportsclub, where the children have possibilities to take part in sportcourses and competitions in basketball, karate, thriatlon and rhythmic gymnastics.

Material and methods

Forty-two children aged 7–8 were selected for the investigation (20 boys and 22 girls). In accordance with the regulations of protecting human subjects in research [3], the participants were selected on a voluntary basis, and procedures and risks of the examinations were explained to them and their parents. The altogether 42 children were grouped by the level of their habitual physical activity into an athletic group (AG N=31), which consisted of children engaged in sessions lasting one hour

occasionally or more in sport training at least twice a week, the other, non-athletic group (NAG N=11) served as a contrast group. Both groups had physical education classes every day (five times a week). Each subject was measured both in 1997 (grade 1, 7–8 years) and 1998 (grade 2, 8–9 years) in the Spiroergometry Laboratory of the Hungarian University of Physical Education.

Anthropometry

Twenty-four anthropometric measurements were taken conforming to the suggestions of the International Biological Program [11]. Robustness of the body was described by the plastic index (PLX) of Conrad's growth type [2]; morphological age (MORF AGE) was estimated as the developmental level of the skeleto-muscular system by using the method of Mészáros [5]. To estimate body composition Drinkwater and Ross' body mass fractionation technique [4] was used, fractional masses expressed as percentages of bone (BONE%), muscle (M%), fat (FAT%) and residual mass.

Protocol of the endurance test

The exercise was a continuous running test performed on a Jaeger 6000 LE model treadmill by using a "vita maxima" (all-out) testing protocol. The protocol has begun at 5 km/h speed, and only speed was increased by 1/2 km/h after each 100 m running. A Jaeger μ-Dataspir model gas analyser was used to determine the composition of expired air. Thoracic leads of a Hellige ECG were employed to monitor heart rates before, during and after the exercise. Before the exercise test heart rate was measured at rest in a sitting position (HRR). The functional status of the subjects' cardiopulmonary system was estimated by using the maximum exercise values of the following spiroergometric parameters: heart rate (HR, beat/min), minute ventilation (MV, l/min), fractional oxygen utilisation (FO_2%), absolute (VO_2, l/min) and body mass related oxygen consumption (VO_2 KG, ml/kg/min), oxygen pulse (O_2P, ml/beat), respiratory exchange ratio (RER), breathing rate (BR, l/min), ventilatory equivalent for oxygen (EQO_2, l/min) and total mechanical horizontal work performed (WORK, joule). Recovery heart rates (HRR1–HRR6) were also measured every thirty seconds for three minutes after the subject stopped running.

To calculate the significance ($p < 0.05$) of the differences between the groups *t*-tests for independent samples were applied and ones for dependent samples for the changes between 1997 and 1998 within each group. The role of the variables in the variance of the total sample was analysed by factor analysis with extraction of principal components. Eigenvalues of the extracted factors were >1. Factor loadings were rotated by the varimax method. Essential variables in each factor were emphasized by their factor loadings of correlation coefficients >0.7.

Results

Table I shows that the AG had lower fat values, greater muscle mass, displayed higher HR, and a larger amount of MET in 1997 than that of the NAG. After a year the AG had a similar body composition, a larger amount of PLX, and their endurance performance improved more than in the NAG, as it could be seen from the larger MV, VO_2, O_2P, HR, VO_2KG and WORK data.

Factors held responsible for the change in maximal aerobic power are demonstrated in Figure 1. Three main factors (Factor 1, Factor 2 and Factor 3) could be separated. In the non-athletic group in 1997 Factor 1 was labelled as circulatory regulation (HR, HRR1-6, HRR), Factor 2 was labelled as body dimension and working ability (BH, BW, MV, VO_2, MET, VO_2KG), Factor 3 as body maturity, age and breathing economy (AGE, MORF. AGE, FO_2, EQO_2). In 1998 the structure of factors changed a little: Factor 1 contained age-dependent metabolic regulation (AGE, BF, HRR1-6), Factor 2 contained body maturity and work output (BW, PLX, DIST, WORK), Factor 3 body composition and physiological working capacity (FAT%, M%, MV, VO_2, MET, HR, VO_2KG) as dominant variables.

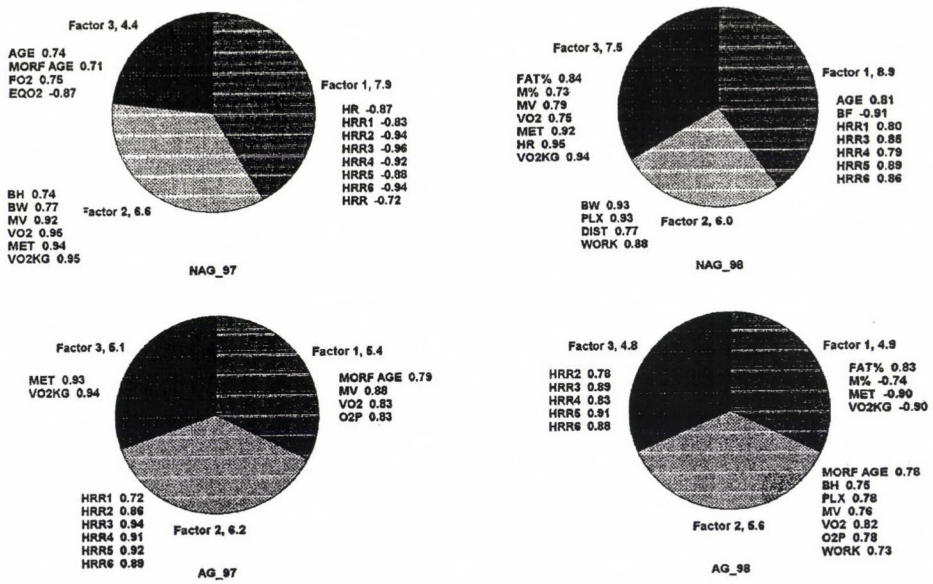

Fig. 1. Changes in the structure of the ratated factors in the groups

Factor 3,4.4; factor 2,6.6 and so on: factor, eigenvalue
AGE 0.74, MORF AGE 0.71 and so on: essential variables and correlation coefficients between factor and variables

Table I

Observed means of the studied variables in the two groups of dissimilar habitual physical activity

Variables	AG 97	NAG 97	t value	p	AG 98	NAG 98	t value	p
AGE	7.58	7.63	–0.3922	0.6971	8.60	8.78	–1.1147	0.2716
MORF. AGE	7.56	7.66	–0.5319	0.5978	8.54	8.17	1.8172	0.0792
BH	129.42	128.86	0.2798	0.7811	135.21	133.77	0.6729	0.5049
BM	24.60	25.82	–0.9331	0.3565	28.30	25.48	2.2071*	0.0331
FAT %	13.98	16.87	–2.0853*	0.0436	15.76	15.81	–0.0298	0.9764
BONE %	20.36	20.26	0.2268	0.8219	19.82	19.52	0.4617	0.6475
M %	41.65	40.29	2.5578*	0.0150	41.46	40.80	0.8277	0.4142
PLX	59.25	59.85	–0.6426	0.5242	62.77	59.93	2.7513*	0.0098
MV	35.36	33.11	0.9486	0.3488	51.40	42.01	3.3953*	0.0016
FO_2	3.74	3.68	0.5168	0.6083	3.73	3.79	–0.5870	0.5605
RER	0.98	0.98	–0.0611	0.9516	1.11	1.13	–0.3737	0.7106
VO_2	1.00	0.94	0.7769	0.4420	1.39	1.14	3.4644*	0.0013
O_2P	5.84	6.41	–1.3725	0.1779	7.46	6.28	2.9956*	0.0047
MET	11.50	10.28	2.0578*	0.0467	13.85	12.65	2.0169	0.0505
BF	62.96	59.90	0.8079	0.4242	69.77	65.73	0.9914	0.3275
HR	184.58	171.27	2.9174*	0.0059	199.26	191.91	2.0778*	0.0442
HRR1	149.21	146.27	0.3528	0.7262	175.65	174.80	0.1182	0.9065
HRR2	126.78	110.90	1.9940	0.0536	144.40	140.70	0.5236	0.6036
HRR3	120.78	104.72	2.3527*	0.0241	130.13	129.10	0.1734	0.8633
HRR4	119.67	105.63	2.1895*	0.0349	123.63	126.20	–0.5453	0.5887
HRR5	117.80	104.18	2.2451*	0.0314	119.13	124.89	–1.3056	0.1998
HRR6	116.36	102.00	2.0819*	0.0463	115.83	119.75	–0.7579	0.4536
VO_2KG	40.23	36.31	1.9291	0.0612	48.60	44.25	2.0791*	0.0441
EQO_2	36.98	36.03	0.8008	0.4282	46.58	50.54	–1.34010	0.1878
HRR	93.89	89.18	0.8533	0.3989	88.39	91.36	–0.6141	0.5426
WORK	24910.05	22532.64	0.7697	0.4462	48593.42	34624.91	3.6059*	0.0009

*: $p<0.05$

AG: athletic group

NAG: non-athletic group

AGE: decimal age (years)
MORF.AGE: morphological age (years)
BH: body height (cm)
BM: body mass (kg)
FAT%: percentage of fat
BONE%: percentage of bone
M%: percentage of muscle
PLX: plastic index
MV: minute ventilation (l/min)
FO_2: fractional oxygen utilisation (%)
RER: respiratory exchange ratio (l/min)
VO_2: volume of oxygen consumption (l/min)
O_2P: oxygen pulse (ml/beat)
MET: number of multiples of the resting metabolic rate (l/min)
BF: breathing rate (l/min)
HR: heart rate maximum (l/min)
HRR1-6: recovery heart rates (l/min)
VO_2KG: body mass related oxygen consumption (ml/kgxmin)
EQO_2: ventilatory equivalent for oxygen (l/min)
HRR: heart rate in rest (l/min)
WORK: total mechanical work (kJ)

Athletic group showed quite a different picture. In 1997 Factor 1 was called as body maturity and physiological work (MORF. AGE, MV, VO_2, O_2P), Factor 2 as circulatory regulation (HRR1-6), and Factor 3 as body mass dependent working capacity (VO_2KG, MET).

The structure of factors in 1998 was as follows: Factor 1 contained marked variables responsible for body compositon and body mass dependent physiological work (FAT%, M%, MET, VO_2KG), Factor 2 body maturity and aerobic working capacity (MORF. AGE, BH, PLX, MV, VO_2, O_2P, WORK), and Factor 3 circulatory regulation (HRR2-6).

Discussion

The athletic group performed more mechanical work and displayed a better economy in running than the non-athletic group: this improved running performance was associated with a comparable respiratory exchange ratio (RER). Rowland [8] found that running efficiency in children improved with growth, i.e., when performance was similar, the contribution of aerobic metabolism to work became larger. Our data showed that this tendency was stronger in the more active children (AG). Increase in maximal aerobic power has been reported to be about 200 ml/min per year in the prepubertal years [6].

The increase in VO_2max was almost 400 ml/min in the athletic group between 1997 and 1998, while this value slightly exceeded 200 ml/min in the non-athletic group. The higher level of endurance fitness was not only affected by growth in size, but by an additional improvement of cardiorespiratory performance, as it could be seen from the factor analysis of the athletic group data in 1998: greater number of variables characterizing the cardiorespiratoric performance gathered into Factor 2 ('background' variable was called as body maturity and aerobic working capacity).

Rowland [9] states that "Differences in trends of VO_2max between individual longitudinal studies could be explained by the variable influences of other factors affecting aerobic fitness:

1. differences in body composition;
2. differences in daily physical activities and sport participation."

This can be seen in our investigation as well, though the variability of factor structure in the groups can be explained partially by the relatively small number of cases. The fact is more important that differences in factors characterizing the two groups were realised by the changes of the variables within one year. The unequal changes of the variables caused by the different way of life and somatic development of the children modify the correlation matrix itself, which is the base of factor analysis.

Oxygen pulse, as Norris et al. [7] demonstrated, is an acceptable estimate of stroke volume. The larger oxygen pulse of the athletic group in 1998 was an evidence for an additional increase in stroke volume.

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Exhaled carbon monoxide concentration increases after exercise in children with cystic fibrosis

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Oxidative stress and hypoxia, which may occur in cystic fibrosis patients (CF) at rest and may be worsened by exercise, induce the expression of heme oxygenase (HO)-1, resulting in increased carbon monoxide (CO) formation. We tested that exhaled CO level (eCO) was higher in CF patients than in healthy subjects, and that exercise increased CO production. Exhaled CO was measured electrochemically in 15 CF patients and 15 control subjects at rest (T_0), immediately (T_1) and 60 minutes after a symptom-limited incremental bicycle exercise test (T_{60}). Arterial oxygen saturation (TcO_2) was monitored transcutaneously. Data are given as mean±SEM. Baseline eCO was 1.90±0.26 ppm in the control and 1.93±0.27 ppm in the CF group. In both groups eCO was lower at T_1 than at rest. In the control group eCO was also low at T_{60}, but in the CF group it was increased compared to baseline level at this timepoint. Exercise caused oxyhemoglobin desaturation in CF patients which was related to the increase in eCO measured at T_{60} ($r=-0.67$, $p<0.01$). Our findings suggest that exercise modulates the level of exhaled CO partly by worsening oxygenisation in CF patients.

Keywords: heme oxygenase-1, hypoxia, exhaled carbon monoxide, inflammation, oxidative stress, cystic fibrosis, exercise

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Cystic fibrosis (CF) is a common autosomal recessive disease associated with abnormal exocrine gland secretion leading to repeated endobronchial bacterial infections. Pulmonary infections and associated inflammatory responses result in oxidative stress and lead to damage of the airways with gradual deterioration of pulmonary function [1]. Deterioration of the clinical condition leads to decreased exercise tolerance and patients may develop hypoxia during exercise [2, 3]. Non-invasive markers of disease activity are searched to help monitoring airway inflammation and improve care for this patient group. Mediators detected in exhaled breath have great advantage, because sample collection is easy, it can be repeated several times and even severe patients able to perform the required type of expiration. Recently, exhaled carbon monoxide (CO) has been suggested to be a marker of airway inflammation/oxidative stress in different inflammatory lung diseases including asthma and bronchiectasis [4–6]. CO is released during the degradation of heme to biliverdin by the heme oxygenase (HO) enzyme family [7]. HO-1 can be induced/activated by inflammatory mediators, oxidants and also by hypoxia under experimental conditions [7].

Because CF is also associated with oxidative stress and non-invasive markers for monitoring disease activity and severity are still lacking, we tested if measurement of exhaled CO could serve such a role. We measured exhaled CO concentration in CF patients and in age-, and weight-matched healthy control subjects under resting conditions and during and after symptom-limited maximal exercise. Because exercise may modulate the concentration of exhaled CO by increasing minute ventilation and diluting CO concentration in it and also by changing CO production in the lung, we measured minute ventilation and calculated CO output as the product of minute ventilation and CO concentration in some subjects. Factors known to modulate CO production including the development of hypoxia may cause rapid induction of the expression of HO-1 enzyme and result in maximal functional response approximately 60 minutes after exposure. Therefore, we measured exhaled CO level not only immediately after the end of exercise, but also 60 minutes after the end of test. Finally, we analysed the relation between exercise-induced changes in arterial O_2 saturation and in exhaled CO concentration.

Patients and methods

Fifteen children with CF were recruited from the patients of the Svábhegy Children Hospital in Budapest, Hungary (Table I). All CF patients had pulmonary symptoms, signs, and radiological changes consistent with CF and were diagnosed by pilocarpine iontophoretic sweat chloride tests. They were receiving pancreatic enzyme replacement therapy, oral supplementation with Vitamin A, C, D and E, N-acetyl-

cystein (NAC), but no any other medication except two patients receiving inhaled corticosteroid (200 and 400 μg/day budenoside, respectively). All patients were in clinically stable condition and were not receiving any antibiotics for at least two weeks prior to the study. Fifteen age- and weight-matched healthy children were also recruited who served as controls (Table I). None of the subjects smoked or were exposed to smoke.

The study was approved by the ethics committee of the Svábhegy Hospital and informed consent was obtained from the patients' legal guardians.

Measurement of exhaled CO

Exhaled CO was measured by a modified analyser (EC50-MICRO Smokerlyzer CO monitor, Bedfont Scientific Ltd, UK) sensitive to CO from 0 to 500 parts per million (ppm, by volume). The subject exhaled slowly from functional vital capacity with a constant flow (5–6 l/min) over 10–15 s into the analyser.

Exercise test

At rest forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), blood pressure (BP), heart rate (HR) and eCO were determined. Then all subjects performed an incremental exercise test on an electronically braked bicycle ergometer to individual maximal effort. The first stage consisted of four minutes exercise at a workload of 30 watts. The workload was then increased to 60 watts and maintained at that level for 6 minutes. From this timepoint the workload was progressively increased by 25 watts in every two minutes until maximal effort was exerted and the subject asked to terminate the test.

To determine minute ventilation (MV) and CO output in 8 control subjects expired air was collected in plastic bags for two minutes at rest, and during the last minute of each progressive workload. The volume of expired air was measured at a constant flow using a Wright respirometer. Concentrations of CO in breath samples were analysed electrochemically.

Cardiac frequency (of 3-leads electrocardiograph (ECG); Hellige EK-26, Freiburg, Germany) and transcutaneous oxyhemoglobin saturation (TcO_2; pulse oximetry; Nelcorr 200 E; Breda, the Netherlands) were measured throughout the test. Exhaled CO was measured at rest (T_0), immediately (within 30 seconds) after the test (T_1) and 60 minutes after exercise (T_{60}). We measured eCO 60 min after the test, because after inhalation of hemin, an active substrate known to cause activation and induction of HO-1, a peak increase in eCO occured at this timepoint in healthy humans [8].

Statistical analysis

Data are given as mean (SE). Baseline variables of the control and the CF groups were compared by unpaired *t*-test. Changes in eCO ($eCOT_1$–$eCOT_0$ and $eCOT_{60}$–$eCOT_0$) in the CF and the control groups were compared by two-way analysis of variance. Spearman correlation test was performed to assess the relationship between exercise-induced changes in eCO and in TcO_2. A two-tailed $p<0.05$ was considered significant.

Results

Baseline measurements

At rest, lung function values and oxygen saturation were lower in the CF than in the control group (Table I). Exhaled CO was detectable in all subjects. Baseline eCO was not different between the two groups (1.93 (0.27) ppm vs. 1.90 (0.26) ppm).

Effect of exercise

All subjects completed the test uneventfully. Peak workload was significantly lower, exercise time shorter and HR at peak exercise significantly higher in CF patients than in control subjects (Table I). Oxygen desaturation (≥3% decrease in TcO_2) occurred in 6 of the CF patients and in none of the control subjects. Mean TcO_2 at exhaustion was 91.6 (1.7)% in the CF and 97.0 (0.3)% in the control group.

Table I

Data at rest and at peak exercise in control and CF subjects

	Control	CF
Gender (Male/female)	7/5	8/4
Age (yr)	14.2 ± 1.2	13.9 ± 0.7
Weight (kg)	49 ± 4.4	40 ± 2.2
Height (cm)	161 ± 5.7	156 ± 2.8
FEV_1 (% predicted)	97.2 ± 2.3	71.0 ± 7.6*
FVC (% predicted)	98.0 ± 1.3	78.6 ± 5.7*
TcO_2 at rest (%)	97 ± 0.3	94 ± 1.0*
Peak workload (W)	185 ± 14	104 ± 8*
Exercise time (min)	18.6 ± 1.2	12.1 ± 1.2*
HR at peak exercise (b/min)	181 ± 3	160 ± 3*
TcO_2 at peak exercise (%)	97 ± 0.3	91.6 ± 1.7*

Values are mean ±SE. *$p<0.05$ between control and CF groups. FVC: functional vital capacity; FEV_1: forced expiratory volume in 1 second; HR: heart rate; b/min: beats/minute; TcO_2: transcutaneous oxygen saturation

Changes in eCO with time were negligible at rest. Exercise caused a decrease in the concentration of exhaled CO both in the control and the CF group immediately after the exercise. In the control group this decrease remained detectable even 60 min later, while in the CF group exhaled CO was higher than at rest 60 min after the end of exercise test (Fig. 1).

Significant correlation was found in CF patients between changes in TcO_2 (T_1–T_0) and changes in eCO induced by exercise (T_{60}–T_0; r=–0.67, p<0.01) (Fig. 2), but similar relation was not found in control subjects. There was one CF patient who gave excessive eCO (+6 ppm) and TcO_2 (–14%) responses, therefore, data were analysed without the values of this patient as well, and the correlation between changes in TcO_2 and changes in eCO was found still significant (r=–0.58, p<0.05).

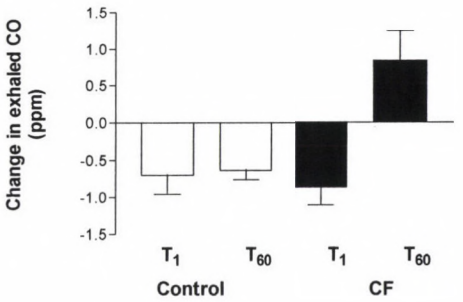

Fig. 1. Changes in exhaled carbon monoxide concentration in control subjects (control, open bars) and in patients with cystic fibrosis (CF, filled bars). *p<0.05 between control and CF groups. T_1: immediately after the test; T_{60}: 60 minutes after the test

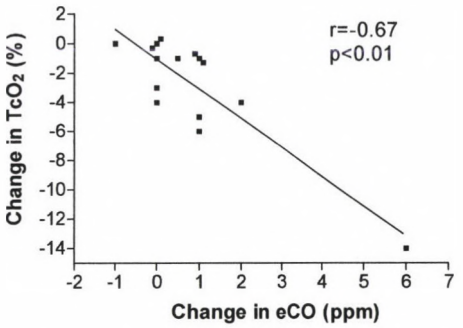

Fig. 2. Relationship between changes in exhaled carbon monoxide level and changes in TcO_2 caused by exercise in CF patients

Table II

Changes in CO output during exercise in healthy subjects

	eCO (ppm)	MV (l/min)	CO output (µl/min)
Rest	2.1 ± 0.23	11.5 ± 2.20	27.4 ± 1.73
60 W	2.0 ± 0.19	34.6 ± 1.15*	72.1 ± 6.65*
Peak exercise	1.4 ± 0.19	66.4 ± 10.42*	98.2 ± 6.92*
Recovery (5min)	1.5 ± 0.15	21.0 ± 8.07	36.2 ± 2.19

Data are given as mean ± SE*; $p<0.05$ vs rest by two-2 way analysis of variance (n=8). MV: minute ventilation; recovery: 5 min after the end of exercise test while resting

In 8 control subjects, the exercise test was repeated in a setting when minute ventilation was also measured during the test and CO output was calculated. CO output increased by exercise and returned to baseline value 5 min after the end of exercise (Table II).

Discussion

Elevated levels of exhaled CO have been shown in inflammatory lung diseases and it was suggested that oxidative stress accompanying the inflammation resulted in HO-1 induction leading to excess CO formation. In the present study we measured eCO in cystic fibrosis, a condition associated with chronic airway inflammation, and demonstrated that although eCO was normal in these clinically stable patients at resting condition, eCO increased after exercise in patients developing oxygen desaturation.

In the present study we investigated a group of CF patients and compared their results to data obtained in a group of healthy age-, height- and weight-matched children. Lung function and oxygen saturation values and exercise tolerance of the CF patients were lower than those of healthy children agreeing with the clinically well-known feature of the disease and also with previously published studies [1–3]. We found normal eCO in children with clinically stable CF, even in those presented with pronounced impairment in lung function. Further studies should be taken to analyse the relation between ongoing airway inflammation/oxidative stress and the level of exhaled CO in these patients to determine how the degree of disease activity/severity is reflected by exhaled CO level.

Immediately after exercise (at T_1) eCO was reduced in both groups. Reduction of eCO was likely the result of increased minute ventilation during exercise "diluting" the endogenously produced CO. Due to technical limitations we were unable to measure minute ventilation in our study. We, however, repeated the test in 8 healthy subjects

later to investigate the effect of exercise on minute ventilation and CO output. CO output actually increased by exercise and returned to resting values 5 min after exercise in healthy subjects (Table II) implying that CO production increases during physical exercise. Although CO is known to have vasodilating capacity [7], further studies should be undertaken to determine its exact role in the lung during exercise. Unfortunately we could only repeat the exercise test with measuring minute ventilation in 3 patients, in whom an increase of CO output was observed immediately after the exercise returning to resting values similarly to the values obtained from healthy control subjects. Their minute ventilation was not different from resting values at 60 min after exercise test, but eCO was higher than at rest (data not shown).

Sixty minutes after exercise was terminated (and subject's ventilation pattern and volume was normal), eCO was found to be increased in the CF, but not in the control group. This increase in CF patients was related to exercise-induced oxygen desaturation suggesting that the development of hypoxia may play a role in the increase of eCO after exercise. HO-1 is a stress-response protein and the expression of protein can be detected 10 to 60 minutes after exposure to inducers under experimental conditions [7]. In our previous study with healthy subjects, inhaled hemin was able to increase the concentration of exhaled CO 30–90 min after inhalation, with the maximal effect being at 60 min [8]. The observed increase in eCO in some CF patients at T_{60} may be explained therefore by relatively rapid induction of the expression of HO-1, but post-transcriptional regulation causing an increase in enzyme activity may also happened. In experimental studies, CO production was suggested to have a protective role against hypoxia [9–10]. This protective function needs to be further investigated and explored in human studies, because it may be a potential target in treatment of different conditions associated with hypoxia.

In summary, the findings of this study showed that although exhaled CO level was normal in clinically stable CF patients, exercise caused increase in CO level in those patients who developed hypoxia during exercise. This observation implies that pulmonary CO production, beside other factors, may be modulated by hypoxia in CF patients and this feature needs to be taken account when exhaled CO level is planned to be used as a marker of the disease.

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Cold pressor test and retinal capillary perfusion in vasospastic subjects with and without capsular glaucoma

(A preliminary study)

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Purpose: Using the cold pressor test the authors investigated the change in retinal and neuroretinal capillary perfusion in vasospastic patients suffering from capsular glaucoma (CG) and in vasospastic control subjects. **Methods:** Changes in retinal and optic nerve head capillary perfusion induced by the cold pressor test (one hand immersed in 4 °C water for 30 seconds, then in 30 °C water for 2 minutes) was measured using the Heidelberg Retina Flowmeter in 4 patients with CG and in 5 healthy control subjects. Previously all subjects showed a reduction of cutaneous capillary flow higher than 70% in the cold pressor test (vasospastic reaction). One eye per subject was investigated. Two images were obtained for each phase (baseline, cold phase and warm phase), and the better quality image from each phase was selected for the measurements. One location on the temporal neuroretinal rim and one location on the temporal retina outside the peripapillary area were selected for the HRF measurements. **Results:** In the CG group neuroretinal rim "Volume" decreased by 26.05%, "Flow" by 25.82% and "Velocity" by 23.91% ($p<0.05$), retinal "Volume" decreased by 12.30% ($p=0.051$), and retinal "Flow" by 22.36% ($p=0.01$) in the

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cold phase. All these parameters returned to the corresponding baseline values in the warm phase. In the control group a significant decrease was observed in retinal "Volume" (15.96%), "Flow" (17.81%), and "Velocity" (16.11%) in the cold phase ($p<0.05$), which diminished in the warm phase but remained still significant for "Flow" and "Velocity". **Conclusion:** Cutaneous cold provocation can induce an immediate decrease in retinal and optic nerve head capillary perfusion at least in a part of the vasospastic subjects with or without capsular glaucoma. This decrease diminishes or disappears quickly when the hand is immersed in warm water. To evaluate the potential role of cold-induced retinal and optic nerve head vasoconstriction in the pathogenesis of capsular glaucoma further investigations are necessary since this reaction was also present in the vasospastic control subjects.

Keywords: cold pressor test, glaucoma, scanning laser Doppler flowmetry, vasospasm

Damaged peripapillary and optic nerve head perfusion seems to be an important factor in the development and progression of glaucomatous optic neuropathy [1–3, 9]. Conditions associated with increased vasoconstrictive reactions, e.g. migraine and cold-induced cutaneous vasoconstriction, have been shown to be frequent among patients suffering from normal pressure glaucoma [3, 8, 10]. In these studies retinal capillary perfusion was not investigated. Recently, since the development of some new and non-invasive clinical methods for the evaluation of ocular perfusion, it became possible to investigate the connection between the regulation of cutaneous and ocular microcirculation.

In another study [6] using the cold pressor test we investigated cutaneous capillary perfusion in patients suffering from capsular glaucoma (CG) and in healthy control subjects of similar age. The most important result of this study was that in the CG group both the time to the maximal cold-induced flow reduction and the recovery time were significantly longer than in the control group. Venous endothelin-1 levels did not differ between the groups. These results indicated that in CG, which is a disease with systemic vascular alterations due to the accumulation of the pseudoexfoliative material, the cutaneous vasoregulation is altered, and this is independent from the venous concentration of the powerful vasoconstrictor, endothelin-1. The cold-induced reduction of cutaneous capillary flow did not differ between the groups, and in each group several subjects showed a pronounced flow reduction, i.e. a reduction higher than 70% of the baseline value (vasospastic reaction).

At the time of the investigation we did not have the possibility to study the potential cold-induced changes in the retinal and optic nerve head capillary perfusion. In the present study these reactions were investigated. Our main goal was to evaluate whether the potential cold-induced alterations are detectable with scanning laser Doppler flowmetry. Since we presumed that pronounced retinal circulatory changes are more probable in subjects showing pronounced cutaneous flow reduction for cold stimulation, only the subjects who showed a vasospastic reaction in the previous study were involved in the present investigation.

Materials and methods

The study protocol was approved by the ethical board of the university, and an informed consent was obtained from all participants. CG patients as well as healthy control subjects, who showed a reduction of 70% or higher in cutaneous capillary flow for cold provocation of the contralateral hand in our previous study [6] were involved the investigation. The 5 CG patients had mean diurnal intraocular pressure higher than 21 mm Hg before treatment, typical glaucomatous visual field loss, optic nerve head damage, pseudoexfoliative material on the iris, and crystalline lens with characteristic iris atrophy and pigmentation of the open chamber angle. The 10 control subjects had intraocular pressure consistently below 22 mm Hg, intact optic nerve heads, no visual field defects, and no signs of pseudoexfoliation. No participants experienced any other ophthalmologic disease. All subjects had normal laboratory results at the time of the investigation.

For 24 hours before scanning laser Doppler flowmetry and the simultaneous cold pressor test all medications including eye drops were stopped. Scanning laser Doppler flowmetry with the Heidelberg Retina Flowmeter, Software Version 1.02 (Heidelberg Engineering GmbH, Heidelberg, Germany) was performed on one randomly selected eye of each participant. The cutaneous cold pressor test was identical with the test used in our previous study [6]. The subjects rested in a quiet room with a temperature of 23± 1 °C for 10 minutes sitting in the chair of the flowmeter. Brachial blood pressure and heart rate were registered. In the cold pressor test the left hand was immersed in cold water (4 °C) for 30 to 60 seconds, followed immediately by an immersion in warm water (30 °C) for two minutes. The position of the body and the head remained unchanged during the investigation.

The Heidelberg Retina Flowmeter is a scanning laser Doppler flowmeter which scans the retina over a 2.7×0.7 mm area in an approximately 0.3 mm deep layer for Doppler shift caused by moving red blood cells. The principle and the technical details have been described elsewhere [5, 7]. The image acquisition time is 2.048 seconds. Dilated pupils are not required for the measurement. During the image analysis, after a fast Fourier transformation a topographical image and three corresponding, brightness coded perfusion images ("Volume", "Flow" and "Velocity" surface maps) are provided automatically. The perfusion maps are derived from the Doppler shift. The numerical measurements are obtained within the software provided measuring frames of different size. The perfusion maps can be used to determine the position of the frames. The parameters provided by the instrument are in arbitrary units, and differ from the parameters characterized by similar names in hemorheology. Parameter "Volume" is a value which is proportional to the number of the moving red blood cells inside the sample volume, parameter "Flow" is a value which is proportional to the total number of red blood cells times their velocity, i.e. the total distance traveled by all the moving

red blood cells inside the sample volume per unit time. The parameter "Velocity" is the actual "Flow" value divided by the actual "Volume" value.

In our investigation the participants' head was fixed in the head rest. Pupils were undilated, and an external fixating light was used to the contralateral eye. Since the measuring unit of the device was fixed after positioning the optic nerve head in the center of the image, the distance between the cornea and the detector of the flowmeter remained constant in each individual during the measurements. The 10×2.5 degrees field size was applied. Two baseline images were acquired first, then the subject's hand was immersed in cold water. Between 30 and 60 seconds of the cold provocation 2 images were obtained (cold phase images). After this the hand was immersed into warm water, and at 2 minutes of warm stimulation 2 images were acquired (warm phase images). The better quality image from each phase was used for the measurements. The 10×10 pixel-sized measuring frame was used for the numerical measurements. One location on the temporal neuroretinal rim and one location on the temporal retina outside the peripapillary area were selected, both of them were characterized by clearly visible capillary crossings in all the three phases. This made possible to place the measuring frame exactly on the same location in all the three phases.

A careful qualitative image analysis was performed before the numerical analysis in all cases. This resulted in exclusion of 1 of the CG patients and 5 of the healthy volunteers from the evaluation due to eye movements, blinking and insufficient image quality. After this process 4 CG patients (mean age 70.5 years, range 58–78 years) and 5 healthy volunteers (mean age 63.0 years, range 52–71 years) with good quality images were included in the numerical analysis, and the calculations. Blood pressure and heart rate were measured immediately before the cold pressor test, i.e. after a 24 hours period without any medication.

The relative changes of the perfusion values (in percent) induced by the cold and warm stimuli were compared to the corresponding baseline values (100%) using the one-sample *t*-test. Covariance analysis was used to evaluate the influence of age, systolic blood pressure, diastolic blood pressure and heart rate on the perfusion changes. P values less than 0.05 were considered significant.

Results

In the CG group neuroretinal rim "Volume", "Flow", and "Velocity" decreased in a statistically significant manner for cold stimulation, and returned to the corresponding baseline values for warm stimulation (Table I, Fig. 1/A). Although statistically significant changes were not measured, a tendency to decrease in the cold phase, and a tendency to increase in the warm phase were seen for neuroretinal rim "Flow" and "Velocity" in the control group (Table I, Fig. 1/A).

Table I

Cold-induced and warm-induced percentile changes compared to the corresponding mean baseline values (100%)

Group	Parameter	Cold-induced mean change (%)	95% confidence interval (%)		p value	Warm-induced mean change (%)	95% confidence interval (%)		p value
Temporal neuroretinal rim									
CG	"Volume"	–26.05	–47.90	–4.20	0.036	+0.30	–13.45	+14.06	0.933
n=3	"Flow"	–25.82	–46.55	–5.08	0.033	–5.02	–12.16	+2.13	0.094
	"Velocity"	–23.91	–45.47	–2.36	0.041	–4.81	–11.60	+1.99	0.093
Control	"Volume"	–0.01	–40.31	+40.29	1.000	+29.94	–44.84	+104.72	0.329
n=5	"Flow"	–15.97	–47.97	+16.02	0.238	+18.19	–33.21	+69.58	0.382
	"Velocity"	–15.85	–46.59	+14.89	0.226	+15.97	–29.22	+61.17	0.382
Temporal retina									
CG	"Volume"	–12.30	–24.71	+0.12	0.051	+12.24	–20.86	+45.34	0.324
n=4	"Flow"	–22.36	–34.41	–10.32	0.010	+3.21	–22.39	+28.82	0.716
	"Velocity"	–8.30	–47.44	+30.84	0.548	+23.04	–62.10	+108.18	0.452
Control	"Volume"	–15.96	–27.63	–4.29	0.019	–4.68	–20.40	+11.05	0.455
n=5	"Flow"	–17.81	–32.45	–3.16	0.028	–10.81	–21.07	–0.55	0.043
	"Velocity"	–16.11	–30.07	–2.14	0.033	–11.21	–20.52	–1.91	0.029

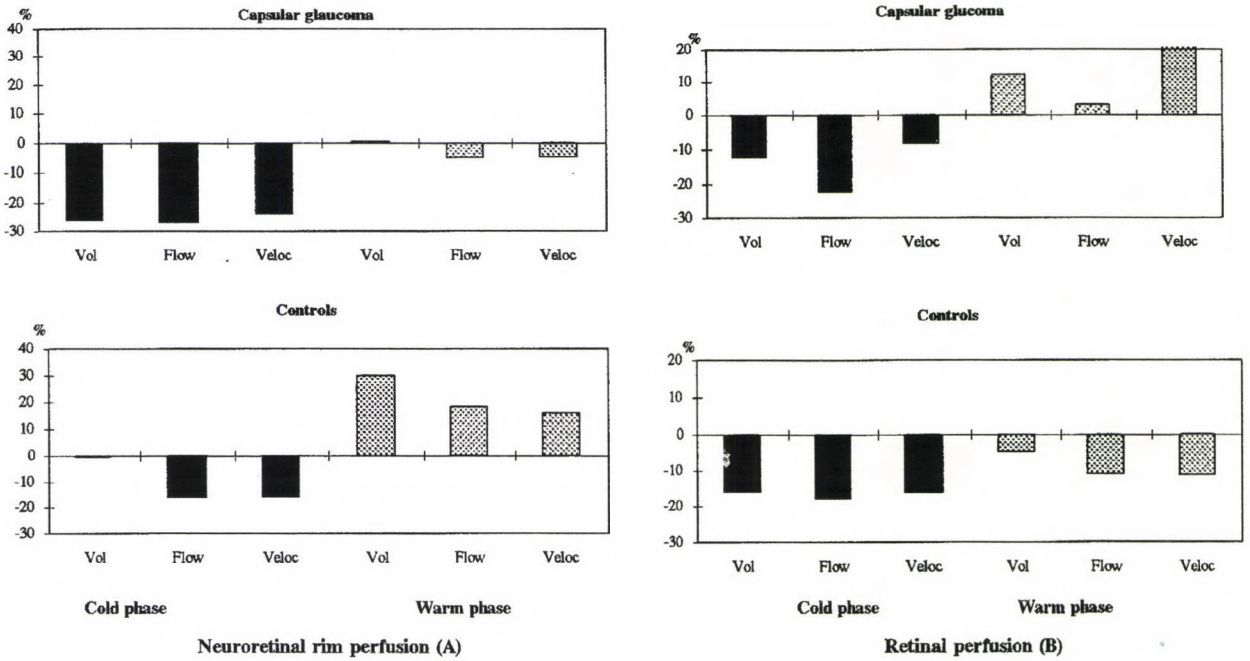

Fig. 1. Cold-induced and warm-induced mean percentile changes of neruroretinal rim capillary perfusion (A) and retinal capillary perfusion (B) compared to the corresponding baseline values

In the CG patients retinal "Flow" decreased significantly for cold stimulation. The decrease of retinal "Volume" had borderline significance in the cold phase. For warm stimulation both parameters returned to the corresponding baseline values (Table I, Fig. 1/B). In the control group retinal "Volume", "Flow", and "Velocity" decreased in a statistically significant manner for cold stimulation (Table I). All these parameters tended to return to the corresponding baseline values, however, "Flow", and "Velocity" remained still significantly reduced after warm stimulation (Table I, Fig. 1/B).

In the CG group systolic blood pressure (mean (SD) was 162.5 (15.0) mm Hg, diastolic blood pressure was 92.5 (6.5) mm Hg and heart rate was 80.0 (4.1) bpm. In the control group the figures were 154.0 (8.9) mm Hg, 82.0 (4.5) mm Hg and 74.0 (7.0) bpm, respectively. Neither these parameters nor age were found to have a significant influence on the cold-induced perfusion changes (covariance analysis, $p > 0.05$).

Discussion

In this study cold-induced and warm-induced changes of optic nerve head and retinal capillary perfusion were investigated in CG patients and healthy control subjects, who previously all showed a vasospastic cutaneous reaction for cold stimulation in the cold pressor test. The reason for selecting subjects with vasospastic reaction (i.e. reduction of cutaneous capillary flow of a finger by 70% or more for immersion of the other hand into 4 °C water for 30 seconds) was our assumption that pronounced changes in the ocular microcirculation are more probable in subjects with pronounced microcirculatory reaction in the skin. It has been shown that in some glaucoma patients with a history of cold hands visual field deteriorates during cold provocation of the fingers [4]. However, as far as we know no direct evidence for cold-induced retinal microcirculatory changes has been published. In this study our main goal was to investigate whether cutaneous cold stimulation induces any measurable change in the retinal and optic nerve head capillary perfusion.

In CG both retinal and optic nerve head capillary perfusion decreased in a statistically significant manner for cold provocation. The parameters returned to the baseline for warm stimulation. In the control group a similar, statistically significant cold-induced decrease of retinal perfusion was observed, which tended to diminish for warm stimulation, but "Flow" and "Velocity" still remained significantly reduced compared to the corresponding baseline values. In this group neuroretinal rim "Flow" and "Velocity" tended to diminish in the cold phase and tended to increase in the warm

phase compared to the baseline values, which can be interpreted as a similar pattern of the perfusion changes.

Our findings show that at least in some vasospastic subjects with or without capsular glaucoma a decrease of retinal and optic nerve head capillary perfusion can be induced by cutaneous cold provocation. However, further studies are necessary to evaluate the potential role of this type cold-induced reaction in the pathogenesis of the glaucomatous optic neuropathy.

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Application of an anti-HQIgY antibody for the measurement of IgY concentrations of hen's and quail's serum and yolk

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The development of a sensitive ELISA for the measurement of quail IgY (QIgY) was the main purpose of our study. The suitable antibody (AB) was prepared in rabbits. Both quail IgY (QIgY) and hen IgY (HIgY) were precipitated by this developed AB. For this reason it was marked as anti-hen-quail-IgY (a-HQIgY). The purified AB was conjugated with horseradish peroxidase (aHQIgY-HRP) and a sensitive direct ELISA was developed, based on this labeled AB.

The prepared aHQIgY AB which was used in this developed ELISA method was suitable for the measurement of total and specific IgY concentration in domestic hen (*Gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*) either.

As a result of our experiments it is very likely that there are identical sequences of IgYs of both species. This part of IgY has good antigen character at the same time. Probably, this phenomenon has occurrence in other *Galliform* species, too. Further investigations will be carried out in this field.

Keywords: anti-HQIgY antibody, IgY concentrations of hen's and quail's serum and yolk, quail IgY (QIgY), hen IgY (HIgY), anti-hen-quail-IgY (a-HQIgY), horseradish peroxidase (HRP), *Gallus domesticus*, *Coturnix coturnix japonica*

The main immunoglobulin fraction of fowls is called IgY, in order to distinguish it from the mammalian IgG. There are some crucial points of difference between IgG and IgY. Avian IgY is more resistant to heat, low pH and/or ion strength of environ-

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ment than IgG. IgY has no reactivity to mammalian auto-antibodies, Fc-receptors and red blood cells. In assays, these cross-reactions can be eliminated using avian-originated antibodies (ABs). Serum IgY is identical to yolk AB, as it has been proven in comparative studies. The isolation and purification for further different uses of AB has great potential. The extraction and purification of immunoglobulin fraction (IgY) from egg yolk of domestic hens and Japanese quail hens is described earlier [3, 4, 7].

According to the literature [1, 3] and our experience [4], advantages of the IgY extracted from egg yolk are the following: 1. birds (*chicken, quail,* etc.) produce ABs against highly conservative mammalian protein, too; 2. the amount of antigen needed for immune response is very low; 3. collecting and storing eggs are non-invasive and not expensive in contrast to bleeding animals and deep frozen storage of sera; 4. AB isolation and purification of yolks by combined methods are quick and inexpensive, 5. the avian AB is acid- and heat resistant, for these reasons it might be used for oral immunotherapy as well.

With regard to animal welfare (i.e. *blood sampling*), ABs, purified from egg yolks of immunized chickens can play an increasing role as an alternative to classic (*mammalian*) ABs. They can be used in immunodiagnostics [1, 5] and therapy as well [8, 9].

On the field of immunology the Japanese quail as a model animal could have an increasing importance. But it should be mentioned that specific anti quail antibodies (ABs) are not aviable on the market, although the production of AB in Japanese quail has special benefits. The quail's advantages are smaller body weigh, less food intake and smaller room in the batteries. Sexual maturity of the female quail is 35–40 days in contrast with that of the domestic hen, which start laying at the age of 130–150 days.

The development of a sensitive ELISA for the measurement of quail IgY (QIgY) was the main purpose of our study.

Materials and methods

Preparation of quail's IgY

The QIgY was prepared by combined water dilution (WD) and dextrane sulfate (DS) precipitation, based on the methods developed by Kokko et al. [3]. The purification was followed by gel filtration and ion-exchange chromatography [4, 7].

Production of antibody

Two New Zealand white female rabbits were immunized. Animals were injected intradermaly into their interscapular region by 200 μg antigen (QIgY) in 250 μl complete Freund adjuvant (*Sigma, St. Louis*). The booster immunizations were

administrated with 100 μg antigen dissolved in incomplete Freund adjuvant three times with two weeks intervals. The blood samples were taken from the marginal ear vein into heparinized tubes.

The AB (anti-quail-IgY = aQIgY) content of sera was tested against QIgY as antigen by immundiffusion technique [6]. The samples containing antibodies were collected and the pool was refrigerated.

Immunoaffinity chromatography

For the purification of AB a HIgY-Sepharose 4B resign was prepared by using CN-Br Sepharosre 4B (Pharmacia Biotech, Uppsala). The prepared immunoaffiny column (vol.: 10 ml) was used and treated according to the manual of Pharmacia Biotech.

ELISA

The purified antibody was conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis) by two step glutaraldehyde reaction [2a].

The ELISA technique was carried out on standard 96-well flat bottom microtiter plates (Greiner, Germany). Both HIgY and QIgY were used as capture antigens. Wells were blocked for nonspecific binding with normal rabbit serum. The labeled AB (aHQIgY-HRP) was used in 1:20 000 to 1:40 000 dilution. The coating (0.05 M carbonate pH 9.6), dilution and washing (PBS) buffers, the incubation times, and the color developing techniques were the same as the usual direct ELISA, which uses HRP as enzyme conjugate [9].

Results and discussion

The suitable AB was prepared in rabbits. As a result of the immunization protocol, high titre of anti-QIgY was produced in the rabbits. In the immundiffusion tests an unambiguous cross-reaction was detectable between anti-QIgY and HIgY, too. Both quail IgY (QIgY) and hen IgY (HIgY) were precipitated by this developed AB. For this reason it was marked as anti-hen-quail-IgY (a-HQIgY). Therefore, it is possible that these (quail and hen) IgYs contain homologue sequences, having good immunogen character.

The pooled rabbit serum (6.1 ml) containing anti IgY AB and 5 ml $NaHCO_3$ buffer (pH 8.3) was taken up to the CN-Br-Sepharose 4B-HIgY immunaffinity column. After an overnight incubation (20 °C) the column was eluted by 0.1 M Glycine-HCl

buffer (pH 2.5). The effluent was monitored at 280 nm and the fractions having higher density than 0.5 OD_{280} were collected into tubes containing 0.5 ml 1 M Tris buffer (pH 8.0) [2b]. The specific antibody content of these pooled fraction (12 ml) was 7.05 mg (i.e.: 1.16 mg/ml).

The purified AB was conjugated with horseradish peroxidase (aHQIgY-HRP) and a sensitive direct ELISA was developed, based on this labeled AB.

The optimal concentrations were determined for the ELISA in the case of both capture antigens (HIgY and QIgY). The investigations were carried out with 16–2000 ng antigens. The antibody (aHQIgY-HRP) dilutions were 20, 30 and 40 thousand, respectively. It was found that there was a linear binding region (LBR) in the range of 125–250 ng/well-antigen concentrations in both cases of capture antigens (Fig. 1).

The quality control parameters of the assay using five parallel samples were the following: sensitivity 1 ng, range of measurement 1–20 ng, reproducibility >95%.

The IgY concentrations of quail's serum and diluted egg yolk samples were compared with the standard QIgY dilutions by ELISA titration (Fig. 2). The dilutions ranged from 1:5000 up to 1:160 000. The character of lines indicates that the antigen binding potential of aHQIgY-HRP conjugate represents the same nature in our ELISA system, apart from the source of antigen.

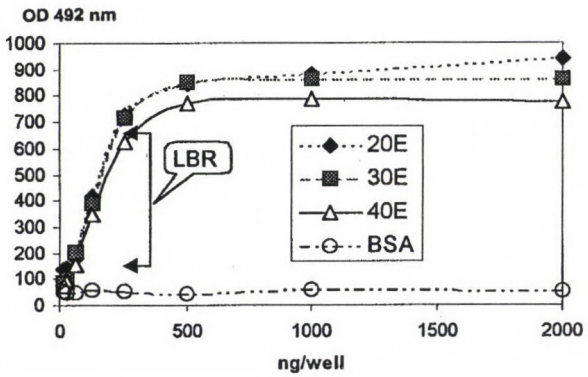

Fig. 1. Determination of linear bindig region (LBR) of ELISA
X axis represents the amount of capture antigen (QIgY) in wells

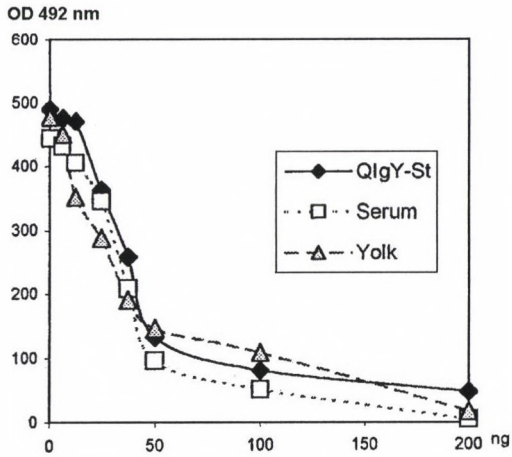

Fig. 2. IgY titration of serum and yolk in Japanese quail
X axis represents the amount of IgY of samples

Conclusion

The prepared aHQIgY AB, which was used in this developed ELISA method was suitable for the measurement of total and specific IgY concentration in domestic hen (*Gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*) either.

As a result of our experiments it is very likely that there are identical sequences of IgYs of both species. This part of IgY has good antigen character at the same time. Probably, this phenomenon has occurrence in other *Galliform* species, too. Further investigations will be carried out in this field.

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Correlation of EEG asymmetry and hypnotic susceptibility

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Hypnosis research of the last decades confirmed that some cortical regions show characteristic modification of spontaneous brain electrical activity as a function of hypnotic responsiveness. Using FFT spectrum of 16 channel EEG recording, it was demonstrated that in highly susceptible subjects the right parieto-temporal region show more electric power than the left one while the low susceptibles have left side predominance or equilibrated power in all derivations. If a specific (Ericksonian) indirect hypnosis induction was administered, the same right side preponderance could be recorded in low susceptibles, too.

On the basis of these results we can confirm the importance of the right parieto-temporal associative area in the alteration of consciousness characterizing hypnotic state.

Keywords: EEG asymmetry, hypnotic susceptibility, spontaneous brain electrical activity, FFT spectrum, indirect hypnosis induction

Research of the last three decades for possible EEG correlates of hypnotic state has resulted in contradictory findings. While some studies have reported greater power in alpha band in high susceptible subjects to hypnosis, others have not. It became clear in the early eighties that there is an important difference between the activity of the two cerebral hemispheres [4, 1]. While in low susceptible subjects in waking state the more rational, cognitive left hemisphere is responsibe for the processing of incoming signals and shows higher electrical activity, in highly susceptibles (especially in hypnosis) the

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holistic, emotional, spatial right hemispheric activity is accentuated. That is why hypnosis was mentioned in the literature as a "right hemispheric" phenomenon [2].

The present study evaluated the EEG patterns in waking and hypnotic state as moderated by different hypnotic level in terms of different cerebral regions as well as left versus right dominance of EEG power.

Subjects and methods

Volunteer students from Kossuth Lajos University were stringently controlled for hypnotic susceptibility on the Harvard Group Scale of Hypnotic Susceptibility, Form A [7], and Stanford Hypnotic Susceptibility Scale, Form C (SHSS/C) [8]. Third-one subjects participated in the experiment: 15 highly susceptibles, scoring 10–12 on the SHSS/C and 16 low susceptibles, scoring 0–2 on the SHSS/C. All subjects were strongly right handed, and had no medical problems.

Each experiment consisted of two part: waking and hypnosis in counterbalanced conditions. During the waking condition subjects had to listen to a tape recorded magazine passage, while in the hypnotic part the SHSS/C induction was administered from magnetic tape. In both conditions a 5 minutes baseline period of EEG was recorded for the analysis of brain electrical activity. In this part the subjects were told to remain relaxed with eyes closed and let their thoughts come and go (other parts of the experiment as imagination of a walk, mathematical task, and verbal-spatial discrimination task will be reported elsewhere).

In five experiments on low susceptibles the induction of hypnosis was carried out by indirect Ericksonian method. This induction procedure cannot be standardized because it is fully personalized but lasted approximately the same period as the other induction (12 minutes). After this procedure the hypnotically altered state of consciousness characterizing hypnosis on mental and behavioral level can be recorded in low susceptible subjects. This induction procedure was never controlled in any electrophysiological experiment before.

Silver-silver chloride electrodes fixed by collodium according to 10–20 system were used for recording the EEG. Bipolar or monopolar (reference on the linked earlobes) derivation was used. Resistance of electrodes were below 5kΩ and kept as equal as possible across symmetric electrodes. As bioamplifier a 16 canal Medicor EEG was used with 0.3 Hz time constant and 70 Hz filter. The data were A/D converted with 200 samples per second and stored on PC. Off-line analysis of different parts of experiment consisted of Fast Fourier Analysis (FFT) of different frequency bands as β, α, τ and δ. On the basis of the power of a frequency band a laterality quotient was calculated between the symmetric electrodes using the formula: $Q=(Lx–Rx)/((Lx+Rx)/2)$

where Lx is the group averaged power of the left side electrode and Rx is that of the right electrode. According to that if the Q is a positive value the left electrode is more active, the negative number shows the preponderance of the right hemispheric electrode location.

Results

According to our earlier research findings [1, 3, 5, 6] a marked asymmetry can be recorded between the power of the two hemispheres. In the α and β frequency band a characteristic difference could be observed as a function of hypnotic susceptibility. Using the traditional direct permissive technique of hypnotic induction (SHSS/C) the hypnotic state could be evoked in high hypnotizables but not in lows. In both frequency bands the frontal derivations showed left hemisphere predominance independently of susceptibility or hypnotic state, while in the parieto-temporal region in high susceptibles the right hemisphere was more active in waking state and hypnotic state as well (see Fig. 1). The analysis of power side by side demonstrated an augmentation of the right parieto-temporal activity in high hypnotizables. On the group averaged data other derivations were more or less equilibrated between the two hemispheres (bipolar recording).

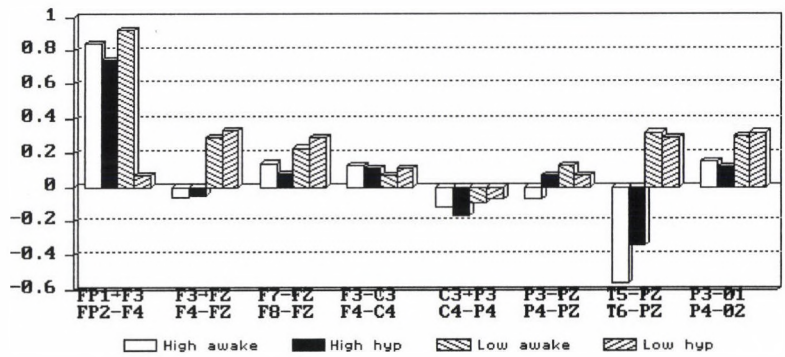

Fig. 1. EEG laterality quotients alpha

Group averaged (N: 15 and 16) laterality quotients according the formula: Q=(Lx–Rx)/((Lx+Rx)/2). Lx: power of left derivation, Rx: power of right derivation. Q+: left dominance, Q–: right dominance. High awake: high susceptible group in waking state. High hip: high susceptible group in hypnotic state. Low awake: low susceptible group without hypnotic induction. Low hip: low susceptible group after formal (ineffective) hypnotic induction. Symmetric bipolar derivations according to 10-20 system.

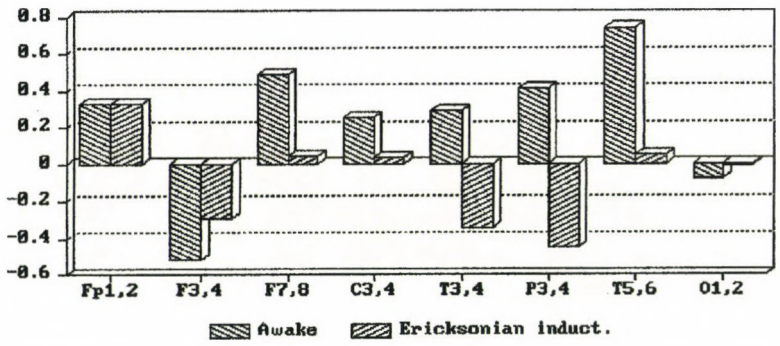

Fig. 2. EEG laterality quotients. Alpha, low susceptibility
Group averaged (N 5) laterality quotients. Awake: without hypnotic induction. Ericksonian induct: after indirect induction of hypnosis. Symmetric monopolar derivations according to 10-20 system. For other codes see Figure 1.

In 5 low susceptible subjects indirect Ericksonian hypnotic induction technique was used. The subjective reports and the observed behavior of subjects both supported the hypnotic alteration of consciousness. The EEG data obtained in monopolar derivation showed similar results as in high susceptibles: left side preponderance in fronto-polar electrodes independently of hypnotic state but right hemispheric predominance in parietal and temporal region after Ericksonian hypnotic induction (see Fig. 2). In waking state all derivations showed left side predominance (except the non-significant F4 electrode), or they were equilibrated.

The β activity showed similar right parieto-temporal predominance in high susceptible subjects but in lows only after indirect hypnotic induction.

Conclusion

The present research confirmed our earlier finding that there is difference between the EEG activity of two hemispheres as a function of hypnotic susceptibility. While in low susceptibles the signal processing is more left hemispheric, cognitive, rational approach, characterized by higher left electric power, the highs showed a more holistic, emotional, spatial mode of thinking with predominant activity in the right parieto-temporal associative cortical region. The fact that if we use Ericksonian induction to evoke hypnotic state, similarly to the highs the low susceptibles also showed higher right parieto-temporal EEG activity underlines the importance of this cortical area in hypnotic alteration of consciousness.

We suggest that the accentuated emotionality, the holistic mode of thinking and the partially inhibited rational signal processing is a result of the augmented activity of right parieto-temporal associative cortex. These findings may explain why the emotion-focused hypnotherapy as kind of psychotherapy is one of the most effective ways to treat different kind of diseases.

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Validity of viscoelastic models of blood vessel wall

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Quantitative description of biomechanical behaviour of the blood vessel wall is needed for understanding normal and pathologic functions of the vascular system. In this study we investigated the stress relaxation of blood vessels. Strips were cut from the vessel wall and they were stretched in a stepwise manner until they torn. The mechanical stress (~10^5–10^6 Pa) induced in the strips was determined. This showed an initial steep rise and then a slow, near exponential decay (stress relaxation) after each step of stretch. For the mathematical description we used two technical models (the Maxwell and the Kelvin model – they are built of two elastic elements and of one viscous element), as well as a one-dimensional continuum mechanical model. The moduli of the models were identified with a curve fitting method. Our aim was to compare these models in order to elucidate, which one describes the rheological behaviour of the blood vessel more correctly. The results of our analysis show that the corresponding moduli diverge from each other in the Maxwell and Kelvin models: that is, they depend on the mode of the assumed connection between the mechanical units. In contrast, moduli of the continuous model are unambiguous. We conclude that the continuous model properly describes the stress relaxation of the vascular wall.

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Its advantage over the technical models is that moduli describing the viscoelastic behaviour of the tissue do not depend on the not wholly well-known structure of the wall components.

Keywords: viscoelastic model, blood vessel wall, stress relaxation

Characterising biomechanical behaviour of the blood vessel wall is important to understand physiological control processes and pathological states of the vascular system. Biomechanical properties of the blood vessel wall are determined by complicated viscoelasticity of the composing passive and active elements which appreciably change under different physiological and pathological conditions [1, 2].

A possible approach to describe the viscoelastic behaviour of a complex soft material like the vascular wall is the use of technical models composed of well defined elastic and viscous elements (springs and dashpots). The governing differential equations can be determined, the solutions are given by routine mathematical techniques, and they can be applied to evaluate measured data. Most frequently the 3 element Maxwell model (also known as Standard Linear Solid model) and the Kelvin model are used [3, 4, 5].

With the application of these models, however, some theoretical and interpretational problems may appear [4, 6]. Therefore, we compared the above models and a third "continuous" one in order to decide how effectively they are applicable to describe the viscoelastic properties of the vessel wall. This latter one dimensional continuum mechanical model proved to be mathematically equivalent to the technical models but its application eliminated the problem of interpretation of the computed moduli.

Materials and methods

a) Measurements

Circumferential strips with a width of approximately 1.3 mm were cut from the descendent thoracic aorta. They were mounted in between two holders with a free length of 3.0 mm in a tissue bath containing nKR solution. One of the holder was fixed, the other connected to a strain gauge force measuring device. The length of the segment could be adjusted with a micrometer screw. Signal from the force-measuring device was preamplified and continuously recorded with a digital pen recorder (SOLTEC Corporation 500 Signal Processor). Sensitivity was approximately 10 mN. Segment length could be set with an accuracy of approximately 10 μm. Resting length of the segment was determined by carefully elevating the distance between the two holders with the micrometer screw, and minimal force development marked the value of ℓ_0.

After ℓ_0 has been identified, the length of the segment was increased in sudden steps (400 μm). The force was continuously recorded. One step of stretch uniformly included two phases: a short stretch (≅0.1 s) and a long relaxation at the new length. The measured stress showed a sudden elastic rise to a local maximum, then while the length was kept constant for 2 minutes, it slowly decayed to a local minimum in time nearly exponentially (stress relaxation). Such steps were repeated until the segment was definitively torn. Maximal stress values during the whole process were used to characterise tensile strength. To compute stress, cross section of the segment was computed from the measured wet weight of the specimen (Cahn C-31 Ultramicrobalance) applying a value of 1.06 g/cm^3 for the density.

b) Models

There are several possibilities to describe the rheological behaviour of the viscoelastic vessel wall. We used the above mentioned Maxwell and Kelvin models as well as the continuous (one-dimensional continuum mechanical) model (Fig. 1).

The Maxwell model consists of an η viscous element (dashpot) and an E_s elastic element (spring) connected in series, and an other elastic element (E_p) connected parallel to them.

In the Kelvin model we connect the spring and the dashpot parallel with each other and an other spring in series to them.

The continuous model is a thin, long homogeneous strip, which can be characterised with the E elastic (Young) module, the η viscosity and the τ relaxation time.

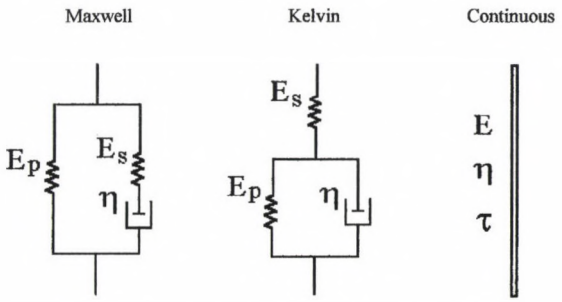

Fig. 1. Two technical and the continuous models E_p is the parallel elastic modulus, E_s is the series elastic modulus, E is the elastic (Young) modulus, η is the coefficient of viscosity, τ is the relaxation time

c) Calculations

The governing differential equation of viscoelastic models given for the (1) order is as follows:

$$\sigma + p_1 \frac{d\sigma}{dt} + \ldots = q_0 \varepsilon + q_1 \frac{d\varepsilon}{dt} + \ldots \tag{1}$$

where $\sigma = F/A$ is the mechanical stress, $\varepsilon = (l - l_0)/l_0$ is the relative strain, q_0, q_1, p_1 are parameters characterising the mechanical properties of the material [3].

The solution of Eq. (1) with the assumption that ε = const. describes the stress relaxation process:

$$\sigma(t) = c_1 + c_2 \exp\left(-\frac{t}{c_3}\right) \tag{2}$$

where t is the time, c_1, c_2 and c_3 are parameters to be determined using the measured stress-time plots.

The σ stresses as function of time during stress relaxation have the forms in the two technical models as derived from Eq. (2) [5]:

$$\sigma(t) = \varepsilon E_p + \varepsilon E_s e^{-\frac{E_s}{\eta} t} \qquad \text{(for the Maxwell model)} \tag{3}$$

$$\sigma(t) = \varepsilon \frac{E_p E_s}{E_p + E_s} + \varepsilon \frac{E_s^2}{E_p + E_s} e^{-\frac{E_p + E_s}{\eta} t} \qquad \text{(for the Kelvin model)} \tag{4}$$

Stress-relaxation in the continuous model

The solution of Eq. (1) for this model can be expressed as follows:

$$\sigma(t) = \varepsilon E + (\sigma_0 - \varepsilon E) e^{-\frac{t}{\tau}} \qquad \text{(for the continuous model)} \tag{5}$$

where σ_0 is the stress induced in the vessel wall after the moment of the stretching, τ is the relaxation time, where the initial stress decreases to the $1/e$ part. It is interesting, that in contrast to the technical models, this solution of the equation contains only the single E elastic (Young) module and the τ relaxation time but not the η viscosity. To determine the value of the viscosity we have to solve Eq. (1) for an other situation, when the strain is not kept constant, e.g. when it changes with time at a definite rate. Such a case will be discussed later.

Stretching in the continuous model

When the vessel strip is stretched, stress relaxation and creep occur at the same time. Let the value of the stress be equal to σ_I in the vessel wall before the stretch (initial stress), and let it be equal to σ_F after the stretch (final stress). Similarly, ε_I is the strain before the stretch, ε_F is the strain after the stretch.

Supposing that the stretching happens at a $\nu_\varepsilon = d\varepsilon/dt$ constant rate, the relative strain can be written in the form of $\varepsilon(t) = \nu_\varepsilon t + \varepsilon_I$. With this condition the solution of Eq. (1) can be written as follows:

$$\sigma(t) = E\nu_\varepsilon t + \sigma_I e^{-\frac{t}{\tau}} + (\eta\nu_\varepsilon - E\nu_\varepsilon\tau + E\varepsilon_I)\left(1 - e^{-\frac{t}{\tau}}\right) \tag{6}$$

Results and discussion

The parameters of the models were identified using the curve fitting methods of the MATLAB program system. In the case of the technical models the three viscoelastic moduli could be identified from the relaxation phases. In case of the continuous model the moduli could be identified in two steps. First we have identified the E elastic modulus and τ relaxation time using the relaxation phase. Then η viscosity was determined from the stretching phase. Strain rate was supposed to be quick but linear as described above. It is interesting to note here that we could have identified all the three moduli in one step if measurements with slow linear stretch were at hand.

The results of the calculations show that all the three models describe the same stress relaxation process with acceptable accuracy, but the numerical values of the corresponding moduli are different in the different models (Fig. 2).

The η viscosity appears at the continuous model only in the stretching phase, the relaxation phase includes only the τ relaxation time. It is the consequence that the viscosity was defined for macroscopic motion only, but in the case of stress relaxation there is no macroscopic motion. We can say that the tissue exerts less elastic resistance against stretch. In this model τ also may contain information connected with molecular level friction.

It is obvious that the values of the viscoelastic moduli of blood vessel wall for the same stress relaxation plot are not the same if computed for the two different technical models. That is, they depend on the assumption how the three elements are connected to each other.

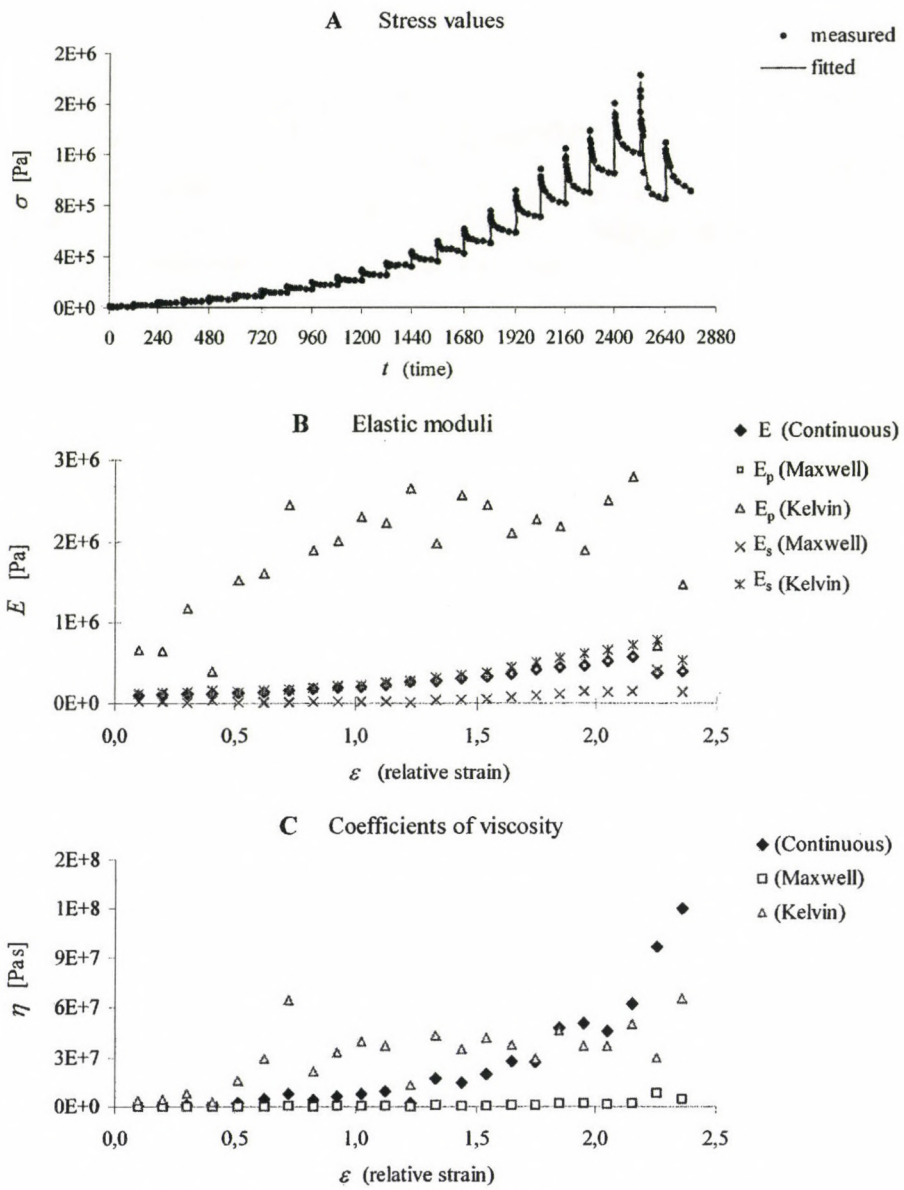

Fig. 2. Stress values and viscoelastic moduli of the various models.
A: Measured and fitted values for the stepwise stress relaxation. σ is stress, t is the elapsed time.
B: E, E_p, E_s are elastic moduli of the different models, $\varepsilon = (l - l_0)/l_0$ is the relative strain.
C: μ is coefficient of viscosity of the different models

Thus, the moduli characterise not only the mechanical properties of the tissue subjected to the measurement; they are also a function of the technical model, which has been chosen. In contrast to this the moduli of the continuous model are unambiguous, they do not depend on unproved considerations related to the vessel wall structure.

Therefore we conclude that stress relaxation of the vascular wall can be described using any of the three above discussed models with acceptable accuracy. Their mathematical equations can be arranged to an analogue form. From the point of view of the biomechanical interpretation the continuous model seems to be less ambiguous. The mechanical parameters obtained in this model are less dependent on the supposed structure of the wall components. A further advantage is that extrapolation for a three dimensional situation seems to be less problematic. At the same time we should note that the models of the vessel wall studied in this paper are the simplest. Some other works discuss more sophisticated structure and mechanical behaviour [7, 8]. The present study shows that the continuous mathematical model is a promising starting point for development of a more complex model, which takes into consideration anisotrop, non-linear properties, and fibrous structure of the vessel wall.

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Gender differences in the echocardiographic characteristics of the athletic heart

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Differences between males and females in the effects of regular physical training on the heart were investigated by echocardiography in 579 male (nonathletes: 122, athletes: 457) and in 336 (nonathletes: 84, athletes: 252) female subjects of variable age. The age groups were: children (<15 yr.), adolescent-young (15–18 yr.), young adult (19–30 yr.), adult (31–45 yr.) and people elder than 45 yr older (45<).

Hypertrophy characterised by relative left ventricular muscle mass (rel.LVMM) of the athletic heart was manifest also in the females, but in comparison with the males female values were much lower both in the unconditioned and in the conditioned groups. In peaple belonging to the oldest groups no differences were seen either between the males and females or between athletes and nonathletes. In comparing nonathletic subjects, females showed smaller left ventricular contractility as reflected by a higher LVET/QT ratio and an increased ability of diastolic relaxation as indicated by a higher E/A ratio. Physical training induced a more marked development when the initial level was lower, namely, in the contractility of the females, and in the compliance of the males. No intersex difference was seen in the modified regulation of the athletic heart, characterised by a lower resting heart rate and slower circumferential shortening velocity (VCF).

Keywords: echocardiography, athletic heart, ageing, gender differences, physical training

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In the authors' previous study made in males [7] echocardiographic characteristics of the athletic heart were divided into morphological, functional and regulative signs. Most of the reports on the athletic heart have referred to males, there are relatively few extensive studies that contain data referring to female athletes [8], although several studies suggest that some basic differences can be supposed between males and females in their response to regular exercise training and also in their vulnerability to different cardiovascular diseases [8, 9].

In the present study an attempt was made to obtain further data on the differences existing between males and females in respect of the morphological, functional and regulative characteristics of the athletic heart at different ages. Morphology, namely, myocardial hypertrophy was described as the relative amount of left ventricular muscle mass (rel.LVMM). According to earlier experiences [2], contractile dynamism, i.e. the systolic function of the heart, was expressed by a quotient relating the duration of a mechanical event during systole (left ventricular ejection time: LVET) and the electrical systole (QT). The lower the ratio, the higher is myocardial contractility. As in several other publications [3, 5], diastolic function can be suitably described by the ratio of the peak velocities of the early and late phases of the transmitral flow (E/A). Autonomous regulation can be estimated by counting resting heart rate in which resting sympathetic and parasympathetic activities are equally involved, and by the circumferential shortening velocity that only refers to the ventricular myocardium, and is thus indicative of only sympathetic activity [4].

Materials and methods

Examinations were made in 579 male (unconditioned: 122, conditioned: 457) and in 336 (unconditioned: 84, conditioned: 252) female subjects of various ages: children (<15 yr.), adolescent-young (15–18 yr.), young adult (19–30 yr.), adults (31–45 yr.) and older (45<) groups.

Echocardiographic measurements were made by using a Dornier AI 4800 type echocardiograph with a 2.5 MHz transducer. All measurements were taken in the morning at absolute rest in a half-sinistral recumbent position.

Two-dimensionally guided M-mode recordings were obtained parasternally; left ventricular wall thickness and internal diameters were measured on the monitor by using the trackball.

In a similar way as in our previous works [7], left ventricular muscle mass (LVMM) was calculated by cubing the respective diameters [10]: $LVMM = \{(IVST+PWT+EDD)^3 - EDV\} \cdot 1.05$, where IVST is the interventricular septum thickness, PWT is the posterior wall thickness, EDD is the left ventricular end

diastolic diameter, EDV is the end-diastolic volume = EDD^3, 1.05 the density of the cardiac wall. In order to use indices in which the exponents of the numerator and denominator match, LVMM was related to the third power of the square root of BSA [6, 7].

To obtain the E/A quotient, transmitral peak flow velocities were estimated in an apical four chamber view by pulsatile Doppler measurement. To estimate the LVET/QT ratio, QT was measured in the ecg and LVET was measured from transaortic flow by the continuous Doppler method. Circumferential shortening velocity was calculated by the formula: VCF = (EDD–ESD)/EDD·LVET, where ESD is the end-systolic diameter.

Always several cardiac cycles were recorded. In estimating peak velocities the fastest one of the 3–5 recorded speeds was taken into account; in calculating the morphological parameters the mean of 6–10 cardiac cycles was used.

The differences between the athletic and non-athletic groups, and those between the comparable male and female groups were tested for significance by using *t*-tests for independent samples.

Results

Average values and standard deviations are demonstrated in Table I. Rel.LVMM of the athletic groups differed significantly in either sex from that of the nonathletes, though female means were smaller. This intersex difference increased with age until young adult age (30 yr.), then decreased, and in the oldest group no difference was seen. In the oldest groups there was no difference between the conditioned and unconditioned groups either.

The LVET/QT ratio was lower, i.e. myocardial contractility was higher, in the athletic groups of the males, the differences were significant in the children and in the young adults. In the nonconditioned females higher ratios were seen which differed significantly from those of the male groups in the adolescent-young and in the young adult subjects. Regular training, however, elicited a greater decrease so that athletic means were much nearer to each other in the two genders.

Diastolic function (ventricular compliance) was investigated by the E/A ratio. In the males, higher values i.e. a better ability to relax were seen in the athletic groups than in the controls. The ratios of the nonathletic females were markedly higher than those of the nonathletic males, revealing a better compliance. The differences in the young adult and in the adult groups were significant, while no difference was seen in the older subjects. Regular physical training did not elicit a further elevation from the high values of the young adult and adult nonathletic females, it was the older female group only in which compliance was markedly and significantly better than in the control women.

Table I

Echocardiographic parameters of male and female nonathletic and athletic subjects (mean ± S.D).

Parameter	Age	Males	Males	Females	Females
		Nonathletic	Athletic	Nonathletic	Athletic
Rel.LVMM	<15	75.7 ± 12.2	**82.2 ± 14.1 o**	67.6 ± 5.2 +	**80.9 ± 13.8 +**
	15–18	66.0 ± 13.3	**85.4 ± 17.5 ***	62.8 ± 6.6	***74.9 ± 10.2 * ***
	19–30	73.1 ± 9.4	**94.6 ± 17.0 ***	***60.2 ± 7.5 ****	***76.2 ± 11.4 * ***
	31–45	75.9 ± 11.3	**91.3 ± 12.7 ***	70.5 ± 15.5	**84.9 ± 9.3 o**
	45<	86.3 ± 23.1	84.5 ± 18.3	85.6 ± 16.0	82.1 ± 11.2
LVET/QT	<15	0.80 ± 0.06	**0.75 ± 0.06 ***	0.81 ± 0.08	**0.76 ± 0.06 o**
	15–18	0.74 ± 0.04	0.74 ± 0.06	***0.82 ± 0.06 !***	**0.75 ± 0.05 ***
	19–30	0.75 ± 0.05	**0.73 ± 0.05 o**	***0.83 ± 0.05 !***	***0.75 ± 0.06 * !***
	31–45	0.76 ± 0.04	0.76 ± 0.07	0.78 ± 0.04	0.74 ± 0.04
	45<	0.76 ± 0.05	0.76 ± 0.06	0.78 ± 0.07	0.77 ± 0.04
E/A	<15	2.00 ± 0.38	2.04 ± 0.36	2.20 ± 0.32	2.04 ± 0.38
	15–18	1.81 ± 0.37	**2.23 ± 0.59 o**	2.05 ± 0.62	2.45 ± 0.87 +
	19–30	1.91 ± 0.38	**2.08 ± 0.51 o**	***2.26 ± 0.43 ****	***2.27 ± 0.70 o***
	31–45	1.43 ± 0.27	**1.73 ± 0.35 !**	***1.71 ± 0.43 o***	1.87 ± 0.32
	45<	1.24 ± 0.36	1.56 ± 0.40 +	1.14 ± 0.30	**1.52 ± 0.37 !**
Heart rate	<15	82.1 ± 13.0	**73.1 ± 11.4 ***	82.7 ± 10.8	74.5 ± 12.4 +
	15–18	70.1 ± 9.7	**60.4 ± 10.2 !**	78.0 ± 12.0 +	**60.3 ± 9.5 ***
	19–30	76.2 ± 14.6	**58.8 ± 9.9 ***	72.6 ± 12.7	**60.6 ± 9.8 ***
	31–45	71.6 ± 13.1	**57.5 ± 10.7 ***	72.1 ± 12.0	**54.0 ± 6.5 ***
	45<	69.3 ± 8.4	64.0 ± 8.5	73.7 ± 7.3	**61.8 ± 7.2 ***
VCF	<15	1.35 ± 0.17	1.29 ± 0.17	1.39 ± 0.11	1.35 ± 0.23 +
	15–18	1.27 ± 0.11	1.18 ± 0.15 +	1.35 ± 0.16	**1.17 ± 0.14 ***
	19–30	1.26 ± 0,15	**1.16 ± 0.17 ***	1.30 ± 0.26	**1.18 ± 0.15 !**
	31–45	1.19 ± 0.16	1.18 ± 0.18	1.24 ± 0.13	1.14 ± 0.11 +
	45<	1.13 ± 0.20	1.22 ± 0.15	1.30 ± 0.21 +	1.23 ± 0.14

rel.LVMM: relative left ventricular muscle mass, LVET: left ventricular ejection time, QT: electric systole, E/A: ratio of peak velocities during the early and late phases of transmitral flow, VCF: circumferential shortening velocity. Bold numbers: significant difference between nonathletic and athletic subjects, shaded cells: significant difference between males and females. +: $p<0.1$, o: $p<0.05$, !: $p<0.01$, *: $p<0.001$

There was no difference between males and females in resting heart rate. In both sexes a slight age-dependent decrease was seen in the control groups, the athletic groups displayed a definite resting bradycardia.

A similar tendency was seen in VCF: a slight decrease with advancing age, smaller values in the athletic groups, no marked intersex differences, only a slight tendency that females would produce somewhat higher values.

Discussion

Differences between males and females in the effects of regular physical training on the morphological, functional and regulative characteristics of the heart were investigated in athletic and nonathletic groups of variable age.

Corresponding to other data [6], the exercise-induced increase of LVMM was very similar in the males and females. The body size related LVMM of females was, however, lower than that of males, both in the athletic and in the non-athletic groups. To choose one of the several possible explanations, such as differences in body structure, or dissimilar haemodynamic response to physical exercise [6], the simplest explanation arises from the hormonal differences as both testosterone and oestrogen sensitive receptors are described in the cardiac muscle, the former increasing cardiac muscle mass, the latter protecting against cardiac hypertrophy [1, 9]. The suggested role of the hormonal effects can be supported also by our data for the oldest groups in which intersex differences were no longer present.

Another remarkable observation made in our oldest groups was the missing difference between the athletic and non-athletic groups. The similarity in the rel.LVMM of the differently conditioned groups was not due to a lack of hypertrophy of the athletic hearts, but to an elevation of the nontrained subjects' cardiac weight. The suggestion that myocardial hypertrophy would no longer be a favourable feature of the athletic heart at an old age can be accepted, all the more so since at an older age cardiac hypertrophy, especially an increased wall thickness is often coupled with impaired cardiac, especially diastolic function, very often as a consequence of borderline or severe systemic hypertension, obesity, etc.

Several of the functional characteristics of the males and females athletic heart might be regarded as being a consequence of morphological variability. A more massive left ventricular musculature can produce higher dynamism during systole, but a decreased ability to relax during diastole in the non-trained males in comparison with the non-trained females. The effect of regular exercise training seems to depend on the initial level of these functions: it will bring about a more marked improvement in the male diastolic functions and in the systolic activity of females. Our data seem to support

the observations of other studies [3, 5] in that regular physical training is likely to protect against an age-dependent decrease of LV compliance, because the E/A ratio was higher in both genders in the oldest athletic groups than in the non-athletic ones.

Resting heart rate is supposed to be controlled mostly by both systems of autonomous nervous control (sympathetic as well as parasympathetic activity), while VCF as a reflection of ventricular myocardial contractility is governed by sympathetic activity alone. The similarity of these parameters in the male and female subjects suggests that there is no marked intersex difference in the autonomous nervous component during adaptation to regular exercise training.

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Galanin mediated inhibitory nervous modulation of cutaneous vascular reactions

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Noxious stimulation induces local inflammatory responses in a variety of mammals but these reactions are only faint in avian species. The possibility that endogenous galanin inhibits neurogenic vascular responses in avians was tested in the wing skin of anaesthetized pigeons. Intraarterial infusion of nanomolar concentrations of the specific galanin antagonist M35 dose dependently enhanced the small mustard oil induced increase of skin blood flow measured by means of a Laser Doppler Imager. Similarly, the small transient vasodilatation following electrical stimulation of a cutaneous nerve was also enhanced by M35. The effect of M35 was not observed after chronic denervation. Coperfusion of M35 dose dependently augmented the histamine and bradykinin induced plasma extravasation revealed by skin microdialyses, but this effect was abolished in the chronically denervated skin. However, chronic denervation *per se* enhanced the plasma extravasation induced by histamine but not by bradykinin and this effect was diminished by coperfusion of galanin. The results suggest an inhibitory modulation of cutaneous neurogenic inflammatory reactions by endogenous galanin in the pigeon.

Keywords: galanin, inhibitory nervous modulation, cutaneous vascular reactions, avian species, galanin antagonist M 35, cutaneous nerve, histamine, bradykinin, pigeon

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A significant population of mammalian cutaneous afferent nerves involved in the transmission of noxious information also possesses local regulatory or "sensory efferent" functions. Sensory nerve-mediated neurogenic inflammatory responses such as involving vasodilatation and plasma protein extravasation are the most prominent features of sensory efferent functions. These tissue reactions are mediated primarily by tachykinins and calcitonin gene-related peptide (CGRP) released from stimulated sensory nerve terminals. In addition, a modulatory, pro-inflammatory effect of these sensory neuropeptides has also been documented [6]. In contrast to mammals, neurogenic inflammatory responses are only moderately expressed in avian species [3, 8]. This species difference cannot be explained by an inappropriate level of sensory neuropeptides. Immuno-histochemical studies revealed that avian dorsal root ganglion neurones contain tachykinins and CGRP similar to mammalian sensory cells [5, 8]. However, in pigeon dorsal root ganglia the proportion of galanin-immunoreactive neurones is considerably higher than in mammals [5]. Previous studies have shown that exogenous galanin inhibited neurogenic inflammatory reactions in the rat by inhibition of peptide release from afferent nerves [4, 10]. We hypothesised that in the pigeon endogenous galanin may interfere with the effect of sensory neuropeptides or inflammatory mediators leading to a reduction of their vascular actions. To test this hypothesis, in the present experiments we studied the effect of a specific galanin antagonist on sensory nerve-mediated cutaneous vascular responses.

Materials and methods

Experiments were performed on 76 domestic pigeons anaesthetised with an isoflurane-oxygen gas mixture. The dorsal surface of the wing was defeathered one day prior to the experiments. The arterial blood pressure and the body temperature were continuously monitored. In 30 animals the ulnar artery was canulated for close arterial injection of galanin antagonist (galanin (1–13) – bradykinin (2–9) amide, M35, Neosystem) dissolved in physiological saline. Skin blood flux (SBF) was measured on the defeathered wing skin by a Laser Doppler Imager (LDI). In the first series of experiments different doses of M35 and its vehicle were administered into the ulnar artery (50 μl/min). Sixteen consecutive LDI images were recorded from a standardised skin area at 1 min intervals. After determination of the baseline perfusion a piece of filter paper moistened with 5% mustard oil dissolved in liquid paraffin was placed onto the skin. In 6 cases the radial nerve innervating the investigated skin area was transected 4–6 days before the experiments (chronic denervation). In the second series of experiments antidromic nerve stimulation was used to elicit cutaneous vascular responses in guanethidin pretreated animals (20 mg/kg s.c.). The nervus cutaneous

antebrachii lateralis was exposed for electrical nerve stimulation. Following an intra-arterial infusion of M35 or its vehicle sequential LDI images were taken with a frequency of 8/min for 8 min. Nerve stimulation was started after the 1st min with rectangular pulses of 10 Hz, 20 V and 0.5 ms for 20 s. Values of SBF were determined by calculation of the average flux values of single images. For a detailed description of the data analysis see reference [9]. Statistical analysis of the experimental data was performed with ANOVA.

In 46 animals plasmapheresis capillaries (diameter 0.4 mms, cut off size 300 kDa, Asahi Japan) were inserted intracutaneously with the aid of a guiding cannula and connected through fine plastic tubes (Tygon) to a perfusion pump. The capillaries were perfused with Ringer's solution at a flow rate of 3.25 μl/min. The effluent fractions were collected every 20 min for photometric determination of their protein content using Bradford's method [2]. After a 60 min equilibration period histamine or bradykinin dissolved in Ringer's solution were perfused for 40 min. To study a possible modulatory role of galanin on cutaneous vasodilatation and plasma extravasation the galanin antagonist M35 was co-perfused with histamine and bradykinin. SBF was measured with LDI as described above. To study the contribution of nervous elements to the mechanism of inflammatory responses chronic cutaneous denervation was produced by sectioning the radial and ulnar nerves 5–7 days prior to the experiments.

Results

Epicutaneous application of mustard oil induced a slight increase in SBF (15.27± 2.39%). Intraarterial infusion of 1 or 5 nM M35 significantly and dose-dependently increased mustard oil-induced enhancement of SBF (1 nM : 29.74±4.33% and 5 nM : 40.4±5.85%) calculated by the area under the curve method (Fig. 1). Neither infusion of vehicle nor infusion of 5 nM M35 alone altered significantly the SBF. In the chronically denervated skin, mustard oil evoked responses similar to that was seen in intact skin (16.12±3.56%). In contrast, administration of M35 failed to result in an enhancement of mustard oil-induced cutaneous vasodilatation (Fig. 1).

Antidromic electrical stimulation of the cutaneous antebrachii lateralis nerve with a short train of suprathreshold impulses elicited a transient increase in SBF (max. 5.15± 2.51%) in guanethidin pretreated animals. Intraarterial infusion of 1 nM M35 resulted in an increase of the immediate vasodilatatory response (9.91±1.22%) followed by a further increase in the SBF (10.99±1.24%) after a latency of 60s. Infusion of 5 nM M35 produced a further enhancement (13.79±0.71%) of these vasodilatatory responses.

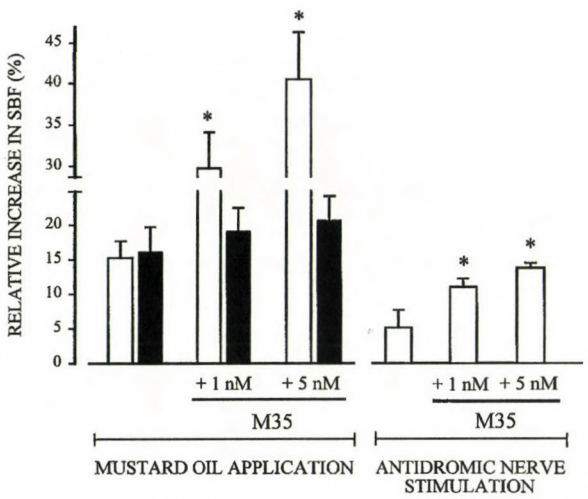

Fig. 1. Effect of M35, a galanin antagonist on relative increases in skin blood flow (mean ± S.E.M.) induced by mustard oil application or antidromic nerve stimulation in intact (open columns) and chronically denervated (black columns) skin. *: significantly different from corresponding control value, $p<0.05$

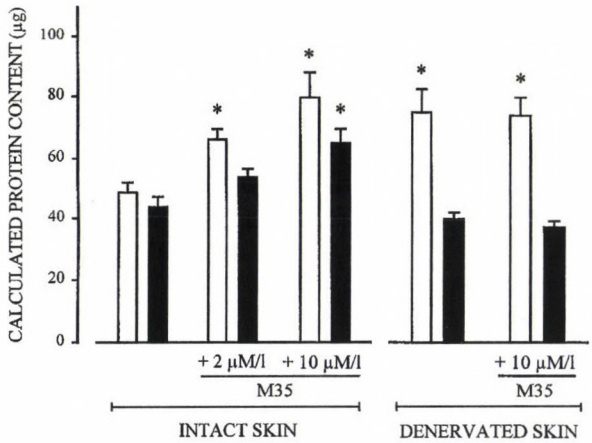

Fig. 2. Effect of M35, a galanin antagonist on histamine- (500 µM/l, open columns) and bradykinin- (200 µM/l, black columns) induced cutaneous plasma protein extravasation in intact and denervated skin. Values represent the total protein content of the perfusate (mean ± S.E.M.). *: significantly different from corresponding control value, $p<0.05$

To elicit plasma extravasation, histamine and bradykinin were perfused at constant doses of 500 and 200 μM/l, respectively, which have been shown to produce similar and reproducible increases in vascular permeability. The calculated protein content of the fractions collected during a 60 min period following the start of the perfusion of histamine and bradykinin amounted to 48.65±3.36 μg and 43.73±3.36 μg, respectively, which were significantly higher than compared to the control values obtained after perfusion of Ringer's solution (20.91±1.29 μg). Co-administration of increasing doses (2 and 10 μM/l) of M35 dose-dependently enhanced the permeability increasing effects both of histamine and bradykinin by 63% and 48%, respectively (Fig. 2).

Chronic denervation *per se* significantly enhanced the permeability increasing effect of histamine but was without effect on the action of bradykinin. Co-perfusion of galanin (10 μM/l) reduced this augmented response following histamine application. Co-administration of M35 failed to affect significantly the permeability increasing effect of either histamine or bradykinin (Fig. 2).

Perfusion of both histamine and bradykinin increased cutaneous blood flow by 28.43±4.19% and 22.04±2.9%. Co-administration of M35 significantly augmented the vasodilatatory effect of histamine (SBF increase: 40.92±6.25%) but not that of bradykinin (26.41±4.82%). Chronic denervation reduced the vasodilatation elicited by histamine (17.81±5.33%) but failed to affect the response to bradykinin (21.2±3.7%). However, in the chronically denervated skin M35 was without any effect on irritant induced vasodilatation (histamine: 19.32±3.1%; bradykinin: 22.62±3.23%).

Discussion

Neurogenic cutaneous vascular responses elicited by chemical irritants and mediated by activation of afferent nerves are common in mammalian species. In contrast, in the pigeon only moderate vascular responses were evoked by epicutaneous application of mustard oil in the present study. This weak vasodilatatory response was greatly augmented by a prior close arterial injection of M35, a high affinity galanin antagonist. Similarly, the modest cutaneous vasodilatation produced by antidromic nerve stimulation was also significantly enhanced after the administration of the galanin antagonist. These findings strongly indicate that endogenous galanin exerts a tonic inhibitory action on sensory nerve-mediated cutaneous vasodilatation. Furthermore, the findings showing an abolition of the pro-inflammatory effect of the galanin antagonist in the chronically denervated skin suggest that inhibitory modulation of the vasodilatatory response by galanin is neurogenic in nature.

Histamine and bradykinin produced distinct plasma protein extravasation in the pigeon skin. The amount of extravasated plasma protein was greatly increased after a prior administration of M35. These results suggest that endogenous galanin may exert an inhibitory effect on histamine- and bradykinin-evoked increases in vascular permeability. In the chronically denervated skin administration of M35 was without effect on histamine or bradykinin elicited vascular responses. This suggests that the inhibitory modulation of the permeability enhancing effects of these vasoactive agents involves a neurogenic link. It is worthy to mention that chronic denervation resulted in a marked augmentation of the vascular permeability enhancing effect of histamine but not bradykinin. This may be explained by a possible difference in the (neural) regulation of vascular histamine and bradykinin receptors, respectively. Alternatively, these changes may be related to denervation-induced alterations in the prejunctional modulation of histamine-induced neuropeptide release from sensory nerves [7].

In conclusion, the present experiments revealed a marked inhibitory modulation of cutaneous vascular responses involving neurogenic vasodilatation and plasma extravasation by endogenous galanin. The results provide evidence for a new, hitherto unrecognized inhibitory local efferent function of peptidergic cutaneous sensory nerves. Inhibition of peptide release from sensory nerves by endogenous agents, e.g. by galanin, may explain the inability of chemical irritants or antidromic nerve stimulation to evoke sensory nerve-mediated neurogenic vascular responses in species other than small laboratory rodents.

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Hyperphagia in cold-adapted rats: A possible role for neuropeptide Y

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The feeding response to intracerebroventricular injection of neuropeptide Y or to starvation is greater in cold-adapted than in non-adapted rats, suggesting that with cold-adaptation the central sensitivity to this peptide is increased. Hypometabolism and hypothermia (which usually follow the administration of neuropeptide Y) cannot, however, be demonstrated in the course of cold-adaptation *per se*.

Keywords: cold-adaptation, food intake, fasting, neuropeptide Y, thermoregulation

Hyperphagia is known to be a necessary accompaniment of cold-adaptation [6], in order to sustain a high metabolic rate in face of continuous heat loss to the cold environment. A great number of substances might contribute to any particular alteration in the pattern of food intake [5]. One possible candidate for the mediation of hyperphagia induced by cold-adaptation could be neuropeptide Y (NPY), since a) centrally applied (exogenous) NPY was reported to elicit food intake [4], b) fasting was demonstrated to result in elevated (endogenous) central NPY-levels [8] and finally, c) some data [7] indicated elevated central NPY-levels during cold-exposure. This is the first study for the analysis of the possible role of NPY in the regulation of food intake in animals that were exposed to cold not only acutely, but long enough to develop cold-adaptation (including the adaptive hyperphagia).

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In the studies presented here, food intake was assessed in cold-adapted and non-adapted rats following central administration of NPY, or following a period of food withdrawal, in order to see the responsiveness to exogenous or endogenous NPY in cold-adapted as compared to non-adapted rats.

Materials and methods

Adult (250–270 g) female Wistar rats were kept, for over three weeks, in a cold chamber of 3–5 °C (cold-adapted, CA group), or at room temperature of 22–25 °C (non-adapted, NA group), with lights on between 6.00 and 18.00 h. Standard laboratory chow and water were available *ad libitum*. The animals were habituated to handling and daily measurements of body weight.

In both CA and NA rats a metal guide cannula was implanted into a lateral cerebral ventricle (ICV) at least a week before the feeding tests. At tests 2 μg NPY was injected ICV (according the methods that had been used previously, 10) in a volume of 5 μl to normally satiated rats at 9.00 h. Body weights were measured every 30 min for a 3-h period. Although concurrent water intake and urinary/fecal loss also modified body weights, most of the weight changes were assumed to reflect food intake (ICV saline injections were followed by no change or a slight fall in body weight). At other tests no ICV injections were given, but a food withdrawal period (at the corresponding adaptation environment) preceded the re-feeding period, whilst water remained accessible. During re-feeding the food intake was assessed again from changes of body weight. In CA rats fasting lasted for 48-h and it resulted in a weight loss of 14–15%, in NA rats either the duration of fasting was the same as that in CA rats (48-h food whitdrawal, that resulted in 7–8% weight loss), or the severity of fasting was similar (15–16% weight loss in the course of 120-h fasting). The initial body weights were similar in the different groups.

Student's *t*-tests were used for statistical analysis. In the Figures mean values ± S.E.M. are demonstrated.

Results and discussion

Central injections of 0.9% NaCl were invariably followed by no change (or a slight fall) of body weight both in CA and in NA rats, *i.e.*, normally satiated rats did not start eating. Increase in body weight (*i.e.*, food intake) was observed in both CA and NA rats following ICV injections of 2 μg NPY, and our preliminary data show a dose-dependence for the feeding response to the substance. The NPY-induced food intake

was consistently higher in CA than in NA rats (Fig. 1), and the cumulative food intake of CA rats, by the end of the 3-h observation period, significantly exceeded that of NA rats.

In CA rats re-fed after 48-h food deprivation the food consumption exceeded the consumption seen in NA rats, irrespective whether the duration or the severity of deprivation was comparable in the two groups (Fig. 2): NA rats, deprived either for 48 or 120 hours, ate less upon re-feeding than the re-fed CA rats.

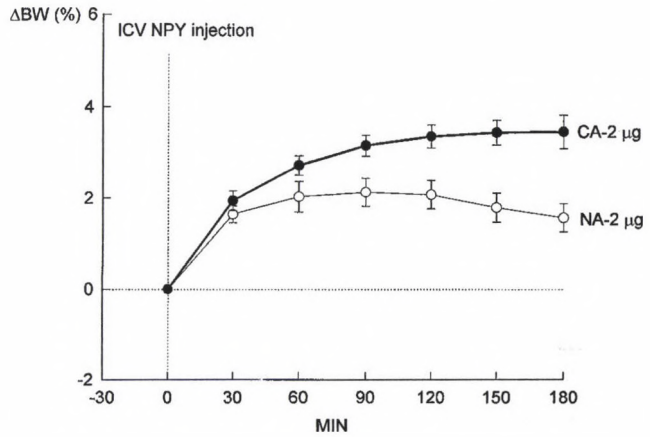

Fig. 1. Changes of body weight (in % of the starting value) following ICV injection of 2 µg NPY to non-adapted (open circles, n=13) and cold-adapted (closed circles, n=11) rats

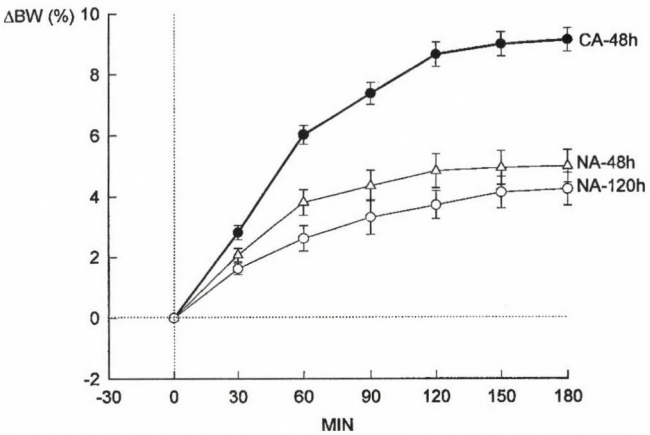

Fig. 2. Changes of body weight (in % of the fasting value) in the course of re-feeding non-adapted rats fasting for 48-h (open triangles, n=12) or for 120-h (open circles, n=11), and in the course of re-feeding cold-adapted rats fasting for 48-h (closed circles, n=14)

Characteristically, NPY has dual action. On the one hand, it influences mechanisms of food intake regulation, namely, it increases food consumption [4]. A presumed enhancement of such NPY activity might thus contribute to the hyperphagia observed during cold-adaptation. On the other hand, NPY-administration also influences the regulatory system of thermal homeostasis: it initiates an energy-saving behaviour, including hypometabolism and hypothermia [1, 4]. The latter effects would be unfavourable, or even deleterious, in the course of cold-adaptation. In reality, cold-adaptation is accompanied not by a decrease, but by an increase of metabolic rate and thermal responsiveness [9].

In lean Zucker rats the NPY release in the paraventricular nucleus was found suppressed [3] during cold-exposure. Another study demonstrated decreased plasma leptin-, but unchanged central NPY-concentrations in rats exposed to cold [2]. These data seem to indicate that the cold-induced hyperphagia might not involve NPY as a single important causative factor. However, contrary to the mentioned data, elevated hypothalamic NPY-levels during cold-exposure were also reported (7). Besides, the present data demonstrate that, even if the same NPY dose is applied, the feeding response is greater in CA than in NA rats, suggesting an enhanced responsiveness to NPY in this regulatory system. Fasting-induced endogenous NPY-activation is also more efficient in CA than in NA rats, during their re-feeding. Apparently, an enhanced NPY-activity seems to contribute to the development of hyperphagia in cold-adaptation. In contrast, in the metabolic/thermoregulatory system cold-adaptation does not appears to induce any change resembling an increased NPY activity, *i.e.*, there is no tendency for hypometabolism and hypothermia [9].

Thus, although NPY has a potential to alter both food intake and body temperature, in cold adaptation the effects of the high NPY-activity are limited to the regulation of food intake and do not influence the regulation of body temperature. This is probably due to the fact that neither the central regulatory pathways, nor their afferent signals are identical in the two systems.

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Exercise-physiological approach in the analysis of blood glucose curves

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The effect of a single bout of oral glucose administration on the blood glucose level during three hours of observation was studied at rest in 6 physical education students and in 6 elite walkers with different physical condition. The influence of the physical condition on the observed data was also investigated.

The evaluation of the glucose tolerance test (OGTT) was first built on a mathematical model and it was developed by a new method. The point of the brand-new method is the interrelationship between the relative percentage differences of blood glucose values, which is analysed as a function of time. The data of the new method better reflect the alterations of blood glucose regulation after oral administration of glucose.

The new method contributes to the more accurate analysis of OGTT curves for beside estimation of the absolute values, it considers the relative differences, thus a thorough examination of the glucose metabolism can be carried out.

It opens new potentialities in the analysis of the individual, absorptive, hormonal and fitness-dependent effects of different carbohydrates that are used abundantly by athletes.

Keywords: blood glucose level, physical education, physical condition, glucose tolerance test, carbohydrates, athletes

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Pharmacologists have endeavoured for many years to work out relatively simple methods to follow the route of the exogenous substances (medicines) in the human body. Sports scientists, on the other hand, investigate how to maintain the constant optimum level of blood glucose during physical exercise, which ensures the quick mobilisation of energy supply, which is of crucial importance in successful sports performance.

With this study we try to contribute to both endeavours. The classic pharmacokinetic investigation studies the absorption, the maximal concentration, the half-time, the metabolism and the elimination of a medicine [2]. In athletes metabolic changes during physical exercise and the effects of acute physical exercise are also studied.

Schwartz et al. [7] investigated the interrelationship between acute physical exercise and pharmacokinetics. Pharmacokinetic parameters of Doxycyclin, Tetracyclin and Sulfomethiazol were studied in 20–28 year-old healthy, sedentary persons. Higher concentration of all the three medicines was observed after physical exercise than at rest. The urinary excretion of the medicines were lower in the exercising group, than in the control one.

In our previous studies we observed changes in the pharmacokinetic parameters in regularly exercising persons [3]. After the oral administration in case of the exercising group the metronidazol absorbed faster, in lower concentration in serum with shorter half-time and quicker elimination of the medicine than in the control group [8].

The estimation of blood glucose level is mainly used in the diagnosis of diabetes mellitus. The glucose tolerance test (OGTT) includes the measurement of fasting blood glucose value, then those measured with 30, 60 and 120 minutes after oral administration of glucose solution, thus helping to establish the diagnosis.

In our previous studies blood glucose values were evaluated by Ackermann's method built on mathematical model improved by Jansson et al. [4] and by our team. It has been used in the diagnosis of diabetes mellitus so far.

In athletic groups of different physical condition the route of the various carbohydrates have been followed at rest and during acute physical exercise (ergometry and field tests) by the parameters of OGTT built on a mathematical model. Differences were observed between the groups and the various situations, which, within narrow limits, can give information on the fitness level. These investigations supported our previous observations, namely, that the biopharmatical conditions of medicines and other exogenous substances vary in athletes of different physical condition, witch can be explained by the phenomena of metabolic and biopharmatical adaptations [9, 10].

Though the energy, coming from carbohydrates is relatively low, in the majority of sports it is one of the limiting factors of sports performance. In endurance sports the consumption of carbohydrates before and during competitions is very common. In sport

games during matches it is also widespread to consume carbohydrates for quick energy supplementation. To follow the route of the supplementary carbohydrate and to study its absorption, metabolism and excretion are necessary to known, because it makes possible to choose the adequate carbohydrate for that covers each athlete's special demands.

Noakes et al. [5] do not recommend the consumption of carbohydrates before physical exercise. According to their study, consumption of concentrated glucose solution half an hour before physical exercise resulted in the impairment of the performance.

Bonen et al. [1] compared blood glucose values and insulin levels in well-trained and sedentary persons after administration of glucose. Glucose administration during physical exercise did not produced any decrease in blood glucose levels in either group.

The above conflicting results made us study blood glucose levels after the administration of glucose solution at rest by our new, more accurate method in endurance-trained elite walkers and in physical education students of lower physical condition.

Materials and methods

Twelve healthy male volunteers between the age of 19–24 years were observed. Their body height and weight ranged between 170–175 cms and 65–70 kgs, respectively. Six of them were specialised for endurance training (mean value of relative aerobic capacity: 65.1±2.3 ml/kg/min), the other 6 were less trained university students (56.2±4.1 ml/kg/min). Blood glucose was measured at rest and after oral administration of glucose solution (75 g of glucose dissolved in 300 ml of water). All the measurements were made under the same circumstances. Carbohydrate consumption of the subjects was not restricted during the days preceding the study and they performed the same physical exercise as before. The examinations were carried out in the mornings at 8 o'clock after a 12-hour starvation and one drop of blood samples were taken from finger-tip at 0, 15, 30, 45, 60, 75, 90, 120, 150 and 180 minutes. Blood glucose was measured by MediSense ExaTech instrument [6].

The blood glucose values were analysed both by the new method elaborated by our team and by the traditional one.

Statistical analysis was made by Student-test. The statistical significance was set at a p value smaller than 0.05.

The point of the process is that beyond the estimation of the absolute blood glucose values, the measured data are expressed in percents in relation to the previous values and they are studied as the function of time.

Results and discussion

Figure 1/a includes mean blood glucose values after administration of glucose solution. The starting value of walkers is lower, the increase is steeper, the maximal concentration is observed later compared to the students of physical education. The starting concentration is restored around the 80th minute.

The starting value of the less trained students of physical education is higher, the slope of the curve is lower, the maximal concentration is reached earlier, the restoration to the starting value occurs at about the 40th minute, and then, following a stagnation, it rises then falls again. No significant differences were found between the two curves.

Figure 1/b demonstrates the new analytical method that makes a detailed analysis of the physiological changes possible. Each point represents the mean values of the groups.

Thick drawn and thick dotted lines demonstrate the increasing while thin drawn and thin dotted lines show the decreasing blood glucose values in relation to the preceding ones. Points above the X-axis represent increasing, while points below the X-axis show decreasing blood glucose values, compared to the preceding values in every case. Where the thick line changes into thin, the direction of the vector of contrainsular and insular effects changes, to this point contrainsular effects dominate and after that the regulation is modified. It is also true inversely, where the thin line turns into thick, the regulation changes again, and contra-insular effects will dominate again. Statistically significant difference between the two groups was found only in the time (30th minute of the study) when the maximal blood glucose level was reached.

It can be observed that these changes of direction occur at different time in the groups of different physical condition. The time and the rate of insular and contra-insular effects can be estimated by means of the periods, belonging to thick and thin phases and by the time of the changes. The number of direction changes shows the dynamics of regulation, namely how often the blood glucose curve changes direction during the examination. The effects last for 90–90 minutes in both group. Direction changes can be observed four times in the case of walkers (points C, G, H, I), but only twice in university students (points C, H).

On the curves given by the values the relative differences can be seen. These data show the regulation of blood glucose level and the alterations of it more accurately. The time, direction and quantity of blood glucose regulation should be specified by means of these new method. With the help of this new method we can obtain data that can better reflect the changes. The speed of changes between blood sampling periods is also measured, which can contribute to further understanding. The percentage of the increasing and decreasing blood glucose values, compared in every case to the

preceding ones, and the addition of them, can help to reveal the dominating effects during the examination.

The speed of the changes can be estimated, if the percentage differences of ABC points are divided by the time, belonging to them (d%/dt). By means of these data (in %/min) the two groups of different physical condition can be characterised. Table I contains the data which represent the percentage changes of the blood glucose values.

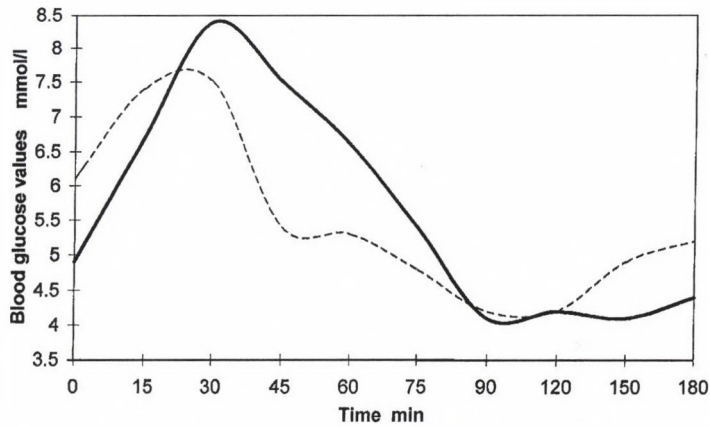

Fig. 1/a. Traditional representation of blood glucose values (mean) after administration of glucose at rest

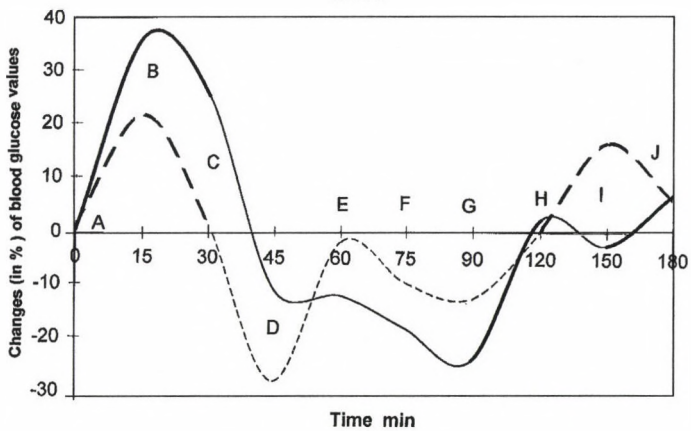

Fig. 1/b. Percentage changes of blood glucose values, compared to the preceding ones after administration of glucose at rest
Drawn line: Curve of the elite walkers, dotted line: curve of the physical education students

Table I

Percentage changes per minute of mean blood glucose values after administration of glucose at rest in physical education students and elite walkers (%/minute)

Phases	University students	Walkers
A–B	**1.42**	**2.44**
B–C	**0.09**	**1.69**
C–D	–1.86	–0.73
D–E	–0.13	–0.80
E–F	–0.63	–1.2
F–G	–0.83	–1.6
G–H	0.0	**0.08**
H–I	**0.56**	–0.08
I–J	**0.20**	**0.24**
Σ+ changes:	**+45.5%**	**+71.8%**
Σ-changes	–52.0%	–67.4%
Δ changes	–6.6%	**+4.4%**

Marked differences can be observed in the two groups. In walkers the absorption is far quicker (2.44 %/min at A–B phases and 1.68 %/min at B–C phases) while in the less trained students the corresponding values are 1.42, 0.09 %/min, respectively. However, a higher decrease can be observed in walkers in the middle of the curve (phases D–G). At the final phase of the curve a small increase, followed by a small decrease, then a greater increase can be observed in walkers. On the curve of the other group stagnation and certain increase can be registered in the same periods of time.

Summarizing the positive and the negative percentages – Σ+and Σ– changes – (here only the totals are represented) these indicates whether insular or contrainsular effects dominate during the studied period. If the results of the changes are considered (Δ changes) it can be established that in walkers mainly blood glucose level increasing effects (+4.4%) while in the other group decreasing effects (–6.6%) are dominant.

With this new method, beyond the traditional analysis of blood glucose curve, we can get more information on the route of carbohydrates in the human body.

The method can eliminate the differences of the various starting blood glucose concentrations.

As blood glucose values are greatly influenced by the preceding data, percentage representation of the changes can ensure more accurate estimation.

By means of the new method the speed of the changes, the direction change of the regulation resultant and the frequency of it, can be followed as well as the dynamics of the regulation.

By the use of the new method difference can also be revealed between the two groups at rest.

The new method is advised to complete the valuation of the traditional OGTT and the modified Ackermann's.

The measured differences between the groups are explained, among others, by the size of carbohydrate stores, by vegetative state, insulin response which parameters are dependent on different physical conditions e.g. the circulation of the liver and the sensitivity of insulin receptors.

To sum up, independently of the analysing method, the differences between the two athletic groups are explained by the biopharmatical adaptation [9, 10], which includes the absoption of exogenic substances, medicines, their metabolism and excretion, in brief, their whole route in the body. According to our investigations, this phenomenon depends on the physical condition of the examined persons.

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Schmidt, R. F.: Fundamentals of Sensory Physiology. Springer Verlag, New York–Heidelberg–Berlin 1978.

Dettler J. C.: Biochemical variation. In: Textbook of Human Genetics, eds Fraser, O., Mayo. O., Blackwell Scientific Publications, Oxford 1975, p. 115.

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Figures (line drawings, diagrams, photographs). These should be numbered consecutively using Arabic numerals. One original copy and two additional copies should be sent. Please, indicate the figure number, the name of the first author and the top of the figure on the backside. Their approximate place should be indicated in the text. Captions should be provided on a separate page.

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