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Influence of Zinc on Relaxation of Smooth Muscles and Ultrastructure of Cardiomyocytes Under Immobilization Stress

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The aim of the study was to investigate the significance of zinc (as antiatherogenic factor) for the relaxation of smooth muscles and the ultrastructure of cardiomyocytes under immobilization stress (as a factor of ischemic heart disease). A 48-d stress was provoked in Chinchilla rabbits (n = 20) by placing them in metal hutches. Half (n = 10) of the rabbits received daily oral zinc supplementation as zinc acetate at a dose of 0.3 mg/kg body wt. The control rabbits (n = 10) had no intervention and received no supplements. The relaxation of smooth muscles from thoracic aorta as mediated by acetylcholine at a concentration from 10^{-8} to 10^{-4} mol/l was detected using a 6MXIC mechanotron in isometric regime. The response was expressed as the percentage of relaxation to prostaglandin $F_{2\alpha}$ (2.10^{-5} mol/l)-induced precontraction. The ultrastructure of the cardiomyocytes was evaluated by electron microscopy (Philips-300). The data showed that immobilization stress decreased the relaxation of smooth muscles and damaged cardiomyocyte ultrastructure as manifested by caryopyknosis, caryorrhexis, desintegration of myofibrils, vacuolization of mitochondria and endoplasmic reticulum. Zinc supplementation in the food of rabbits under immobilization stress increased the relaxation of smooth muscles, caused hypercontraction of myofibrils, accumulation of mitochondria with dense cristae and single lysosomes in cardiomyocytes.

Key words: immobilization stress, zinc, muscles, relaxation, cardiomyocytes

INTRODUCTION

A stressogenic situation such as conflict at home, at work, ecological catastrophe, hypodynamics, etc. may cause the dysfunction of central nervous system (CNS). There is no doubt that dysfunction of CNS is important for the pathogenesis of ischemic heart disease (IHD) which is related to disorders of coronary blood circulation. This in turn depends on the contractility of smooth muscles, which is controlled by vasoactive agents (such as nitric oxide, prostacyclin, thromboxane, endothelin, etc.) synthesized and released by the vascular endothelium (1, 2) and may determine functional and ultrastructural disturbances in cardiomyocytes. The literature data indicate (3, 4) that antioxidants, scavengers of free radicals positively act on the relaxation of blood vessels in patients with IHD. Zinc, as a critical component of biomembranes as well as activator or inhibitor of numerous enzymes, protecting cells from oxidative stress (5, 6), attending in the mechanism of apoptosis (7, 8), providing antiatherogenic properties (9, 10) may be significant for maintaining the ultrastructure of cells in tissues and for contractility of smooth muscles. In addition, functional analysis using vessel segments from aorta or coronary artery revealed that, similarly to the human situation, these conductance vessels develop a nitric oxide (NO)-dependent endothelial dysfunction (11). Risk factors of ischemic heart disease such as arterial hypertension, hypercholesterolemia, smoking and others are related to endothelium-dependent relaxation as much in coronary artery as in peripheral artery (3, 4, 12). All this allows us to detect the reactivity of coronary artery in a non-invasive way or, as in our experiment, definite changes in the relaxation of thoracic aorta permit to associate changes in the coronary arteries with relaxation.

Thus, the aim of our investigation was to determine the significance of zinc for relaxation of blood

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vessels and in maintaining the ultrastructure of cardiomyocytes under conditions of immobilization stress (as an IHD factor).

MATERIALS AND METHODS

Induction of immobilization stress

The animals were cared, used and killed according to the defined code given by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (License No. 0006).

A 48-d immobilization stress was provoked according to B. M. Fiodorov (13) in male Chinchilla rabbits (weight 2.5–3.0 kg; n = 20) by placing them in metal hutches, which closely shrouded their body, but rabbits could loosely feed and drink. Every day (48 days) 10 rabbits that lived in metal hutches received oral zinc doses of 0.3 mg/kg body wt (in the form of zinc acetate). The remaining rabbits did not receive zinc supplement and were placed in metal hutches for 48 days. The control rabbits (n = = 10), which had no intervention and received no zinc supplement, were kept in vivarium conditions.

All the rabbits used in the experiment received the same food, which was not purified in respect to trace elements. For production of zinc supplement a solution of zinc acetate was used, in which 1 ml of solution contained 1 mg of zinc. The solution was used with respect to body weight of rabbits, a dose of zinc 0.3 mg/kg was dropped with an automatic pipette into one of the food components (in a dough-ball, 8 g weight, made of barley meal). Such dough-balls were given to a half (n = 10) of rabbits (who had an intervention) every day starting from the second day of immobilization.

Vascular studies

Following the immobilization regime (after 48 days) the rabbits were anesthesized using thiopental-sodium (35 mg/kg). On opening the thoracic chest of rabbits, samples of thoracic aorta were taken to test the relaxation of smooth muscles. Isolated preparations of thoracic aorta of experimental and control rabbits for testing the relaxation of smooth muscles were perfused with an oxygenated Ringer solution containing (in mmol/l): NaCl – 139; KCl – 3.5; $CaCl_2$ – 2.5; $NaHPO_4$ – 2.4; $MgCl_2$ – 1.7; NaH_2PO_4 – 0.67; $NaHCO_3$ – 1.5; glucose – 5; ph 7.2–7.4 at 37.5 °C. To elucidate the influence of acetylcholine-mediated relaxing effect of endothelium upon the preparation of thoracic aorta smooth muscles, the latter were precontracted with prostaglandin $F_{2\alpha}$ (2.10^{-5} mol/l). The relaxation of smooth muscles (precontracted with prostaglandin) mediated by acetylcholine at a concentration from 10^{-8} mol/l to 10^{-4} mol/l was determined with the aid of a 6MXIC mechanotron in isometric regime. Responses were expressed as the percentage of relaxation to prostaglandin $F_{2\alpha}$-induced precontraction. The solution of the biologically active substance (acetylcholine and prostaglandin $F_{2\alpha}$) was prepared *ex tempore*.

Ultrastructural studies

For ultrastructural studies of cardiomyocytes, samples of the left ventricle of the heart were immersed into a fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mmol/l cacodylate buffer (ph 7.4) for more than 4 h at room temperature or overnight at 4 °C. The specimens were then postfixed for 2 h with 1% osmium tetraoxide solution in 0.1 mmol/l cacodylate buffer (pH 7.4) dehydrated through a graded ethanol series and embedded in a mixture of Epon 812 and Araldit. Ultrathin sections stained with uranyl acetate and lead citrate were evaluated by electron microscopy (Philips-300).

Statistical analysis

Values are presented as mean ± SEM. Data derived from repeated measurements were analyzed by a two-way ANOVA. Otherwise data were compared with Student's t test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Smooth muscle relaxation as affected by acetylcholine in rabbits not receiving or receiving zinc supplementation under imoobilization stress

The present data show that a long-term immobilization stress (lasting 48 days) caused a disorder of the relaxation of smooth muscles (Fig. 1). For example, in rabbits that received no zinc under immobilization stress (Fig. 1, c), the relaxation of smooth muscles (after precontraction with prostaglandin $F_{2\alpha}$ (2.10^{-5} mol/l)) of thoracic aorta under the influence of acetylcholine at a concentration from 10^{-8} to 10^{-4} mol/l was significantly lower ($p < 0.05$–0.01) than in rabbits given zinc (Fig. 1, b). Besides, in rabbits that received no zinc (Fig. 1, c) supplement during immobilization stress, the endothelium-dependent relaxation of smooth muscles was lower ($p < 0.05$–0.01) than in control rabbits (Fig. 1, a) at any of 10^{-6} mol/l, 10^{-5} mol/l, 10^{-4} mol/l acetylcholine concentration. On the other hand, in rabbits given a zinc supplement (Fig. 1, b) under immobilization stress the contractility of smooth muscles affected by acetylcholine did not

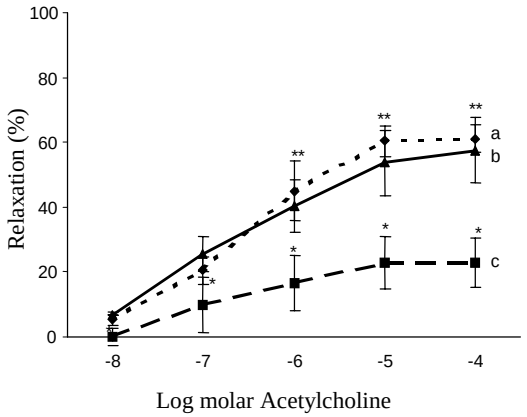

Fig. 1. Endothelium-dependent relaxation of smooth muscles of thoracic aorta:
a – control rabbits, b – rabbits given a zinc supplement under a 48-d immobilization stress, c – rabbits without a zinc supplement under a 48-d immobilization stress, * $p < 0.05–0.01$, *b* compared with *c*; ** $p < 0.05–0.01$, *a* compared with *c*

change as compared to that of control rabbits (Fig. 1, a).

Morphological changes in cardiomyocytes induced by immobilization stress without and with zinc supplementation

In the cardiomyocytes of rabbits receiving no zinc supplement during immoobilization stress the following structural changes were observed: caryopyknosis, caryorrhexis, desintegration of myofibrils, vacuolization of mitochondria and endoplasmic reticulum (Fig. 2). In cardiomyocytes of rabbits that received zinc in the course of the 48-d immobilization stress, hypercontraction of myofibrils, accumulation of mitochondria with dense cristae and single lysosomes were visible (Fig. 3). Figure 4 shows the cardiomyocyte morphology in control rabbits.

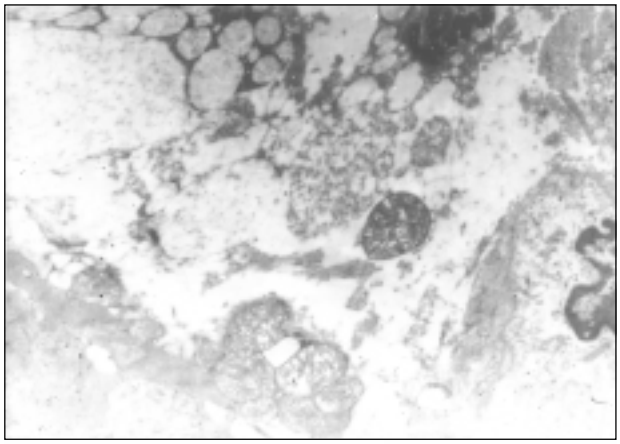

Fig. 2. Caryopyknosis, caryorrhexis, desintegration of myofibrils, vacuolization of mitochondria and endoplasmic reticulum under immobilization stress. ×12000

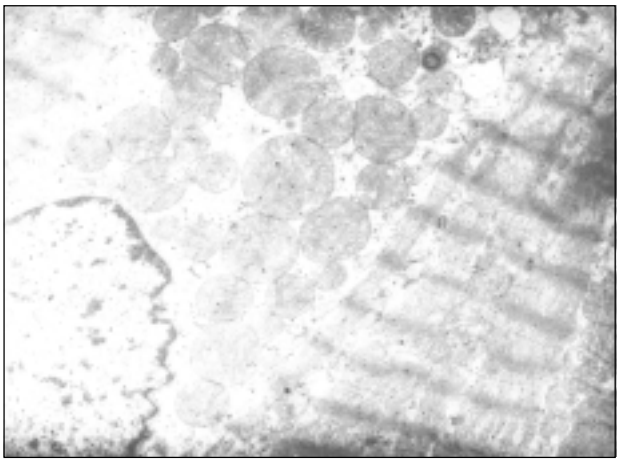

Fig. 3. Hypercontraction of myofibrils, accumulation of mitochondria with dense cristas and single lysosomes in cardiomyocytes under the influence of zinc in immobilization stress. ×12000.

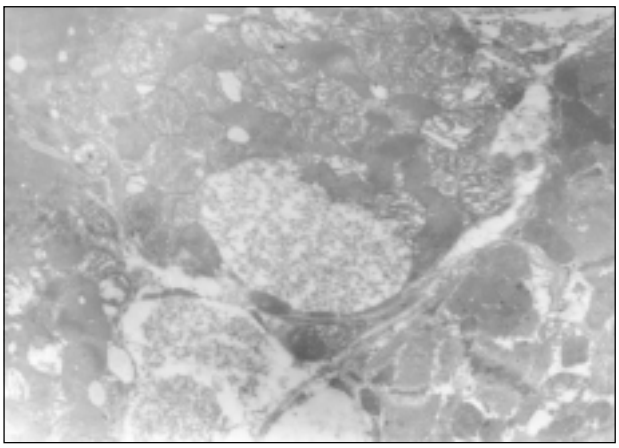

Fig. 4. Morphological view of cardiomyocytes in control rabbits. ×12000

DISCUSSION

The favourable effects of zinc on the relaxation of smooth muscles and on the ultrastructure of cardiomyocytes may be manifested directly or indirectly via many mechanisms. One of the indirect mechanisms may be related to the synthesis and release of the vasodilator, nitrous oxide (NO), in the endothelium. By activating glucose 6-phospatate dehydrogenase (14) zinc takes part in the synthesis of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is required for the catalyzing function of NO synthetase (15). Besides, zinc prolongs the half-life of NO as a cofactor of superoxide dismutase and inhibits the production of peroxynitrite (produced from an interaction of NO with

O^{-}) (16). Peroxynitrite is a strong peroxidant of lipids and causes vasoconstriction. For example, lipid peroxides and/or peroxynitrites activate prostaglandin H synthase, leading to an increased number of prostaglandin endoperoxide and thromboxane, which bind to the same receptor and thus evoke vasoconstriction. On the other hand, prostacyclin is reduced as a result of extensive lipid peroxidation and/or peroxynitrite, which preferentially inhibit prostacyclin synthase activity resulting in reduced vasorelaxation (17). Thus, zinc added to the food of rabbits under immobilization stress might affect the above-mentioned biochemical mechanisms, causing relaxation of blood vessels.

Zinc, also as an antioxidant precipitating in the first antioxidant protection system against reactive oxygen species and as a cofactor of superoxide dismutase and inhibitor of development of apoptosis (8, 9, 16), might predetermine a normal ultrastructure of cardiomyocytes. Zinc, being part of the structure of nuclear acid transcription factors (for example, NF-kB), *i.e.* of their domains called zinc fingers, plays a role in factor binding to DNA (9). In that way inactivated NF-kB could have a positive influence on the ultrastructure of cells (as in our experiment of cardiomyocytes), because activated NF-kB can stimulate expression of genes encoding cytokines, chemokines, adhesion molecules (18). Zinc also inhibits Ca^{2+}/Mg^{2+} endonucleases, which injure poly(ADP-ribose) polymerase (DNA-repairing enzyme). The proper functioning of poly(ADP-ribose) polymerase requires a lot of bioenergy (15). Zinc as an activator of glycolysis enzymes (such as hexokinase, lactate dehydrogenase, malate dehydrogenase, phosphofructokinase) and glucose 6-phosphate dehydrogenase (14) can influence the compensation of bioenergy (ATP) depleted by DNA repair. The active synthesis of bioenergy in cells (as well and in cardiomyocytes) may cause their structural integrity. ATP is also necessary for disintegration of the myosin–actin complex, *i.e.* for relaxation of muscles.

Thus, a positive influence of zinc on the relaxation of smooth muscles and on the ultrastructure of cardiomyocytes under stress may be related to the above-mentioned biochemical property of this element.

CONCLUSIONS

Zinc lowers the tone of smooth muscles in the thoracic aorta and protects the ultrastructure of the cardiomyocyte under immobilization stress.

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Zn POVEIKIS KRÛTINËS AORTOS LYGIØJØ RAUMENØ ATSIPALAIDAVIMUI IR KARDIOMIOCITØ ULTRASTRUKTÛRAI IMOBILIZACIJOS STRESO ATVEJU

S a n t r a u k a

Norëdami ávertinti Zn poveiká krûtinës aortos lygiøjø raumenø relaksacijai ir kardiomiocitø ultrastruktûrai streso atveju, šinðilos veislës triuðiams (ið viso 20) sukëlëme 48 parø stresà juos imobilizuodami metaliniuose narveliuose. Deðimt imobilizuotø triuðiø papildomai maitinome 0,3 mg/kg Zn (Zn acetato forma). Kontroliniai triuðiai, kuriø taip pat buvo 10, buvo laikomi be intervencijos áprastinëmis vivariumo sàlygomis. Lygiøjø raumenø relaksacinæ funkcijà tyrëme mikromechanotronu, veikdami krûtinës aortos segmentus didëjanèiomis acetilcholino koncentracijomis (10^{-8}–10^{-4} mol/l) prieð tai sukëlæ segmentuose pastovø susitraukimà prostaglandinu $F_{2\alpha}$ ($2{,}10^{-5}$ mol/l). Kardiomiocitø ultrastruktûrinius pokyèius vertinome elektroniniu mikroskopu „Philips-300“. Gauti duomenys rodo, jog veikiant acetilcholinu, triuðiø, negavusiø Zn, lygiøjø raumenø atsipalaidavimas buvo reikðmingai sumaþëjæs lyginant su kontroliniais ir Zn gavusiais triuðiais. Be to, 48 parø imobilizacija nulëmë kardiomiocitø miofibriliø lizæ, visiðkà jø suirimà, mitochondrijø ir endoplasminio tinklo vakuolizacijà, mitochondrijø kristø fragmentacijà, homogenizacijà, branduoliø piknozæ, karioreksæ. Tuo tarpu Zn gavusiø triuðiø 48 parø imobilizacijos streso metu kardiomiocitø ultrastruktûriniai pokyèiai buvo ne tokie ryðkûs ir siejosi su jø hiperfunkcija, t. y. miofibriliø hiperkontrakcija, mitochondrijø kristø pagausëjimu.

Raktaþodþiai: imobilizacijos stresas, Zn, raumenys, relaksacija, kardiomiocitai