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Cardioprotective Effects of ATP-Sensitive Potassium Channels Activation in Experiments *in Vivo*: Influence on Biochemical Parameters of Blood Following Ischemia-Reperfusion of Myocardium

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ABSTRACT: In experiments on the anesthetized dogs with modeling of experimental ischemia (90 min) and reperfusion (180 min), participation of biochemical processes in the cardioprotective effect of the preischemic activation of ATP-sensitive potassium (KATP) channels caused by intravenous introduction of flocalin, a new fluorine-containing opener of these channels was shown. Flocalin was introduced in a dose of 0.1 mg/kg of animal body weight which practically did nit change parameters of hemodynamic under normoxia. Thus, experiments on the influence of flocalin on changes of biochemical parameters of arterial blood during ischemia-reperfusion of myocardium revealed certain features of ischemia-reperfusion syndrome development under stimulation of K_{ATP} channels. Analysis of blood biochemical parameters revealed that flocalin suppressed free radicals reactions and manifested anti-oxidative properties: reduced quantity of H_2O_2 and NO_3^- (the latter can be interpreted as a reduction in peroxinitrite formation), prevented the decline of catalase and superoxide dismutase activity. Practically constant content of low-molecular nitrosothiols in blood during the whole experiment and increase in the level of NO_2^- during the reperfusion can be indicative of intact functions of the NO system and protective influence of flocalin during the ischemia-reperfusion of myocardium. Practically the unchanged content of inorganic phosphorus and uric acid in blood during ischemia-reperfusion under conditions of preischemic introduction of flocalin indicates that there is a prevention of ATP degradation and formation of both superoxide anion by xantinoxidase and peroxinitrite by its interaction with nitric oxide. All the mentioned properties of flocalin, related with the changes in biochemical parameters of arterial blood, together with the changes in hemodynamic parameters, result in decrease of infarct size in myocardium after ischemia-reperfusion by 37% versus control experiments.

KEY WORDS: K_{ATP} channels, flocalin, ischemia-reperfusion, free radicals, NO system

I. INTRODUCTION

Adenosine triphosphate sensitive (K_{ATP}) channels are the crucial arm in the cardiopro-

tection under ischemia and reperfusion of myocardium, and pharmacological activators of these channels reveal anti-ischemic and spasmolytic properties.[14] One of the novel with the powerful cardioprotective properties and sufficiently low toxicity is the fluorine-containing activator of these channels, flocalin.[7,8,11] In experiments in vitro and in vivo when having pharmacological preconditioning the flocalin effect on the changes of hemodynamic parameters is well studied. From our point of view to the positive effects of flocalin, which could promote its cardioprotective properties, cold be attributed moderate decrease of the arterial pressure, which slacks the stressing of the injured heart and promotes preservation of the cardiac output in the first hours of ischemia, prevention of the increased resistance of coronary vessels and relative saving of myocardium contractile indexes during reperfusion. One of the characteristic values of cardioprotective flocalin action is the decrease in the infarct zone after ischemia-reperfusion under the preischemic intravenous flocalin injection by 37% as compared with control.[7] Thereat changes in biochemical blood and myocardium parameters under flocalin remain practically uninvestigated.

The aim of our work was to investigate changes in biochemical parameters of arterial blood under pharmacological preconditioning, induced by intravenous injection of the new fluorine-containing opener of K_{ATP} channels from sarcolemmal and mitochondrial membranes – flocalin in vivo experiments in a doses range, which practically have no influence on hemodynamic.

II. METHODS

Experiments were carried out on outbreed dogs with body mass from 15 to 25 kg, anesthetized with chloralose urethane (0.07 g/kg and 0.7 g/kg intravenously) using the method described previously.[4,7] In our work we used method of a retrograde catheterization autoperfusion and a target embolization of a branch of the left coronary artery, which enables to model the local ischemia-reperfusion of myocardium without breast opening and spontaneous breathing maintenance.

For estimation of biochemical parameters during the experiment the blood was taken several times. Intensity of free radical reactions was estimated by the hydrogen peroxide content[12] and by means of H_2O_2-induced chemoluminiscense (HL)[5]. Registration was carried out using "Chemoluminograph EA-1", and HL kinetic parameters processing and visualization – using specially designed software. For estimation of malondialdehyde (MDA) to sample aliquots was added 0.5 ml of 1-% of tiobarbituric acid solution diluted in 50 mM of NaOH and 0.5 ml of 28-% trichloracetic acid solution. Obtained mixture was kept for 20 minutes on the boiling-water bath, than it was cooled-down and the extinction value was measured at 532 nm.[6] Antioxidant system (AOS) state was estimated in heart homogenates using activity parameters of key enzymes of antioxidative defense. Activity of superoxide dismutase (SOD) was studied using Chevari method.[9] Enzyme activity was put in c.u., where 1 c.u. corresponded to 50% blockage of the enzyme activity. Catalase activity was studied using the Korlyuk et al method.[3] Nitrite-anion (NO_2^-) content was established in protein-free aliquots oh the

heart homogenates and blood plasma in calorimetric reaction by means of Giss reagent using the Green method[13], and nitrate-anion (NO_3^-) content – using brucine method in protein-free aliquots by means of spectrophotometry.[10] Nitrothiosoles content was estimated using the Saville method,[2] and ureic acid content – in calorimetric reaction in aliquots of the heart homogenate and blood plasma using the test-kit from "Filicit-Diagnostics" (Dnipropetrovs'k). Availability in the solution of ATP and GTP degradation products – xanthine, inosine and hypoxanthine was estimated by means of spectrophotometry by absorption in protein-free aliquots at $\lambda = 254$ nm. Measurements were conducted on the CF-26 LOMO. Protein content was estimated using conventional Bredford method with Cumassi G-250 ("Freak". Germany). Moreover, in probes was estimated the content of inorganic phosphorus using the Ostrowsky calorimetric method.[1]

We have conducted two series of experiments – the control (n = 11) and the experimental (n = 7). In both series were estimated parameters at regional myocardium ischemia (90 min) and the following reperfusion (180 min), but in the experimental group at the background of intravenous administration of 0.1 mg/kg of K_{ATP}-channels opener – flocalin 10 minutes before ischemia onset.

Obtained results were using methods of variance analysis in Origin 7.0. Results' trustworthy was estimated by t-Student criterion. $P < 0.05$ was taken as statistically significant.

III. RESULTS

Solution of continuity or malfunction of cell membranes plays an important role in pathogenesis cardiovascular diseases and processes of lipid peroxidation (LPO) are the universal mechanisms of damage under whatsoever disease. Participation of free radical processes in pathogenesis of an ischemic and a reperfusion heart damages is nowadays obvious. According to the modern apprehension peroxidative processes, which occur in lipid structures of cell membranes, promote their cohesiveness violation, resulting sometimes in irreversible damage of cardiomyocytes. In our experiments we have established that ischemia-reperfusion in dogs induces increase in oxygen free radicals formation, indicative of which are the considerable intensification of chemo luminescence, increase in production and pools of hydrogen peroxide (H_2O_2), and also the end product of LPO, namely, MDA content (Fig. 1A). Such an intensification of LPO processes is accompanied by simultaneous decrease in antioxidative potential (Fig. 1B,C), in particular by the activity inhibition of the main enzymes of the antioxidative system (AOS) of the organism – catalase and superoxide dismutase (SOD).

In our experiments flocalin considerably decreased LPO intensity and prevented decrease in activity of enzymes of AOS system of the organism catalase and SOD (Fig. 1A,B,C). It should be noted that introduction of flocalin reduces H_2O_2 content in animals' blood already before the ischemia – (1.01 ± 0.095) pM/mg of the protein at the initial conditions as compared with (0.77 ± 0.095) pM/mg of the protein after flocalin introduction. Analogous decrease in H_2O_2 content after flocalin administration was registered during the whole ischemia and the following reperfusion, yet starting from the 3rd hour of reperfusion it practically was indistinguishable from the initial

conditions (Fig. 2). At the same time in control (when modeling ischemia-reperfusion without preliminary flocalin introduction) H_2O_2 content increased during the ischemia and especially during the reperfusion.
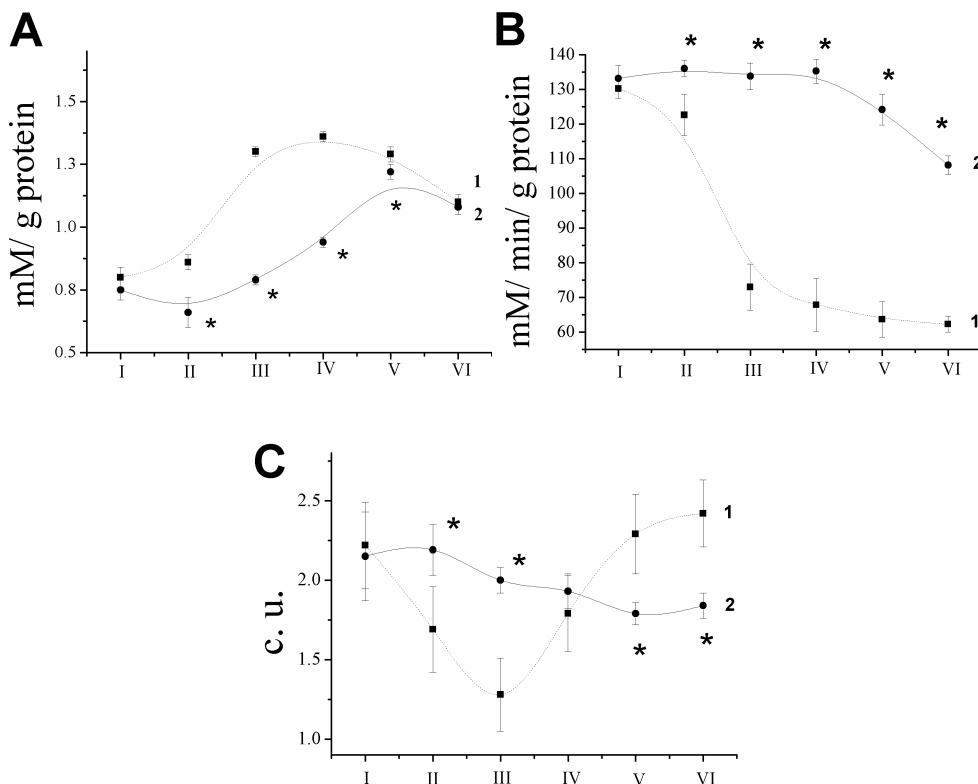
FIG. 1. Flocalin effect on malondialdehyde content (**A**), catalase (**B**) and superoxide dismutase (**C**) activity in dogs' blood plasma at different periods of ischemia-reperfusion of myocardium in the control group – ischemia-reperfusion (1) and ischemia-reperfusion after flocalin introduction (2); I – initial parameters (normoxia); II and III 1 and 90 min of ischemia respectively, IV – VI – 60, 120 and 180 min of reperfusion respectively. *P < 0.05

Thus, preliminary introduction of flocalin decreases H_2O_2 content in animal's blood so during ischemia (by 67% on the 60th minute of ischemia, P < 0.05), as during reperfusion (by 73% on the 60th minute of reperfusion, P < 0.05).

Catalase and SOD activity under preischemic flocalin administration, in contrast to the control, during the whole experiment, apart from the last hour, did not undergo any considerable changes, which could be indicative of flocain promoting balance retension in the system LPO – AOS. This could be also confirmed by the studies of induced chemoluminescence (HL) of blood plasma in dynamics of ischemia-reperfusion. In control experiments was observed a significant increase in HL throughout all the kinetic parameters. On the 90th minute of ischemia the general light sum HL within 5

minutes of registration ($\sum_5$) and the end value of the radiation intensity in 5 minutes (I_5), which cloud be used to estimate HL decay rate and indirectly AOS state increased as compared to the initial value (normoxia) correspondingly by 2.4- and 1.57-fold (P < 0.05). Reperfusion resulted in more intensive reinforcement of oxidative processes. Thus, on the 60th minute of reperfusion the increment in $\sum_5$ comprised 170% (P < 0.05), and on the 120th minute – 185% (P < 0.05) as compared with the initial value. Yet, increase in I_5 was not so rapid: on the 60th minute by 80% (P < 0.05), and on the 120th minute – 85% (P < 0.05) as compared with the initial value (Table 1).
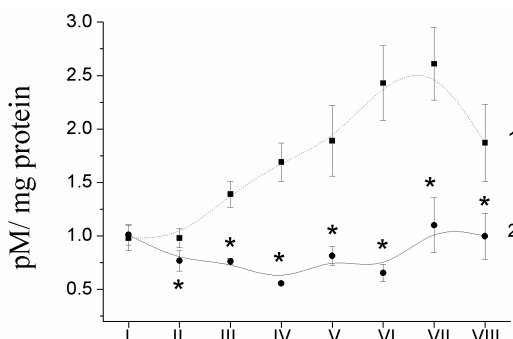
FIG. 2. Flocalin effect on changes in H_2O_2 in the blood under ischemia-reperfusion of myocardium the control group – ischemia-reperfusion (1) and ischemia-reperfusion after flocalin introduction (2); I – initial parameters (normoxia); II - H_2O_2 content at flocalin introduction; III – V – 1, 60 and 90 min of ischemia respectively, VI – VIII – 60, 120 and 180 min of reperfusion respectively. * P < 0.05

Thus, obtained results indicate that increase in peroxidative processes in arterial blood, due to the balance distortion in formation and inactivation of peroxide lipids, which resulted in their excessive accumulation during the process of ischemia-reperfusion. In virtue of the changes in I_5 it could be stated that blood antioxidative activity under ischemia-reperfusion is undersized, yet analyzing the HL parameters, it could be stated the LPO depends also on the other mechanisms.

Flocalin reduced increase in all the kinetic parameters of induced HL in blood plasma under ischemia and the following reperfusion of a heart (Table 1). Thus, under ischemia and at the background of the preliminary flocalin introduction the total HL intensity was smaller than in the control. If on the 180th minute of reperfusion in control $\sum_5$ values were tripled as compared to the initial values, than after flocalin injection this parameter was smaller by 17% (P < 0.05) as compared to the control. In case of the preliminary flocalin introduction I_5 was also smallest so during the ischemia, as during the reperfusion (on the 120th minute by 15%) as compared to the control. It should be noted that flocalin action also reduces I_0 (the HL fast flesh amplitude) under ischemia-reperfusion (on the 180th minute by 17% (P < 0.05) as compared to the control. In other words, in this model of ischemia-reperfusion flocalin reveals antioxidative properties.

TABLE 1. Flocalin effect on the parameters of blood plasma chemoluminescence in dogs in different periods of myocardium ischemia-reperfusion under ($M \pm m$, n = 18). *$P<0.05$ as compared with the control; **$P<0.05$ as compared with initial values (normoxia). IR − ischemia-reperfusion, F&IR − flocalin injection and ischemia-reperfusion

	Normoxia	Ischemia, 90 min	Reperfusion, min		
			60	120	180
The amplitude of the fast flash of chemoluminescence, (I_0, mV)					
IR	63 ± 8	181 ± 30 **	298 ± 42 **	281 ± 13 **	294 ± 10 **
F&IR	79 ± 8	173 ± 24 **	273 ± 49 **	240 ± 17 *,**	243 ± 17 *,**
The total Light sum of chemoluminescence during the 5 minutes of registration (Σ_5, mV/sec)					
IR	17873 ± 1740	42841 ± 1918 **	48308 ± 3801 **	50866 ± 2739 **	53585 ± 2180 **
F&IR	17808 ± 810	32455 ± 3542 *,**	46768 ± 5318 **	43827 ± 2126 *,**	44370 ± 2423 *,**
The end value of the luminiscece intencity within 5 minutes (I_5, mV)					
IR	45 ± 1	71 ± 7 **	81 ± 3 **	83 ± 3 **	103 ± 3 **
F&IR	39 ± 4	63 ± 6 **	74 ± 4 **	71 ± 4 *,**	90 ± 4 *,**

The other evidence of the defensive impact of flocalin under ischemia-reperfusion of myocardium is the changes in the blood pools of (NO_2^-) and (NO_3^-): increase in NO_2^- and slight decrease in NO_3^- under ischemia and reperfusion (especially under reperfusion) of myocardium as compared with normoxia. It is known that NO_2^- content in blood could be a parameter of the normal performance of the nitric oxide system. In our experiments during the whole period of reperfusion after flocalin injection NO_2^- content was higher that at initial conditions and control (Table 2), meanwhile in the control this parameter decreased during the ischemia. Thus, flocalin did not only prevent reduction of NO_2^- content in the blood under ischemia, but also considerably increased its content under reperfusion. To some extend a cardioprotective aspect of flocalin action is also the constant content of NO_3^-, since its increase could be indicative of enhancement of peroxinitrite formation due to interaction of NO with superoxide radical (Table 2).

It is known that low-molecular nitrosothiols (LMNT) are donors for nitric oxide molecules. Their attenuated content in the blood points to the active usage of NO molecules, which could have negative consequences because of the possibility of vasoconstriction emergence. Flocalin has virtually no impact on the LMNT content

(Table 2), which together with the increase in NO_2^- during reperfusion can indicate that nitric oxide system is maintained adequate and that there is a cardioprotective effect of flocain under ischemia-reperfusion of myocardium.

TABLE 2. Flocalin effect on the biochemical blood parameters under ischemia-reperfusion of myocardium ($M \pm m$, $n = 9$). *$P<0.05$ as compared with the control; **$P<0.05$ as compared with initial values (normoxia). IR – ischemia-reperfusion, F&IR – flocalin injection and ischemia-reperfusion

	Normoxia	Ischemia, min			Reperfusion, min		
		10	60	90	60	120	180
NO_2^-, pM/mg of a protein							
IR	62 ± 10	50 ± 11	41 ± 4 **	36 ± 7 **	59 ± 9	79 ± 10	29 ± 6 **
F&IR	63 ± 5	56 ± 3	41 ± 10	67 ± 17 *	101 ± 27 *,**	119 ± 19 *,**	113 ± 32 *,**
NO_3^-, nM/mg of a protein							
IR	32 ± 3	24 ± 3 **	26 ± 2	19 ± 2 **	16 ± 4 **	20 ± 7	75 ± 14 **
F&IR	26 ± 1	22 ± 2 **	18 ± 7	16 ± 1 **	18 ± 3 **	21 ± 4 **	23 ± 3 *
Low-molecular nitrosothiols, pM/mg of a protein							
IR	10 ± 2	11 ± 2	8.8 ± 0.9	7 ± 1 **	12 ± 2	11 ± 2	9 ± 1
F&IR	9.6 ± 0.4	6 ± 2 **	8.8 ± 0.4	7.7 ± 0.7 **	10 ± 3	11 ± 2	8 ± 2
Ureic acid, nM/mg of a protein							
IR	1.26 ± 0.08	1.7 ± 0.2	2.8 ± 0.2 **	3.1 ± 0.6 **	12 ± 1 **	10 ± 1 **	6.3 ± 0.9 **
F&IR	1.6 ± 0.3	1.67 ± 0.08	1.8 ± 0.4 *	1.5 ± 0.5 *	1.9 ± 0.9 *	1.7 ± 0.3 *	2.0 ± 0.6 *
Optical density (D_{254})							
IR	0.27 ± 0.02	0.36 ± 0.07 **	0.43 ± 0.06 **	1.3 ± 0.1 **	3.8 ± 0.5 **	3.5 ± 0.4 **	3.3 ± 0.5 **
F&IR	0.18 ± 0.02	0.8 ± 0.4 **	0.27 ± 0.02 *,**	0.39 ± 0.06 *,**	0.37 ± 0.02 *,**	0.39 ± 0.07 *,**	0.31 ± 0.05 *,**
Inorganic phosphorous, nM/mg of a protein							
IR	25 ± 1	35 ± 4 **	23 ± 15	158 ± 25 **	289 ± 50 **	203 ± 33 **	222 ± 29 **
F&IR	19 ± 2	20 ± 3 *	22 ± 8	18 ± 5 *	20 ± 4 *	24 ± 5 *	22 ± 3 *

To the latter point other biochemical blood parameters: content of ureic acid, inorganic phosphate and the total products of ATP and GTP degradation – inosine, xanthine and hypoxanthine. All of them are markers of ATP molecule degradation, which is especially demonstrative under ischemia. Inorganic phosphate is formed during ATP hydrolysis (ATP→ADP+Ph$_N$→AMP+2 Ph$_N$→adenosine+3Ph$_N$). Ureic acid is the end product of metabolic transformation (degradation) of ATP molecules by xanthine oxidase enzyme. In our experiments was shown that under preischemic administration of flocalin content of inorganic phosphate and ureic acid in blood was virtually unchanged during the experiment as compared with the initial values (Table 2), which points to the protective properties of flocalin and to the inhibition of the complete metabolic conversion of ATP to ureic acid.

At the same time increase in the optical density at $\lambda = 254$ nm as compared with normoxia could be indicative of the increase in the blood content of inosine, xanthine and hypoxanthine, which are the intermediate metabolites of ATP conversion to the ureic acid. It is known that during ATP metabolism inosine turns to hypoxanthine, hypoxanthine turns to xanthine and xanthine turns to ureic acid. The last two metabolic transformations occur under the influence of xanthine oxidase and are accompanied by superoxide anion radical release. Thus, decrease as compared with control (ischemia-reperfusion without flocalin administration) in blood content of inosine and xanthine under flocalin action could be indicative of inhibition of metabolic transformations at the mentioned above stages and of thereto related increase in superoxide anion radical release. Namely, decrease in blood content of ATP degradation metabolites, ureic acid and inorganic phosphate under preischemic administration of flocalin (Table 2) could be indicative of the decrease in the superoxide anion radicals formation under ischemia-reperfusion of myocardium, which in turn points to the possible inhibition by flocalin under ischemia-reperfusion and peroxinitrite formation.

Thus, analysis of blood biochemical parameters has shown that flocalin inhibits free-radical reactions and reveals anti-oxidative properties: decreases H_2O_2 and NO_3^- content (the latter could be indicative of decreased peroxinitrites formation), prevents reduction of catalase and SOD activity. Practically unchanged content of LMNT in the blood during the whole experiment and an increase in NO_2-during the reperfusion could suggest that the nitric oxide system is maintained at the adequate level and there is a protective influence of flocalin under ischemia-reperfusion of myocardium. The fact that under preischemic injection of flocalin the inorganic phosphate and ureic acid content in the blood remains unchanged during ischemia-reperfusion could be indicative of the prevention of ATP degradation and thus of formation of so superoxide anion, as peroxinitrite due to the interaction of the latter with nitric oxide.

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The Association of A^{-1082}→G Polymorphism of IL-10 and (A^{-308}→G) Polymorphism of TNFα Genes with Bone Density in Elderly Women

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ABSTRACT: We studied the association between bone mineral density and (A^{-1082}→G) polymorphism of IL-10 and (A^{-308}→G) polymorphism of TNFα genes. SNP-polymerase chain reaction was used. The study failed to find significant association of A^{-308}→G TNFα polymorphism and bone state in postmenopausal women. The patients with AA genotype of A^{-1082}→G IL-10 gene have significantly lower indices of bone mineral density comparing with G allele carriers (GG/AG). This association remained true for data of bone mineral density of total body, and separate parts of skeleton: lumbar spine, femoral neck, Ward's triangle of total femur. Our study indicates that A^{-308}→G polymorphism of TNFα gene is not associated with indices of bone mineral density in postmenopausal women. However, there is strong evidence that women, carriers of AA genotype of A^{-1082}→G polymorphism of IL-10 gene have significantly lower indices of bone mineral density. This indicates the potential for predictive genetic testing of osteoporosis risk. Analyses of gene combination IL-10 G^{-1082}→G and TNFα G^{-308}→G ('anti-inflammatory genotype') did not show any significant association of this genotype with bone characteristics, which shows low predictive value of this combination for diagnostics of osteoporosis.

KEY WORDS: gene polymorphisms, bone, aging, IL-10, TNFα

I. INTRODUCTION

Bone mineral density (BMD) in people decreases with age. Such phenomenon is called «osteopenia» and can result in osteoporosis, which consequence is a decrease in the bone strength and increase the frequency of low-trauma fracture. In the recent times osteoporosis has become widely distributed, especially among people from countries with mature economies, and it has become a significant medical and social problem.[35,41]

During the last few years a drastic breakthrough was made in understanding of pathogenetic mechanisms of osteoporosis. It was established that bone loss, induced by the age-related changes, sex hormones deficiency or glucocorticoids excess, associated with disbalance of bone remodeling.[41]

It is known that bone remodeling and osteoporosis development can be regulated by a great number of factors of different etiology. Among these there are sex hormones, glucocorticoids[20,25], insulin-like growth factors[42], pro-inflammatory cytokines[1], etc.

Besides this, basing on the family pathology analysis was shown existence of a genetic control of pathology development. According to the data of genealogical examination, presence of osteoporosis in close relatives doubles the risk of this disease development. It was also established that there is a higher risk for osteoporosis in women than in men independently of the race or the country of residence.[36]

The attempts to find changes in genes' structures, which would play a role of essential risk factors in osteoporosis development and also would be essential for increase the incidence of fractures in elderly people, gave contradictory results. It should be noted that search for marker genes was done in several directions, which could be divided into three big groups:

– genes responsible for synthesis of proteins, which directly take part in bone metabolism processes (for example, proteins of bone matrix);

– genes responsible for synthesis of cytokines, which take part in regulation of bone remodeling processes (interleukins and growth factors);

– genes responsible for synthesis of hormones and their receptors.

Our attention was drawn by the second group of investigations, where connections between cytokines genes polymorphism and osteoporosis risk were described. It was established that there is a possible connection between TNF-α polymorphism[3], osteoprotegerin receptor[24], IL-1 and IL-1 receptor antagonist (IL-1 RA)[40], IL-4, EGF-β and TGF-β[23] and osteoporosis development. Yet, depending on the country, were the experiments were conducted, the data can be right opposite.[2] For analysis of the interconnection between cytokines gene polymorphism and postmenopausal osteoporosis development we have chosen TNF-α and IL-10 genes, which play an opposite role in inflammatory processes development and also in regulation of the balance between activity of osteoblasts and osteoclasts, which play a key role of bone remodeling.[29,39]

It was shown that in the development of the postmenopausal osteoporosis an important regulatory role can be played by polymorphism in the promoter of TNF-α gene.[26] Other authors have shown that despite of the analogous values of femoral neck BMD, women with TNF-α AA genotype (A^{-308}→G) have bigger endocortical bone diameter (p = 0.03) and higher bone strength (p = 0.003) as compared to GG carriers. Among 376 cases with femoral fracture during 12 years, 22% of patients were diagnosed with low risk of femoral fracture (percentage risk 0.78, 95% confidence interval 0.63 – 0.96). At the same time polymorphism A^{-308}→G was not connected with lowering of fracture risk.[27]

We haven't found any data on connection between IL-10 polymorphism and bone loss in postmenopausal women. At the same time, it is known that G allele of IL-10 gene (A^{-308}→G) is connected with an increased life span in men.[33] Analysis of IL-10 and TNF-α genes combinations ("anti-inflammatory genotype": IL-10^{-1082}GG – TNF-α^{-308}GG) have shown the same relationship in men, which can indicate high informativity of pro-inflammatory markers in age-related pathology development. Therefore, for analysis of chosen genes polymorphism influence on the development of the age-related bone loss we have chosen an effect of some point mutations in the promoter of TNF-α^{-308}→G and IL-10 A^{-1082}→G genes, which lead to changes in the interleukins' pro-

duction. Genotype AA of TNF-$\alpha^{-308} \to$G gene polymorphism as well as genotype GG of IL-10 A$^{-1082} \to$G gene polymorphism provide a high production of corresponding cytokines, and genotype GG of TNF-$\alpha^{-308} \to$G gene polymorphism as well as genotype AA of IL-10 A$^{-1082} \to$G gene polymorphism provide a low production.[3,43]

Interaction of different genes inside genome, and also co-interaction of human and animal genome with the external environment are the main direction of recent studies. Understanding of molecular physiology of concrete genes will perhaps result in the individual approaches of diseases diagnosing and treating.

The aim of our investigation was to establish correlation between polymorphisms of TNF-$\alpha^{-308} \to$G and IL-10 A$^{-1082} \to$G genes, their possible combinations and structure-functional state of bone in postmenopausal women.

II. METHODS

152 female in-patients took part in the study. Subjects were recruited through the Ukrainian Scientific–Medical Centre for the Problems of Osteoporosis. All subjects were ambulatory, free of chronic diseases (renal, liver, thyroid, parathyroid disorders, cancer), and not taking medications (e.g., glu-cocorticoids, estrogen replacement therapy, bisphosphonates, anticonvulsives, fluoride, heparin, thyroxine, vitamin D metabolites) known to affect skeletal metabolism. According to the WHO osteoporosis criteria, women were divided into three groups: I – with normal indexes of bone state (T-score > -1 SD), II – with osteopenia (T-score ≤ -1.0 to ≥ -2.5 SD) and III – with osteoporosis (T-score < -2.5 SD).

For analysis of the relations between TNF-$\alpha^{-308} \to$G, IL-10 A$^{-1082} \to$G polymorphisms and bone fractures in anamnesis patients were divided into three subgroups: with fractures of the radius bone in the typical place, X-Ray diagnosed deformations in spondile soma and with osteoporotic fractures of lower limbs in postmenopausal women. Only women whose fractures occurred as the result of the fall from a level of their height or lower were diagnosed as osteoporotic fractures.. Healthy women with no fractures compiled the control group.

Anamnesis included accident pathologies, patterns of menstrual function establishment and decay. When collecting anamnesis patients' complaints as well as primary manifestations of distrophycally-distructive changes in bone, existence of fractures or traumas in the past, their number and specificity of the injuries were taken into account. A custom designed questionnaire included known risk factors for osteoporosis, presence of fractures in anamnesis, and also information on fractures among close relatives (father, mother), time of onset of menarche, menopause, duration of a menopausal period, bad habits, physical activity etc.

Bone mineral density were estimated using dual-energy X-ray absorptiometry. Conventional BMD measurements of the spine (L1–L4 in the anterior– posterior position), proximal femur (neck, Ward's triangle, and trochanter regions), and radial shaft (33% site) were determined using a densitometer Prodigy (GE Healthcare, Lunar Prodigy, Madison, WI). Quality control procedures were followed in accordance with the

manufacturer's recommendations. Instrumental variation was determined regularly by a daily calibration routine using a phantom supplied by the manufacturer.

For gene polymorphism determination 100 μL of heparinized blood were diluted in 100 μL of lysing reagent. Samples were kept at -70°C until the DNA extraction, which was conducted according the user manual of the kit tool "DNA-sorb-A" ("AmpliSence", Russia).

The gene polymorphism was established using polymerase chain reaction (SNP-PCR), which assured estimation of the point mutation through matching of two types of the primers, which differ in one nucleotide.

TABLE 1. Primer sequences

Gene name	Sequence	The length of the amplificated fragment, bp
TNF-α	C: 5'-TCT CGG TTT CTT CTC CAT CG	184
	F: 5'- ATA GGT TTT GAG GGG CAT GA	
	R:5'- ATA GGT TTT GAG GGG CAT GG	
IL-10	C: 5'- CTT GGA TTA AAT TGG CCT TAG A	194
	F: 5'-ACT ACT AAG GCT TCT TTG GGA A	
	R: 5'-CTA CTA AGG CTT CTT TGG GAG	

Amplification mixture was prepared using "Fermentas" (Canada) reagents. The final volume of the amplification mixture in each of the samples was 25 μL. Amplification mixture composition: 0.5 μL (1 μg/ μL) C and F (or R) primers of the corresponding gene, 2 μL of dNTP (2 mM), 7 μL of DEPC-treated water, 10 μL of test DNA, 5 μL of PCR buffer (2.2 mM/L $MgCl_2$), 0.2 U of Taq-polymerase. Amplification was conducted using thermocycler PalmCycler (Corbett Research, Australia). Sequences of the used primers are listed in the table 1. Amplification was carried out according to the following program: 96°C – 1 min, 96°C – 15 sec, 65°C – 50 sec, 72°C – 40 sec (10 cycles); 96°C – 1 sec, 60°C – 50 sec, 72°C – 40 sec (20 cycles). Analysis was carried out by means of electrophoresis in 2% agarose gel with ethidium bromide. Occurrence of a point mutation was estimates by the availability of the amplification product (Fig. 1).

In the presented work by means of SNP-PCR we studied the influence of individual point polymorphism in the promoters of TNF-α $A^{-308} \rightarrow G$ and IL-10 $A^{-1082} \rightarrow G$ genes, which result in changes of cytokines production. The mutation frequency in patients are presented in Table 2.

It should be noted that TNF-α $A^{-308} \rightarrow G$ gene AA homozygote and IL-10 $A^{-1082} \rightarrow G$ gene GG homozygote provide a high production of the corresponding cytokines, and GG homozygote of TNF-α $A^{-308} \rightarrow G$ gene and AA homozygote of IL-10 $A^{-1082} \rightarrow G$ gene provide a low production of the corresponding cytokines.
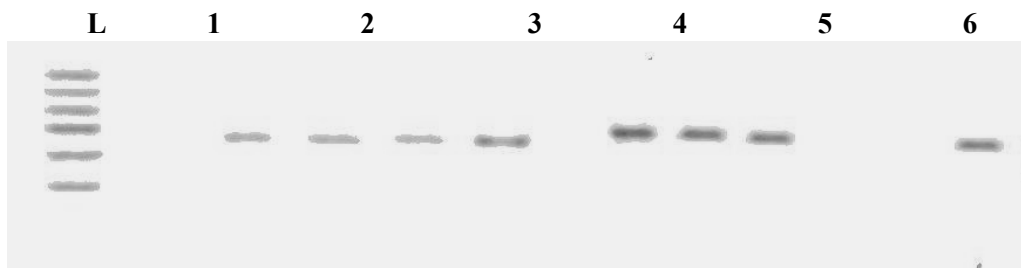
FIG. 1. Electrophoregram of amplification products of TNF-α (-*308 A/G*) (L – homozygote (AA, high expression), 2 – heterozygote (AG), 3 – homozygote (GG, low expression) and IL-10 (10 *(-1082 A/G)* (4 – heterozygote (AG), 5 – homozygote (AA, low expression), 6 – homozygote (GG, high expression)

The genotypes for each SNP were analyzed as a three-group categoric variable (referent model). All data were expressed as percentages or mean ± standard deviation (SD). Comparisons of non-numerical variables were made with Pearson's chi-square test. Comparisons of continuous variables between two groups were performed using Student's t-test; the non-parametric Mann–Whitney U-test was used when normal distribution was skewed. Two-sided $P < 0.05$ was considered statistically significant.

TABLE 2. The polymorphism frequency in patients

Gene name (polymorphism)	Polymorphism variant		
	Homozygote 1	**Heterozygote**	**Homozygote 2**
TNF-α (-308 A/G)	AA: 7 (4.6%)	AG: 34 (22.4%)	GG: 111 (73.0%)
IL-10 (-1082 A/G)	AA: 43 (28.3%)	AG: 70 (46.1%)	GG: 39 (25.7%)

III. RESULTS AND DISCUSSION

In the Table 3 the values for main clinical characteristics and indexes of bone mineral density in patients with the present combination of TNF-α A^{-308}→G polymorphism are presented. Women with different genotype were similar in age, postmenopause duration, weight, height and body mass index. We have analyzed the association between A^{-308}→G polymorphism of TNF-α gene and bone mineral density in postmenopausal women . We have not found any reliable association between different genotypes of TNF-α A^{-308}→G gene and the indexes of structure-functional state of bone, perhaps, due to the small number of patients with AA genotype.

TABLE 3. Characteristics of the patients, who have different genotypes of TNF-α (*-308 A/G*) gene, M ± SD

Parameter	TNF-α			P
	AA	AG	GG	
age, years	59.4 ± 7.2	59.2 ± 1.5	59.4 ± 0.7	0.92
postmenopause duration, years	14.4 ± 4.7	9.2 ± 1.3	10.5 ± 0.5	0.65
weight, kg	76.4 ± 4.9	78.8 ± 3.1	75.2 ± 1.4	0.31
height, cm	163.8 ± 2.3	162.8 ± 1.10	161.5 ± 0.5	0.34
body mass index, kg/m^2	28.5 ± 1.9	29 ± 1 (n = 33)	28.9 ± 0.5	0.62
patients with fractures, %	40.0	44.1	45.7	0.50
women with fractures and duration of postmenopause ≥5 years, %	40.0	27.3	46.2	0.98
patients with low-energy (osteoporotic) fractures verus patients with all fractures, %	100.0	72.7	64.3	0.56
BMD of the total body, g/cm^2	0.96 ± 0.08	0.98 ± 0.03	0.91 ± 0.02	0.53
T-score of the total body, SD	-0.3 ± 0.6	-0.2 ± 0.2	-0.7 ± 0.1	0.11
BMD of the lumbal spine, g/cm^2	0.98 ± 0.08	1.04 ± 0.02	0.98 ± 0.01	0.37
T-score of the lumbal spine, SD	-1.7 ± 0.6	-1.3 ± 0.2	-1.7 ± 0.1	0.19
BMD of the femoral neck, g/cm^2	0.90 ± 0.08	0.88 ± 0.02	0.84 ± 0.01	0.78
T-score of the femoral neck, SD	-1.0 ± 0.6	-1.1 ± 0.1	-1.34 ± 0.08	0.64
BMD of the Ward's triangle, g/cm^2	0.73 ± 0.08	0.70 ± 0.02	0.67 ± 0.01	0.55
T-score of the Ward's triangle, SD	-1.4 ± 0.6	-1.6 ± 0.2	-1.82 ± 0.09	0.27
BMD of the trochanter, g/cm^2	0.81 ± 0.07	0.82 ± 0.03	0.76 ± 0.02	0.41
T-score of the trochanter, SD	-0.3 ± 0.6	-0.3 ± 0.2	-0.8 ± 0.1	0.13

In Tables 4 and 5 the values for main clinical characteristics and indexes of bone mineral density in patients with the investigated polymorphisms of IL-10 A^{-1082}→G gene are presented. Women with different genotype were similar in age, duration of postmenopause, weight, height and body mass index (see Table 2).

TABLE 4. Characteristics of the patients, who have different genotypes of IL-10 (*-1082 A/G*) gene, M ± SD

Parameter	IL-10 (*-1082 A/G*)			P
	AA	**AG**	**GG**	
age, years	58.9 ± 6.3	59.8 ± 7.9	59.7 ± 6.0	0.77
postmenopause duration, years	10.6 ± 6.5	10.9 ± 7.3	10.1 ± 8.0	0.86
weight, kg	75.6 ± 14.7	77.9 ± 15.2	74.4 ± 15.9	0.49
height, cm	162.2 ± 5.0	161.6 ± 6.1	161.6 ± 5.9	0.86
body mass index, kg/m^2	28.8 ± 5.5	29.8 ± 5.5	28.4 ± 5.6	0.39
patients with fractures, %	43.6	35.8	38.5	0.69
women with fractures and duration of post-menopause ≥ 5 years, %	50.0	40.4	37.5	0.57

In our study we have established a reliable connection between AA genotype of IL-10 A^{-1082}→G gene, bone mineral density and the frequency of osteoporotic fractures. It was revealed that in women with AA IL-10 A^{-1082}→G genotype as compared to G allele carriers (AG/GG) values of T-score and BMD both of the total body and separate parts of it – lumbal spine, femoral neck and Ward's triangle, are statistically lower (see Table 3).

The risk of osteoporotic fractures in women with AA IL-10 A^{-1082}→G genotype as compared with G allele carriers (AG/GG) is comparatively low and comprises 1.5 (95% CI: 1.08 – 2.09), yet it is trustworthy (p < 0.04), which points to the existence of the connection between these two indexes.

The mechanism of bone loss in people with AA IL-10 A^{-1082}→G genotype can be eligible by the decreased production of IL-10 by immune cells in general or by the bone microenvironment. It is known that some proinflammatory cytokines, namely IL-1, TNF-α, IL-6, IL-11 stimulate processes of bone resorbtion.[14] It is established that all of them proinflammatory and their production increases with age.

TABLE 5. Characteristics of the bone state in the patients who have different genotypes of IL-10 *(-1082 A/G)* gene, M ± SD

Parameter	IL-10 *(-1082 A/G)*			P
	AA	**AG**	**GG**	
patients with low-energy (osteo-porotic) fractures verus patients with all fractures, %	87.5	61.9	53.3	0.10
	87.5	When combining carriers of G (AG/GG) vs. AA 58.3		0.04
BMD of the total body, g/cm^2	1.04 ± 0.09	1.1 ± 0.1	1.1 ± 0.1	0.04
	1.04 ± 0.09	When combining carriers of G (AG/GG) vs. AA 1.1 ± 0.1		0.01
T-BMD of the total body	-1.1 ± 1.1	-0.3 ± 1.4	-0.4 ± 1.3	0.04
	-1.1 ± 1.1	When combining carriers of G (AG/GG) vs. AA -0.3 ± 1.4		0.01
BMD of the lumbal spine, g/cm^2	0.9 ± 0.5	1.0 ± 0.2	1.0 ± 0.2	0.01
	0.9 ± 0.5	When conbining carriers of G (AG/GG) vs. AA 1.0 ± 0.2		0.003
T-BMD of the lumbal spine	-2.1 ± 1.0	-1.4 ± 1.3	-1.4 ± 1.2	0.01
	-2.1 ± 1.0	When conbining carriers of G (AG/GG) vs. AA -1.4 ± 1.3		0.003
BMD of the femoral neck, g/cm^2	0.8 ± 0.4	0.9 ± 0.2	0.9 ± 0.1	0.12
	0.8 ± 0.4	When combining carriers of G (AG/GG) vs. AA 0.9 ± 0.1		0.04
T-BMD of the femoral neck	-1.4 ± 0.8	-1.1 ± 0.9	-1.2 ± 0.8	0.14
	-1.4 ± 0.8	When combining carriers of G (AG/GG) vs. AA -1.1 ± 0.9		0.05
BMD of the Ward's triangle, g/cm^2	0.6 ± 0.4	0.7 ± 0.2	0.7 ± 0.1	0.03
	0.6 ± 0.4	When combining carriers of G (AG/GG) vs. AA 0.7 ± 0.1		0.009
T-BMD of the Ward's triangle	-1.9 ± 0.9	-1.6 ± 1.1	-1.5 ± 1.0	0.09
	-1.9 ± 0.9	When combining carriers of G (AG/GG) vs. AA -1.6 ± 1.1		0.03

From the published data it is known that manifestation of many age-related pathologies such as atherosclerosis, Alzheimer's disease, osteoporosis, are caused by the development of chronic inflammatory processes and by an increase of proinflammatory cytokines production.[4,13,19,45] Most of them stimulate osteoclasts activity and processes of bone resorbtion, which lead to age-related osteoporosis.[47] Pathology development could be due to the increase in catabolic signals as a result of a chronic inflammatory process, and with no clinical manifestations disease results in osteoblasts apoptosis and in malfunction of bone remodeling processes.[13,34]

Osteoporosis along with other age-related pathologies has expressed gene causation. In different individuals age-related changes in bone mineral density vary greatly. Partially it is caused by the individual fluctuations in the cytokines production level. Such an assumption is confirmed by the investigation of Ferrari et al,[8] who have shown that IL-6 gene polymorphism influences the risk of osteoporosis in women of postmenopausal period. A similar data were obtained on IL-1-β and IL-1Ra gene polymorphism, which correlated with the decrease in the bone mineral density and lumbal osteoporosis development in women.[5]

It is known that production of proinflammatory cytokines depends on estrogen's' content[46] and it is also regulated by antiinflammatory cytokines, which are produced by Th2-cells. It was shown that IL-4 and IL-10 inhibit production of IL-1, TNF-α and other Th1-cytokines which leads to inhibition of bone resorbtion.[23,29]

Thus, the impact of IL-10 A^{-1082}→G gene AA homozygote, the presence of which results in reduction of this cytokine production in the organism, can influence bone remodeling processes by means of increase in the level of proinflammatory cytokines.

As it was mentioned above, IL-10 and TNF-α play an opposite roles in development of inflammatory processes. Thus, it could be assumed that one of the main functions of IL-10 is central regulation of inflammatory process, whereas TNF-α controls the intensity and the duration of local inflammation.[17,22]

Stimulation of blood cells from different individuals with bacterial lipopolysaccharide *in vitro* results in production of different amount of IL-10 and TNF-α, which enables to assume that they are regulated genetically with the probability of 75% and 60% correspondingly.[16] Individual differences in the level of production of these cytokines, can conditioned the duration of inflammatory processes.

It should be noted that isolated estimation genotypes of individual cytokines' genes can lead to the results, which would be of a limited use. Because *in vivo* control of individual cytokines synthesis is regulated not only be the gene structure, but also by the set of external factors, which include a bundle of pro- and antiinflammatory cytokines, hormones, etc.[10]

In our investigation we haven't revealed any connection between the indices of bone mineral density and the possible combinations of studied gene polymorphisms (Table 6). Among genotypes AA/GG and GG/AA of the studied genes no significant difference in the incidence of osteoporotic fractures and BMD was observed.

Thus, polymorphism of TNF-α gene (A^{-308}→G) is not connected with the structure-functional state of bone in postmenopausal women. In women AA carriers of IL-

10 A^{-1082}→G polymorphism BMD indices are statistically lower. This enables to pursue an early diagnostics of osteoporosis risk.

TABLE 6. Characteristics of the patients, who have different genotype combination of TNF-α (*-308 A/G*) and IL-10 *(-1082 A/G)* gene, M ± SD

Parameter	IL-10-1082GG; TNF-α -308GG	IL-10-1082 AAAG; TNF-α -308 AAAG;	P
age, years	59.7 ± 5.6	59.4 ± 7.4	0.84
postmenopause duration, years	10.4 ± 3.7	9.9 ± 2.3	0.65
weight, kg	75.1 ± 16.0	76.7 ± 15.1	0.61
height, cm	161.7 ± 6.3	161.9 ± 5.7	0.86
body mass index, kg/m^2	28.7 ± 5.7	29.3 ± 5.5	0.60
patients with fractures, %	43.3	38.4	0.91
women with fractures and duration of postmenopause ≥5 years, %	50.0	38.8	0.28
patients with low-energy (osteoporotic) fractures verus patients with all fractures, %	61.5	73.7	0.37
BMD of the total body, g/cm^2	0.9 ± 0.1	0.9 ± 0.1	1.00
BMD of the lumbal spine, g/cm^2	1.0 ± 0.1	1.0 ± 0.3	0.40
BMD of the femoral neck, g/cm^2	0.9 ± 0.1	0.8 ± 0.3	0.52
BMD of the Ward's triangle, g/cm^2	0.7 ± 0.1	0.7 ± 0.3	0.48

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The Effect of Neuraminidase Blocker on Gabazine-Induced Seizures in Rat Hippocampus

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ABSTRACT: Concentration of neuraminidase (NEU), an enzyme which cleaves negatively charged sialic acids from carbohydrate moieties of the cellular membrane, could vary depending on physiological conditions. Multiple evidences suggest that fluctuations of NEU extracellular concentrations can influence neuronal activity. In the present study we have examined the effect of down regulation of endogenous NEU activity on seizure-like activity (SLA) induced by gabazine (specific blocker of inhibitory synaptic transmission) in the hippocampal CA1 pyramidal region of cultured slices. We have shown that in slices pretreated with the blocker of endogenous NEU (N-Acetyl-2,3-dehydro-2-deoxyneuraminic acid, NADNA) duration of synchronous oscillations induced by gabazine was considerably increased comparatively to control untreated slices. This study adds further information that changes in the level of NEU activity is an important factor, which can affect neuronal network excitability.

KEY WORDS: polysialic acid, neuraminidase blocker, seizure, gabazine, hippocampus

I. INTRODUCTION

Polysialic acid, a large cell-surface negatively charged carbohydrate, regulates manifold physiological functions including cell migration, axon outgrowth, neurogenesis, synaptogenesis and neuronal excitability.[7,10,16] A key enzyme, which regulates the level of sialic acid in the cell outer membrane, is endogenous NEU.[14, 15] Concentration of NEU in the brain could vary depending on physiological conditions[2] and many studies suggest that increase in the level of the extracellular concentrations of NEU can affect cell-to-cell interactions, synaptogenesis and influence neuronal activity.[7,13,17] However, there is a lack of studies devoted to investigation of the effect of endogenous NEU deficiency on the cellular and neuronal network activity. In our recent study we have shown that seizures induced by infusion of the high-potassium/low magnesium (High-K^+/low Mg^{2+}) artificial cerebrospinal fluid (ACSF) into hippocampus were significantly longer and seizure threshold was decreased in rats pretreated by the NEU blocker.[7] A major goal of the present study was to determine how oversialylation following blockade of endogenous NEU affected the hippocampal seizures evoked by gabazine, a specific $GABA_A$ receptor inhibitor. The mechanism of seizure induction in gabazine model of seizures is based on the blockage of synaptic inhibition. The etiology of temporal lobe epilepsy is closely associated with hippocampal changes in

GABAA receptor expression and function.[3,11,18,20] In the present study we showed that in hippocampal slices pretreated with specific NEU blocker epileptiform activity induced by blockade GABAergic transmission was considerably exacerbated.

II. METHODS

All procedures used in this study were approved by the Animal Care Committee of Bogomoletz Institute of Physiology.

Slice cultures were prepared using the method of Stoppini et al.[12.19] Briefly, Wistar rat pups were anesthetized and decapitated at postnatal day 7. The brains were removed and hippocampi were cut into 350 μm transverse sections using a McIlwain tissue chopper. Slices were then transferred to sterile porous membrane inserts (Millicell, Bedford, MA, USA), which were placed in a 6-well plate containing 1 ml culture medium/well (50% of MEM, 25% horse serum (HS), 25% HBSS, 5 mM Tris, 2 mM NaHCO3, 12.5 mM HEPES, 15mM glucose, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.2) and cultivated at $+35^{o}$C at air atmosphere with 5% CO_2. The culture medium was changed the next day after preparation of the slices and then twice a week. All experiments with organotypic hippocampal slice cultures were performed at 14 – 21 days in vitro. For recordings, slices were transferred to a submersion-type chamber mounted to the microscope (Olympus BX50WI, Japan) and superfused with the oxygenated ACSF of the following composition in (mM): NaCl 126, KCl 3.5, $CaCl_2$ 2.0, $MgCl_2$ 1.3, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11. Extracellular field potentials were recorded from hippocampal CA1 pyramidal layer using borosilicate glass pippetes filled with ACSF. Pipette resistance was 1 – 3 MΩ. Recordings were performed using AC differential amplifier (A-M Systems, Carlsborg, WA, USA) (bandpass 0.1 Hz – 1 kHz; ⨉100) and digitized at 10 kHz online with an analogue-to-digital converter (NI PCI-6221, National Instruments, Austin, TX, USA) and stored on a computer using WinWCP program (Strathclyde Electrophysiology Software, University of Strathclyde, Glasgow, UK). Off-line analysis of the recordings was performed using Clampfit (Axon Instruments, Sunnyvale, CA, USA) and Origin 7.0 (Microcal Software, Northampton, MA, USA).

III. RESULTS AND DISCUSSION

Field potential recordings were performed from hippocampal CA1 pyramidal layer in organotypic slice culture. Bath application of 10 μM of gabazine led to the increase of neuronal activity following spontaneous interictal-like discharges in all control slices (Fig. 1). To examine the effect of downregulation of the endogenous NEU activity cultured hippocampal slices were incubated with NADNA (2 mM) for 2 hours. SLA was induced in all 10 slices pretreated with NADNA (Fig. 1). This activity persisted as long as gabazine was kept in extracellular solution in control as well as in NADNA pretreated slices.
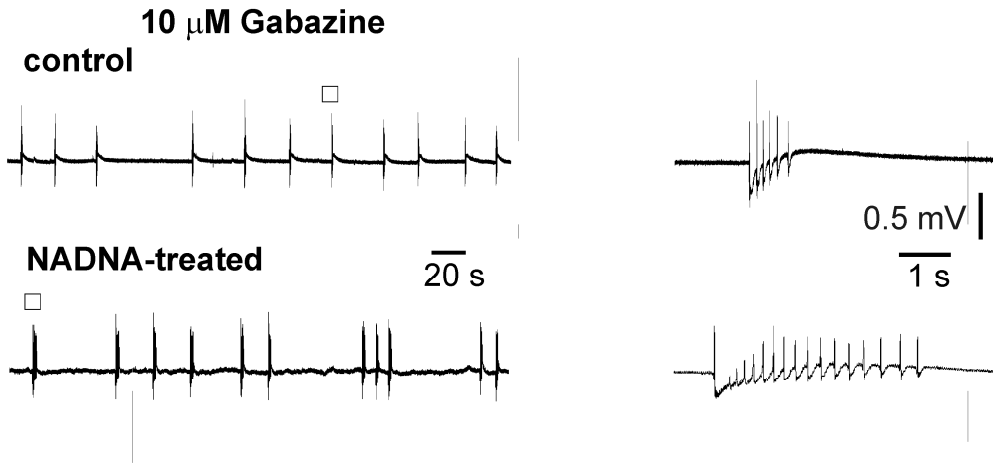
FIG. 1. Effect of NEU activity suppression on gabazine-induced SLA in cultured hippocampal slices. Extracellular field potential recordings from CA1 pyramidal cell layer in the presence of 10μM gabazine in control (upper panel) and NADNA pretreated (lower panel) slices. Spontaneous discharges marked with squares shown in expanded scales in the right panel
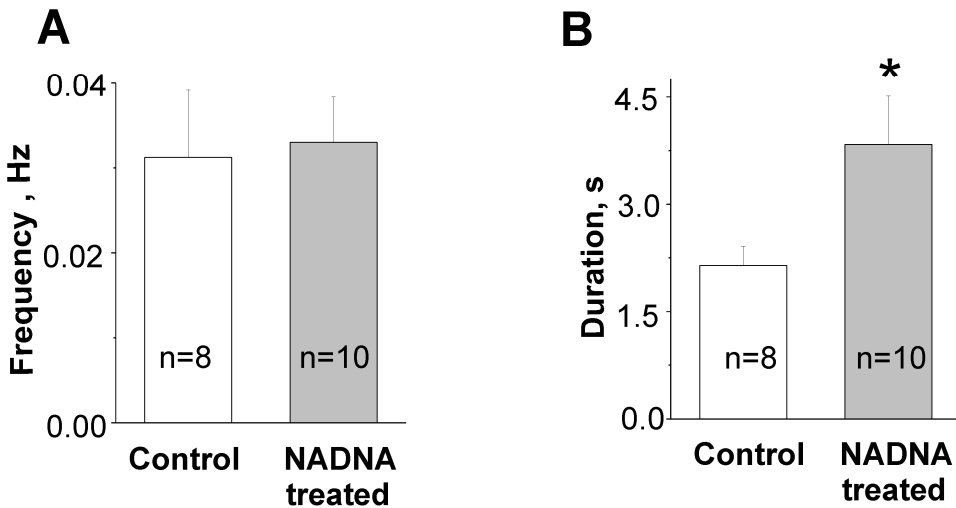

FIG. 2. Effects of NADNA pretreatment on different characteristics of gabazine-induced SLA in CA1 pyramidal layer. Summary plots show the PS frequency (**A**) and duration (**B**) during the epileptiform discharges in control (white) and NADNA pretreated (grey) slices. All values are mean ± SEM, *$P < 0.05$ versus control. N designates the total number of slices in each experimental group

The frequency of synchronous discharges during SLA for both control and NADNA-

pretreated slices was 0.03 ± 0.01 Hz (Fig. 2A). However the duration of synchronous oscillations was significantly increased in NADNA pretreated slices comparatively to controls (2.1 ± 0.3 s in control vs 3.8 ± 0.7 s in NADNA pretreated slices; $p < 0.05$, Fig. 2B). These data are in agreement with our previous report when blockade of endogenous NEU significantly reduced seizure threshold and aggravated hippocampal seizures induced by infusion of High-K^+/low Mg^{2+} solution *in vivo*.[7] Previous studies have shown that pretreatment of slice with NEU significantly altered kinetic properties of the voltage-gated sodium channels.[1,7,15] The authors connected this phenomenon with a presence of the negatively charged sialic acid residues on the extracellular surface region of the channel.[6,9] The charge created by these carbohydrates constantly influences the gating apparatus of the channel. Pretreatment with NEU removes sialic acid residues from the extracellular membrane and as a result shifts channel activation curve to the depolarized direction.[7,15] Further support of the idea that sialic acids contribute to the voltage dependence of sodium channel gating was obtained using recombinant deletion of likely glycosylation sites from the sodium channel sequence.[1] The deletion of the channels glycosylation sites resulted in mutant channels that gated at voltages up to 10 mV more positive than wild-type channels. In our previous study pretreament with NEU led to increase in the action potential threshold following decreasing of neuronal activity.[7] The blockade of the endogenous NEU in our present study has an opposite effect on the neuronal network activity. It was demonstrated that seizure intensity in a kindling model of epilepsy were not altered when NADNA was administered concurrently with NEU. So there is no direct proconvulsant effect of NADNA on SLA.[7] We proposed that NEU deficiency leads to accumulation of sialic acid in extracellular region and as a result increases opening probability of the sodium channels which leads to enhancement of the neuronal excitability. Recent studies support this assumption. It was shown that NEU inhibitors induced paired-pulse facilitation in population spikes without changes in excitatory postsynaptic potentials in the CA1 region of hippocampal slices and enhanced synchronization in rat hippocampal CA3 networks.[8,21] Also the fact that inherited diseases (sialidosis, galactosialidosis, Salla disease etc.) concerned with defective or deficient metabolism of endogenous NEU and sialic acid are often accompanied with epilepsy, exemplifies a substantial role of the level of sialylation in regulation of neuronal activity.[4,5,22] Present study adds further evidence that modulation of NEU activity renders a substantial influence on neuronal network excitability.

IV. ACKNOWLEDGEMENTS

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Effects of Genipin at NO synthesis and Ischemia-Reperfusion Induced Oxidative Stress in Old Rat Hearts

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ABSTRACT: Genipin is aglycone of genipiside, one of the active compounds of Gardenia gasminoides Ellis. The gardenia fruit extract has been used in traditional Chinese medicine to relieve the symptoms of type 2 diabetes which is accompanied by extensive oxidative stress and endothelial dysfunction of NO production. Besides, genipin was shown to inhibit UCP-dependent proton leak through the inner mitochondrial membrane, which leads to the increased mitochondrial potential and ATP production. We have studied the effects of genipin at ischemia/reperfusion-induced oxidative stress and activity of NOS isoenzymes using Langendorf perfused old rat heart model. Ischemia-reperfusion is a well known oxidative agent, which induces significant increase in superoxide radical, hydrogen peroxide and hydroxyl radical content. Genipin administration in a dose of 10^{-5} M for 15 minutes before the prolonged ischemia exerted powerful antiradical and antilipoperoxidative effects. Heart ischemia-reperfusion was accompanied by generation of peroxinitrite and by nitrozative stress. We have demonstrated the inhibitory impact of genipin on iNOS expression, which is possibly, occurs via preoteinkinase A inhibition and via stabilization of I-κB-NF-κB complex. Genipin stimulated cNOS activity perhaps by activating PI3K/Akt signaling pathway. Although, post-ischemic recovery of cardiodinamic parameters of old rat hearts was depressed due to the "switch of" of the NO production by inducible NOS, which is important in early period of reperfusion. Thus, we conclude that genipin is a powerful antioxidant and it processes an insulin-like activity due to its ability to manage NO production at the level of intracellular signaling cascades.

KEY WORDS: ischemia-reperfusion, oxidative stress, genipin, nitric oxide synthase, aging, uncoupling proteins, heart

I. INTRODUCTION

Damaging influence of blood flow renewal in myocardium after prolonged ischemia is conditioned by so called free radical explosion, which is accompanied by formation of the reactive oxygen species (ROS) and reactive nitrogen species (RNS),[5] by damaging of protein and lipid cell structures, which lead to myocardium mitochondria dysfunction. UCP (uncoupling proteins), which are localized in inner mitochondrial membrane, catalyze proton leak from intermembrane space into the mitochondrial matrix, uncoupling oxidative phosphorylation. Published data indicate that activation of UCP-dependent proton leak, which could be committed by superoxide radical,[13] is accompanied by decrease in membrane potential and, thus, inhibiting superoxide production by complex I of respiratory chain.[8] Such a mutual regulation defends the cells from a

free-radical damage induced by ROS and RNS. It is known from the published data that UCP2 or UCP3 deficient mice reveal features of oxidative stress.[29] Recently it was shown[31] that proton-conducting activity of UCP2 can be inhibited with genipin – aglycone of genipiside, one of the active compounds of Gardenia gasminoides Ellis. The gardenia fruit extract has been used in traditional Chinese medicine to relieve the symptoms of type 2 diabetes which is accompanied by extensive oxidative stress and endothelial dysfunction of nitric oxide (NO) production. Similar distortions are seen under aging and heart ischemia-reperfusion. For these reasons the aim of our work was to ascertain effects of genipin – inhibitor of UCP2 activity – on development of oxidative stress and on NO synthesis in the hearts of old rats under ischemia-reperfusion.

II. METHODS

24 month old Wistar male rats (450 – 550 g weight) were studied. A retrograde perfusion of coronary vessels of the isolated heart was done using Kebs-Henseleit solution (in mM): NaCl – 118, KCl – 4.7, $MgSO_4$ – 1.2, $NaHCO_3$ – 24, KH_2PO_4 – 1.2, glucose – 10, $CaCl_2$ – 2.5. The perfusion solution was constantly aerated with carbogen (95% O_2 and 5% CO_2) at 37°C. The total myocardium ischemia was modeled by the 20-min perfusion shut-off with the following reperfusion (40 min). Genipin ("Walco Inc.", USA) was administrated to the perfusion solution in dose of 10^{-5} M for 15 minutes before ischemia. Registration of cardiodynamics parameters and calculation of oxygen cost of myocardial work was done as described earlier.[2]

For biochemical experiments hearts were immediately frozen in liquid nitrogen. Intensity of the oxidative metabolism in heart homogenates of old rats was estimated by the change in generation rate of unstable free radicals – superoxide anion-radical (O_2^-)[21] and *OH-radical,[17] and by changes in the content of stable hydrogen peroxide[18] and the end product of lipid peroxydation (LP) – malonic dialdehyd.[24]

Intensity of *de novo* NO generation was estimated by activity establishment for different isoforms of NO-synthases: calcium-independent inducible NOS and calcium-dependent constitutive NOS by citrullin formation, nitrite-anion content (NO_2^-)[14] and nitrate-anion (NO_3^-).[27]

Intensity of reutilizative NO generation was estimated by establishing nitroreductase activity under changes in NO_3^- in the incubation medium at the abundance of NADH. Arginase activity, which is concurrent to NO-synthase activity and produces polyamines, under enzymatic degradation of which considerable amounts of H_2O_2 and toxic aldehydes are formed, was estimated using photometric method.[4] Citrullin, which is the product of different NO-synthases and is formed simultaneously with NO, being thus the marker of NO generation, was studied using spectrophotometric method,[7] and urea – using the kit "Filicit-Diagnistics", Ukraine. Protein concentration in samples was estimated using Bredford method.[9]

Statistical data processing was done in Excel using difference method. All the results were presented as the mean ± standard deviation. The data were analyzed for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered to be significant.

III. RESULTS

III.A. Genipin Prevents Development of Oxidative Stress under Ischemia-Reperfusion of Myocardium

As it is known that prolonged ischemia and the following reperfusion of myocardium are accompanied by increase in the rate of ROS and RNS formation, which initiated LP.[5] Mitochondria are the one of the main sources for ROS formation at different chronic pathological states and at aging[22] Ischemia-reperfusion is a good experimental model for modulation of mitochondrial functions and for studying of mechanisms of ROS and RNS generation. Our results shows that ischemia-reperfusion induce considerable (three-fold) increase in the rate of superoxide anion generation in the hearts of old rats (Table 1). The reason for this could be activation of xanthine oxidase and lipid oxidases. Moreover, hydrogen peroxide content increased two-fold and the rate of hydroxyl radical – by 2.5. This is accompanied by almost two-fold increase in the malonic dialdehyd content which points to the considerable activation of LP. In our experiments ischemia-reperfusion was accompanied by the simultaneous increase in H_2O_2 and *OH-radical content. Possibly, the main pathway of *OH-radical generation in the heart of old rats under ischemia-reperfusion is not only "conventional" – formation of H_2O_2 in the Fenton reaction amidst metal ions (Fe^{2+}, Cu^{2+}),[1] – but also owing to enhancement of peroxinitrite degradation, which could be formed during the simultaneous intensive generation of superoxide anion and nitric oxide, which occurs in the conditions of our experiments. Thus, ischemia-reperfusion induce oxidative stress in the old rat hearts.

A preliminary perfusion of coronary vessels with the inhibitor of UCP2-depended proton leak, genipin, resulted in considerable, even lower than in the control group, decrease in ROS production in response to ischemia-reperfusion (Table 1). This point to the powerful antioxidative properties of genipin and characterizes it as inhibitor of ROS generation and LP activation (10-fold decrease in malonic aldehyde content). Should be noted excessive inhibition of ROS generation by genipin in the chosen dose. Enhancement of this process with age could be of an adaptive character, because it is known that the amount of ROS and LP products, which is produced under physiological conditions, takes part in some processes, namely in signals transduction,[11,15] calcium mobilization from sarcoplasmic and mitochondrial depots, activation of Na+-H$^+$-exchanger etc. Moreover, there are evidences that increase in H_2O_2 generation leads to enhancement of endothelial NO-synthase (eNOS) expression on transcription and translation levels,[10,32] and superoxide anion is a powerful amplificator of iNOS expression, e.g calcium-independent *de novo* synthesis of nitric oxide.[3] It is known that ROS are one of the mediators of ischemic preconditioning:[28] short ischemic periods stimulate increase of the ROS background level, which "prepares" the heart to the next prolonged hypoxia and, as a consequence, the early reperfusion period proceeds with much lesser morphological and functional damages of myocardium.

Thus, excessive decrease in ROS generation in the heart tissues after ischemia-reperfusion at the background of genipin pretreatment could be a reason for the de-

creased myocardium tolerance to the ischemic damage, which we observed in our experiments (Fig. 1).
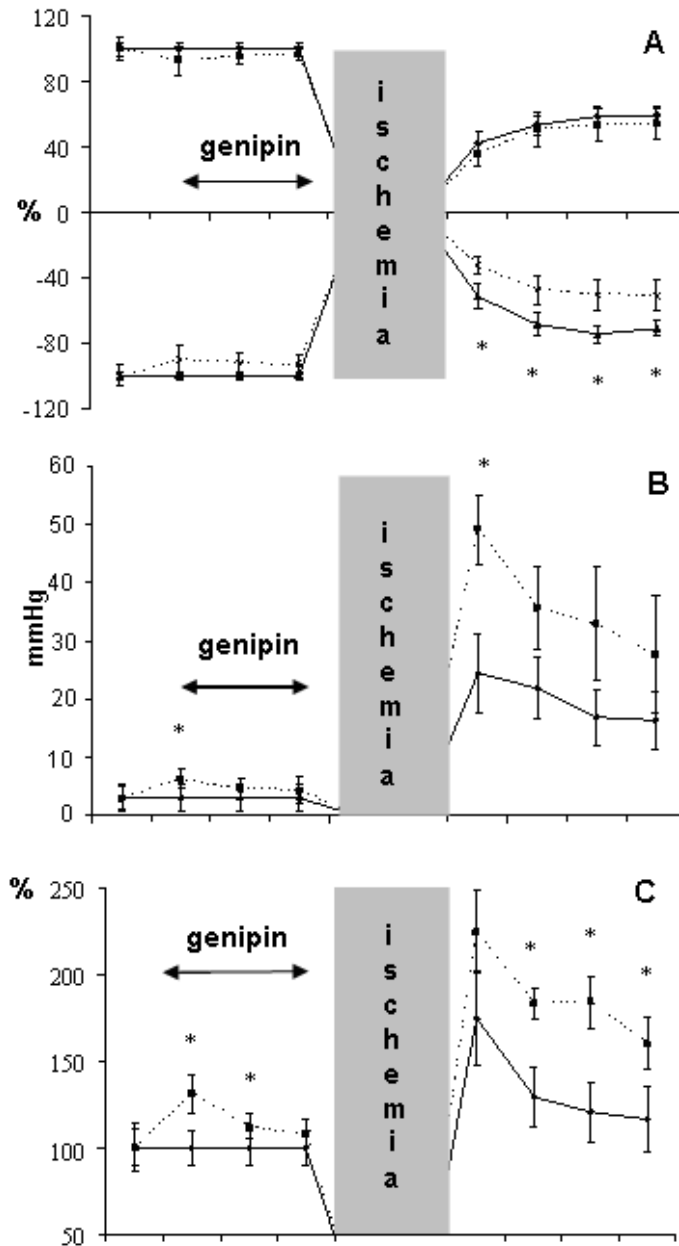

FIG. 1. Cardiodynamic parameters under genipin pretreatment: dP/dt (a), the end-diastolic pressure (b), the oxygen cost of myocardial work (c) of old rats: solid curve – ischemia-reperfusion, dotted curve – genipin pretreatment+ischemia-reperfusion

TABLE 1. Effect of ischemia-reperfusion and genipin pretreatment on the profile of the oxygen free radicals and malonic dialdehyd content in the heart tissues of aging rats ($M \pm m$; $n = 5$ -6). *P<0.05, **P<0.01, ***P<0.001 – as compared to the control. #P<0.05, ##P<0.01, ###P<0.001 – as compared to the values in the group with ischemia-reperfusion

Parameter	Control	Ischemia-reperfusion	Genipin + ischemia-reperfusion
O_2^-, c.u.	5.7 ± 0.6	15.5 ± 1.5 ***	8.3 ± 0.4 **, ###
H_2O_2, pM/mg of a protein	3.9 ± 0.5	7.0 ± 1.2 *	1.1 ± 0.3 ***, ###
$^*OH$, c.u..	1.7 ± 0.2	4.1 ± 0.4 ***	0.4 ± 0.1 ***, ###
Malone dyaldehyde, nM/mg of a protein	18.4 ± 2.6	35.9 ± 3.7 **	3.6 ± 0.5 ***, ###

III.B. Genipin Influence on the System of Nitric Oxide Synthesis under Ischemia-Reperfusion of Myocardium

NO plays one of the main roles in regulation of myocardium function due to its vasodilatory effects.[26] It is known that nitric oxide is generated at L-arginine oxidation by calcium-dependent enzyme cNOS. Calcium-independent NO synthesis by enzyme iNOS is also of a great importance. On the model of transgenic mice was established that hyperexpression of iNOS does not lead to the contractile dysfunction of myocardium or any other irreversible effects.[16,19,30] Yet, concerning the importance of iNOS there are some deprecations. Especially it concerns the early periods of reperfusion. There are some data regarding cardioprotective role of iNOS just in the delayed periods of reperfusion.[6] At the same time the basal activity of iNOS is often being ignored. Therefore, of a great interest for us was studying of different pathways of NO synthesis under ischemia-reperfusion and the effect of genipin perfusion before ischemia.

Table 2 shows that activities of enzymes responsible for *de novo* NO synthesis were not significantly changed after ischemia-reperfusion of old rat hearts. It is known that under increase of arginase activity there is an increase in the content of products of arginine degradation to urea and ornithine with the further formation of the vast amount of polyamines, which in turn are the sours for hydrogen peroxide generation by means of oxidases. Activity of arginase, which competes with cNOS for the common substrate – L-arginine – in our experiments likewise experienced no changes. On the one hand, this points to sufficient content of endogenous L-arginine for calcium-dependent NO synthesis; on the other hand, there is no impact of amioxidase pathway into H_2O_2 generation as a consequence of polyamines degradation under old rat hearts ischemia-reperfusion. Decreased urea content (Table 2) at such conditions does not accomplish any inhibitory impact on the citrullin cycle of L-arginine resynthesis.

At the same time activity of NADH-dependent nitratreductase increased by 1.5-times (Table 2), which points to the intensification of reutilizative NO synthesis not

only in the ischemic period, but also in 40 minutes after reperfusion. It should be noted that nitratreductase is being activated at hypoxic conditions, thus, increase in its activity in our experiments points to the luck of oxygen in the heart tissues and to the effectivity of our model of ischemic heart damage. Also we observed changes in the pools of stable metabolites of NO: NO_3^- increased two-fold, which could be caused by peroxinitrite degradation under excessive simultaneous generation of NO and superoxide anion radical; NO_2^- content decreased by 2.5-fold, which also indicate the heart tissue hypoxia, since its formation from NO is possible just in oxygenated solutions. Thus, high NO_3^- content indicates that there is an intensive formation and degradation of peroxinitrite, pointing to the development of nitrozative oxidative stress under ischemia-reperfusion of isolated rat heart.

TABLE 2. Effect of ischemia-reperfusion and of genipin pretreatment on the nitric oxide system in the heart tissues of aging rats (M ± m; n = 5-6). *P<0.05, **P<0.01, ***P<0.001 – as compared to the control. #P<0.05, ##P<0.01, ###P<0.001 – as compared to the values in the group with ischemia-reperfusion

Parameter	Control	Ischemia-reperfusion	Genipin + ischemia-reperfusion
Inducible NOS	4.1 ± 0.8	3.0 ± 0.3	0.77 ± 0.07 **, ###
Constitutive NOS	1.8 ± 0.2	2.2 ± 0.4	4.7 ± 0.4 ***, ##
Nitratereductase, nM/mg of a protein	1.5 ± 0.1	2.4 ± 0.2 **	1.21 ± 0.04 *, ###
Citrulline, nM/mg of a protein	36.0 ± 3.7	123.3 ± 10.0 ***	26.8 ± 4.3 ###
NO_2^-, pM/mg of a protein	170.4 ± 11.8	69.4 ± 18.1 **	178.0 ± 13.0 ###
NO_3^-, nM/mg of a protein	17.5 ± 1.9	30.2 ± 3.1 **	13.7 ± 0.9 ###
Arginase, nM/mg of a protein	2.6 ± 0.3	2.6 ± 0.1	1.11 ± 0.07 ***, ###
Urea, nM/mg of a protein	193.5 ± 28.0	101.3 ± 4.5 **	52.0 ± 2.8 ***, ###

In experiments with genipin perfusion before the heart ischemia we have seen a considerable decrease in iNOS, arginase and reductase activity (Table 2). At the same time there was a two-fold increase in cNOS activity. Yet, this was probably not enough for restoration of the diastolic function of the left ventricle during the reperfusion, since the rate of myocardium relaxation (dP/dt $_{min}$) was statistically lower than in the control (Fig. 1A) and it did not recovered till the end of the observation. This points to the lack

of nitric oxide in the heart tissues during postischemic period, and also to the calcium overload of cardiomyocytes (contracture). It should be noted that the perfusion with genipin already on the 5th minute of the observation resulted in the increase of the end-diastolic pressure (Fig. 1B), and also to the significant decrease of the coronary flow and increase of the oxygen cost of myocardial work during the whole period of the genipin perfusion. All this points to the complication of left ventricular dilatation and to some extent to the endothelial dysfunction. Perhaps, NO synthesized by iNOS is essential for inhibition of fibroblasts proliferation and for synthesis of constrictive eicosanoids, which are synthesized at inflammatory processes. Thus, it's no wonder that the early stage of postischemic recovery of the heart contractile function in our experiments was accompanied by the excessive contracture of the left ventricle and the coronary vessels. Thus, in the cell is formed a deficit of signaling molecules, which should initiate cascades of the protective programs of cardiomyocytes under hypoxia.

Dual action of genipin is conditioned on the one hand by the excessive inhibition of ROS production under ischemia-reperfusion of the old rat hearts. Antilipoperoxidative activity of genipin was observed in experiments with brain homogenates,[20] where it inhibited malonic dialdehyd formation in response to LP with hydroxyl radical, generated by Fe^{2+}/ascorbate system. In our experiments genipin revealed also antiradical properties, namely it decreased content so of superoxide anion radical, as of hydroxyl radical and, as a consequence-of malonic dialdehyd.

Why would cNOS activation occur, which we observed in our experiments? It is known that cNOS is activated by calcium ions and by phosphorylation with proteinkinase Akt/PKB,[12] which leads to the increase of NO production.[23] Therefore among the possible mechanisms of genipin action are: 1) activation of PI3/Akt signaling pathwaym where proteinkinase Akt/PKB phosphorylates cNOs and thus activates it; or 2) inhibition of proteinkinase C. In both cases increased production of NO by cNOS occurs. Among the wide variability of signaling pathways in the cell there is the one, which starts from membrane receptors sensitive to insulin-like growth factors (relaxin/insulin-like family peptide receptor),[25] which is schematically shown on the Fig. 2.

The second aspect of the genipin action consists in the "turn off" of the *de novo* synthesis of NO by iNOS in the heart tissues of old rats under ishemis-reperfusion. Probably, this is done through the inhibition of iNOS expression. Mechanism which would mediate this process (Fig. 2) could consist in the prevention of degradation of IκB-β protein – inhibitory subunit of the nucleus factor κB (NF-κB) [20], since its nucleus localization depends on the IκB-β degradation. If this protein is decayed, NF-κB comes into the nucleus and binds with the promotor region of iNOS gene, enhancing its expression[25] and NO production. IκB-β degradation occur due to its phosphorylation by proteinkinase A, which is activated by cAMP. In turn cAMP content is controlled by adenylatecyclase, which increases cAMP content and by phosphodiesterase, which metabolizes cAMP and prevents PKA activation. Probably genipin action is directed to inhibition of proteinkinase A activity.

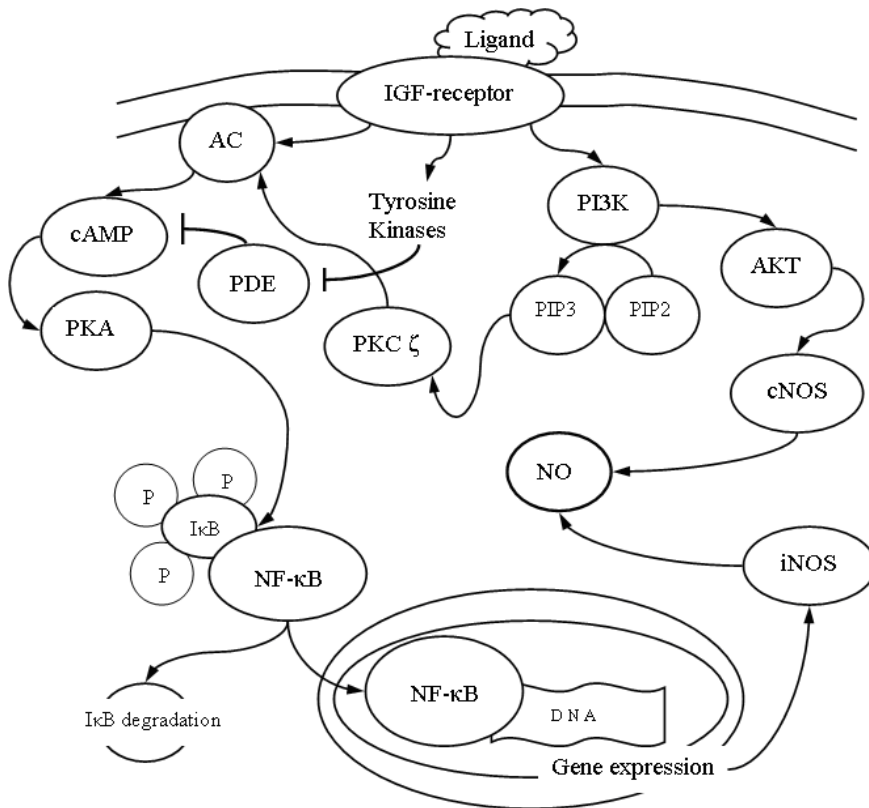Thus, in our experiments genipin revealed a regulatory action on iNOS expression and on cNOS activity.

FIG. 2. Signaling pathway of NO-synthases activation through the receptors sensitive to the insulin-like growth factors

IV. CONCLUSIONS

1) Ischemia-reperfusion of old rat heart is accompanied by an increase in the rate of ROS (H_2O_2, superoxide anion radical, *OH-radical) and RNS formation (high content of NO_3^- indicated an intensive formation and degradation of peroxinitrite), which results in LP initiation.

2) Inhibition of UCP2 activity with genipin in a dose of 10^{-5} M was accompanied by the decrease in the heart contractile activity and by an increase in the end diastolic pressure and the oxygen cost of myocardial work in old rats. Perfusion with genipin before the ischemia increased reperfusional disturbances of heart function in old rat hearts.

3) Genipin reveal powerful antiradical and antilipoperoxidative properties, since pretreatment with genipin resulted in considerable decrease in generation of ROS and prevention of LP activation in response to ischemia-reperfusion of old rat hearts.

4) The possible stimulatory effect of genipin on cNOS activity may consist in PI3K/Akt activation.

5) Genipin considerably lowered iNOS activity in the heart tissues of the old rats, perhaps due to the inhibition of proteinkinase A activation, which resulted in IκB-NF-κB complex stabilization and to the inhibition of iNOS expression.

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Age-Dependence in Alterations in Nitric Oxide Synthesis in Cardiovascular System during Adaptation to Physical Training

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ABSTRACT: Emerging evidence suggests that training-derived cardiovascular preconditioning is mediated by antiapoptotic and antioxidant action of nitric oxide. Nitric oxide has been reported as one of the major down-regulator of ROS generation and apoptosis-mediated cardiovascular dysfunction in aging animals and man. We studied both activity of oxidative de novo and nonoxidative salvage pathways of nitric oxide synthesis and nonoxidative L-arginine degradation by arginase. Our results show (1) the activation of both salvage (by nitratereductase + nitritereductase reduction of nitrate-and nitrite-anions) and de novo (L-arginine oxidation by NOS) pathways of nitric oxide synthesis and (2) rising of nitric oxide bioavailability in training rats. (3) Salvage/ de novo ratio degree in aorta correlate with aortal reactivity to dilation action of nitric oxide suggests that degree of this ratio in plasma can be a potent biochemical marker of training effectively in sportsmen. In aging rats endothelial dysfunction is not eliminated by training, but long-time swimming (as preconditioning) increased endothelial (NO) -dependent vasodilatation of the aortal VSMC of both adult and aging rats. Thus, training-dependent vasoprotection in adult and aging rats can be released by up-regulation of NO synthesis, especially by nonoxidative salvage, but not by oxidative de novo pathways.

KEY WORDS: nitric oxide, vascular tone, endothelium, precondition, training, aging

I. INTRODUCTION

It is known that the prominent role in the adaptation of the heart muscles and of the blood vessels to physical training plays signaling pathway NO/рГЦ/cgmp/pkg. Yet, the details of the NO synthesis itself, which is the most universal regulator of their activity, are still not conclusively established. Recently were found many NO-controlled effectors systems, which control vessel tonus and cardiomyoctes' contraction, angiogenesis and apoptosis, proliferation and differentiation, oxidative metabolism and intracellular calcium content, K_{ATP}-channels and soluble guanilatcyclase activity, Ca^{2+}-atpases and cyclooxygenases etc. Depending on the concentration, compartment and synthesizing pathway NO can be and antagonist. Thus, NO is known to be as pro-, so antistressive, cytoprotective and cytotoxic, vasoprotective and vasotoxic, cardioprotec-

tive and cardiotoxic, neuroprotective and neurotoxic etc. Amidst such a profusive variety the question is lost, whether there is a top-priority NO action, which enables effective realization of incredibly powerful regulatory process initiated by such a small molecule. Nevertheless, incredible as it may be seen, there is a growing numbers of evidence that suggest that there is no more important NO function than regulation of synthesis of itself. But then, there is nothing strange in such a problem presentation, if one would recollect the structure of NO biosynthesis system, which consists of enzymatic constitutive and inducible de novo synthesis with L-arginin oxidation by no-synthases under normoxia (in mitochondria inclusively); nonenzymatic de novo synthesis with L-arginin oxidation under extreme conditions; enzymatic salvage synthesis under recovery of the oxidated stable NO metabolites with the corresponding reductases at hypoxia (in mitochondria also); enzymatic salvage synthesis under recovery of the oxidated NO metabolites in conditions of NADP oversynthesis.

Each of those pathways of NO synthesis has a number of alternatives. There are constitutive calcium-dependent and inducible calcium-independent de novo NO synthesis, NADPH-dependent salvage synthesis, synthesis by dezoxi forms of hemoglobin of erythrocytes and myoglobin of cardiomyocytes, nonenzymatic synthesis under different extreme conditions. Established were molecular, biochemical and physiological mechanisms of NO synthesis regulation by the active oxygen forms (superoxide anion, hydrogen peroxide) and by means of self-regulation, regulation of gene expression and of activity of different NOS isoforms and enzymes of their co-factors biosynthesis, which are essential for enzymes' functioning. At the same time there is still no complex research concerning activity of all the known pathways of NO synthesis under different physiological conditions. Previously we have detected changes in the speed ratio of inducible and constitutive NO synthesis, its de novo and salvage synthesis, oxidative and non-oxidative L-arginin metabolism at aging, arterial hypertension, 1st type diabetes, low doses of radiation.[1-5] According to our results oxidative metabolism and activation of NO-production in the cardio-vascular system are normalized by moderate hypoxia,[5] inhibitor of angiotensin-transforming enzyme enalapril,[4.8] hormones ecdysterone[2.9,10] and melatonin,[7] calcium channel blocker diltiazem,[1] exogenous substrates of the Krebs cycle,[3] and also urea.[6] Data concerning the impact of physical training are rather sporadic and controversial.[20]

The aim of this work is to study characteristic properties of nitrogen oxide synthesis, arginin exchange and oxidative metabolism, endothelium-mediated changes in vessels' tonus under adaptation to the ladled physical training (swimming) in rats of different age.

II. METHODS

Experiments were conducted on adult (12 month) and old (21 month) Wistar male rats before (control) and after ladled physical training (swimming) : training was done in the basin filled with water at 30 – 32ºC five times per week (starting with 2 minutes and ending with 75 minutes of training) during 6 weeks according to the scheme:

TABLE 1. The scheme of rat training by swimming

Week	Day/time (min) of training				
1	1 / 2	2 / 6	3 / 10	4 / 14	5 / 18
2	8 / 22	9 / 26	10 / 30	11 / 34	12 / 38
3	15 / 42	16 / 46	17 / 50	18 / 54	19 / 58
4	22 / 62	23 / 66	24 / 70	5 / 74	26 / 75
5	29 / 75	30 / 75	31 / 75	32 / 75	33 / 75
6	36 / 75	37 / 75	38 / 75	39 / 75	40 / 75

Circular preparations of the isolated aorta (1 – 2 mg) from the chest were placed in the ductal, thermostatic (35 – 36ºC) chamber, which was perfused with the standard buffer Krebs solution, and pulled with the force of 7 – 10 mN.[18] Under the conditions, which were close to isotonic, the contractile activity of aortal smooth muscles (SM) was recorded. For investigation of the impact of vasodilator agonists, SM were previously activated by adding of noradrenalin (HA 10^{-6} M) to the Krebs solution. The basal level of such a contraction of SM was taken as 100% and all further calculations of amplitude changes in response to acetylcholine iodide (10^{-6} M) and sodium nitroprusside (10^{-4} M) (Sigma, USA) were performed with relation to that basal level.

In aorta homogenates and in blood plasma the following activities were measured: (i) calcium-dependent (constitutive – cNOS, which is the sum of endothelial – eNOS and neuronal – nNOS) and calcium-independent (inducible – iNOS) activities of enzymes responsible for de novo NO synthesis; (ii) NADH-dependent nitrate reductase activity, which characterizes intensity of non-oxidative salvage NO synthesis; (iii) arginase activity – the main enzyme of non-oxidative L-arginine degradation.

In the protein-free probes aliquots were estimated pools of stable NO metabolites – (NO_2^-) -and (NO_3^-) -anions; pools of active oxygen metabolites – hydrogen peroxide (H_2O_2); and pools of one of the products of arginine metabolic pathway – urea. Also was estimated the intensity of complete degradation of ATP cleavage products by xanthine oxidase (which is accompanied by production of superoxide-radical) by measuring the uric acid content using chemicals from "Fylysyt-Dyahnostyka" (Dnipropetrovsk, Ukraine). Oxygenation index (OI) in rat aorta and blood plasm was calculated on the basis of urea, nitrite-and nitrate-anions concentrations according to the formula:

$$OI = 1000 * [nitrite] / [nitrate] + [urea], c.u.$$

III. RESULTS AND DISCUSSION

III.A. Biochemical Investigations

The effect of regular physical trainings on NO synthesis, as it is known, in muscle cells

depends on their duration, intensity, specificity, and also on the number of muscle groups, which are being contracted, on the duration and the contractile frequency. NO synthesis induced by physical training is usually occurs at the level of regulation of different NOS isoenzymes expression. Under the condition of long and intensive physical training increases expression of iNOS; in case of moderate physical training – increases mostly cNOS. The signaling mechanisms, which regulate these processes, are already known. For example, it was established that induction of iNOS in the skeletal muscles fulfills due to the action of cytokine interleukin 6 (IL-6), which is produced during the regenerative (after training) period by muscle cells themselves, [19] whilst cNOS expression is stimulated mainly by two signaling cascades – nucleotide AMP-kinase cascade and lipid phosphatidilinositol-3-kinase cascade, through proteinkinase activation. Nucleotide signaling cascade Akt/PKB is important for NO synthesis regulation in the cardiovascular system, especially in large vessels, where AMPK is "launched" by the bias potential.
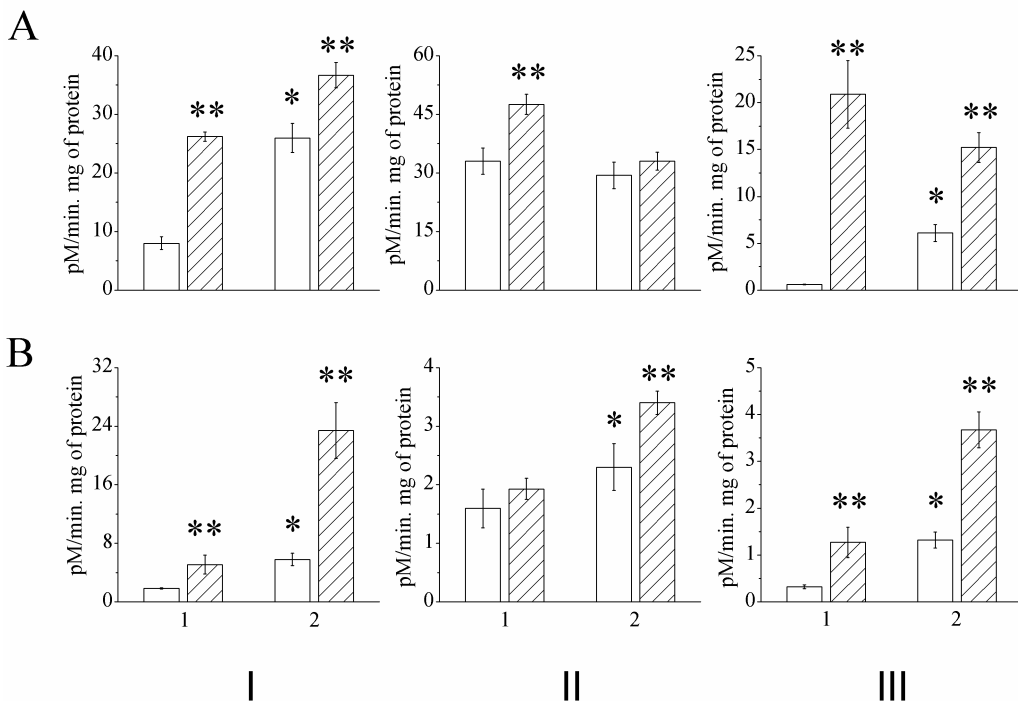
FIG. 1. Effect of training in the form of swimming on the activity of enzymes responsible for nitric oxide synthesis: iNOS (I), cNOS (II) and nitrate reductase (III) in aorta (**A**) and in plasma (**B**) of different age rats: 1 – adult rats; 2 – aging rats; white columns – control; hatched columns – after training; * the difference is statistically significant when comparing adult and aging rats; ** the difference is statistically significant when comparing rats before and after training

It was established that in adult rat aorta after physical training iNOS content increases by more than 3-fold, cNOS – just by 1.4 fold, and nitrate reductase – by more than 30 times. In aging rats in the absence of cNOS activity stimulation, just slightly (by 2.5 times) increased iNOS and nitrate reductase content (Fig. 1A). In blood plasma of adult and aging rats after physical training iNOS content was greater than in control by 2.8 and 4 correspondingly, and nitrate reductase content, vice versa by 4 and 2.8 times. Changes of cNOS content at the same time were insignificant (Fig. 1B).

So, during the period of adaptation to training in rats are seen significant age differences in NO synthesis in aorta and in blood plasma depending on their life duration and regeneration ability. Age-related changes in the NO-synthesis system which occur during training are summarized in the table 2.

TABLE 2. Special features of the impact of training by means of swimming on the changes (y%) in the ratio of parameters of the oxidative and the non-oxidative pathways of NO synthesis and non-oxidative arginine exchange in aorta and plasma of the adult (1) and the aging (2) rats. Sign "+" means increase and "-" means decrease in the ratio as compared to its value in the control untrained rats of the same age, which value was taken as 100%; * - considerable impact of training by means of swimming

Parameter,%	Aorta		Plasma	
	1	2	1	2
Nitratreductase/NOS ratio	+100 *	-61 *	+351 *	+527 *
iNOS share	+82 *	+12	+36	-9
Arginase/NOS ratio	-57 *	-36	-48 *	+21

As a consequence of the training there was a considerable decrease in nitrate-anion content, which is a marker of peroxynitrites formation and degradation intensity, in adult (15-fold) and in aging (2.8-fold) rats. Urea content credibly decreased only in aging rats (Fig. 2A), pointing out the signs of disadaptation of their metabolism. It should be noted that urea is a reliable marker of adaptation, characterizing intensity of arginase non-oxidative arginine metabolism, which promotes formation of a set of powerful low-molecular bioregulators (urea, polyamines), which play role of antioxidants and regulators of NO synthesis (proline and oxyproline). Besides this there are precursors for synthesis of collagen and γ-aminobutyric acid (GABA) as an effective endogenous regulator of mitochondrial pore of variable permeation, which influences processes of apoptosis, necrosis, proliferation and differentiation.

Content of the NO bioavailability marker, nitrite-anion, in aorta of adult rats after physical training increased by 1.5 fold, and in aging rats it increased almost twice, reaching even the control values of adult rats (Fig. 2A). In blood plasma of training

adult rats bioavailability of nitric oxide increased by 3.8 times, whilst in aging rats it remained unchanged. Thereby the rate of oxidative metabolism and the rate of peroxinitrite formation in those rats reliably decreased, which is pointed out the considerable decrease in nitrate-anion pools. On the contrary, training did not influence the urea content in the blood plasma of rats from both groups (Fig. 2B). Possibly, identification of the stationary pools of stable NO metabolites in the plasma compartment of the blood can an essential new criteria/marker of the training process effectivity in sportsmen, not only in swimmers, but also in runners.
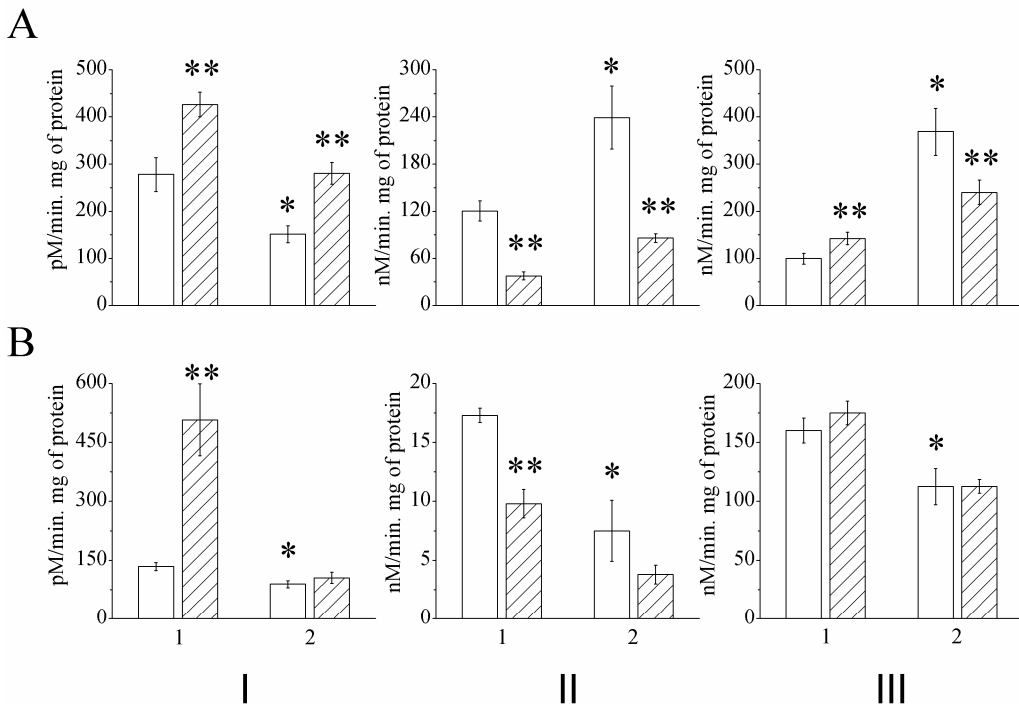
FIG. 2. Effect of training in the form of swimming on the size of the stationary pools of stable metabolites of oxidative and non-oxidative L-arginin degradation – nitrite-anion (I), nitrate-anion (II), urea (III) in aorta (**A**) and in plasma (**B**) of different age rats: 1 – adult rats; 2 – aging rats; white columns – control; hatched columns – after training; * the difference is statistically significant when comparing adult and aging rats; ** the difference is statistically significant when comparing rats before and after training

It should be noted that with age activates a non-oxidative metabolism of L-argigine. Thus, arginase activity in aging rats' aorta (as compared to adult rats) is 1.6 fold greater, and in the blood plasma it is even worth (2.3 fold greater). In the post-training period arginase activity used to decrease in aorta and blood plasma correspondingly by 2.5 and 1.4-fold in adult and by 1.2 and 1.8-fold in aging rats (Fig. 3). So, inhibitory effect of training manifested independently of the animal's age.
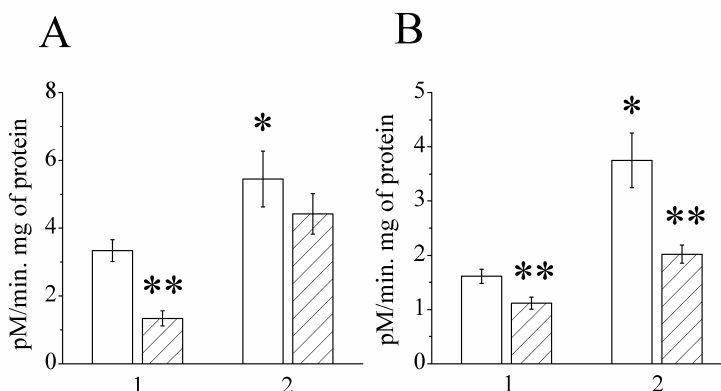
FIG. 3. Effect of training in the form of swimming on arginase activity in aorta (**A**) and in plasma (**B**) of different age rats: 1 – adult rats; 2 – aging rats; white columns – control; hatched columns – after training; * the difference is statistically significant when comparing adult and aging rats; ** the difference is statistically significant when comparing rats before and after training

Interestingly, that urea pools in aorta and blood plasma did not depend on the enzyme activity, which is clear, since the main place of its synthesis, as an end-product of protein metabolism of nitrogen is a liver, kidneys and the enterocytes of small intestine, bit not the cardiovascular system organs. Here urea is used by the cells for synthesis of the main low-molecular bioregulators – polyamines and GABA. Yet, let us get back to the changes in aorta after training urea pools, which is highly dependent on the animal's age. If training effectivity that much depends on changes in aorta not only of different pathway of NO synthesis (see Table), but also on the ration arginasa/NOS, than of a great importance not only an oxidative metabolism of arginine, but also a non-oxidative arginase metabolism of arginine (synthesis of urea, polyamines, proline, oxyproline, GABA), which is still being discussed. Our results are pointing to the important role of arginine exchange (not only oxidative, but also non-oxidative) in the adaptation of cardiovascular system to the long-lasting action of regular physical training, which assumes usage of not only exogenous nitric oxide donors and stimulators of its synthesis, but also regulators of the non-oxidative arginase metabolism, and not only its inhibitors, for regulation of adaptation process (it's intensification, acceleration, prolongation, etc).

Established were also indexes that directly influence NO synthesizing system (pools of H_2O_2 as an enhancer of eNOS expression and stimulator of its activity), or those, which can be taken as markers of oxidative activity of xanthineoxidase (ureic acid pools) (Fig. 4). Thus, H_2O_2 content in aorta and in blood plasma under the conditions of training independently of the age increased 2-3-fold, and ureic acid content, vice versa, decreased in all examined structures. So, physical training led to a drastic increase in OI in adult and to a moderate increase in OI in aging rats.

Thus, in context of adaptation to swimming NO synthesis is being potentiated, one of the main function of which is stimulation of energy production and supply of

demanded for this purpose resources (including oxygen available for mitochondrial respiration). These changes, as it turned out, are age-dependent.
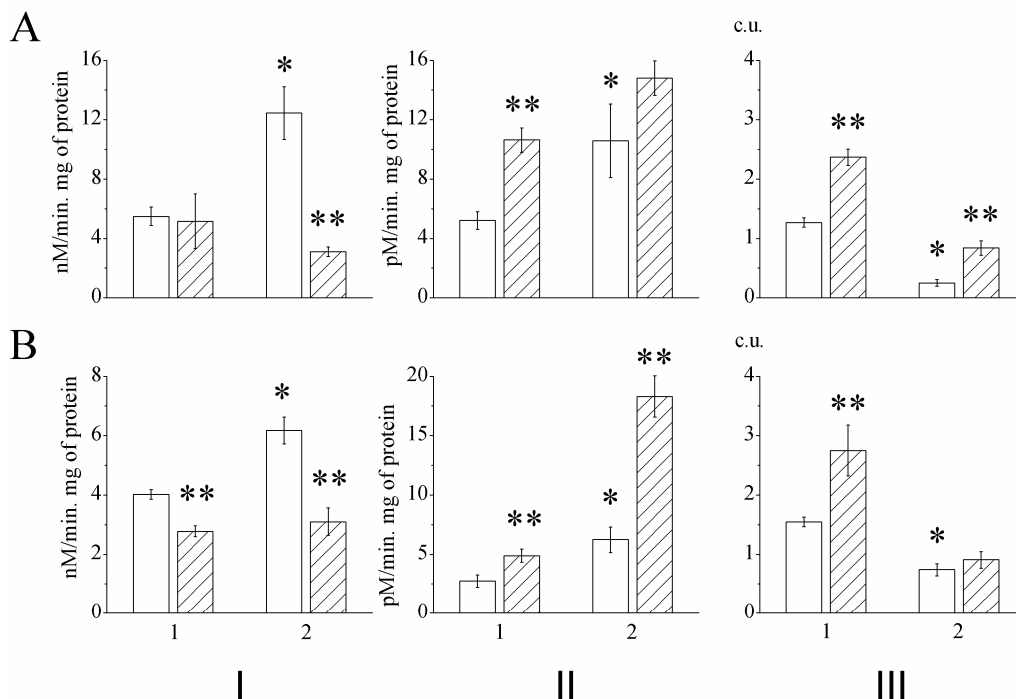
FIG. 4. Effect of training in the form of swimming on the oxidative metabolism – ureic acid (I), H_2O_2 (II), oxygenation index (III) in aorta (**A**) and in plasma (**B**) of different age rats: 1 – adult rats; 2 – aging rats; white columns – control; hatched columns – after training; * the difference is statistically significant when comparing adult and aging rats; ** the difference is statistically significant when comparing rats before and after training

III.B. Physiological Investigations

As a result of conducted investigation was established that after 6 weeks of swimming training of adult rats (n = 6) preactivated with adrenalin SM of the thoracic aorta did respond to acetylcholine application with the standard reaction – relaxation (Fig. 5). It was reproducible in all the experiments with the amplitude of 40 to 65% (in general (63 ± 8) %). In untraining control rats in response to the same agonist the mean values were (41 ± 10) %. In other words, training in the form of swimming promoted an increase in the amplitude of endothelium-dependent relaxation of thoracic aorta SM in adult rats by more than 50%.

On addition of NO donor – sodium nitroprusside (10^{-4} M, n = 7) to the buffer solution upon training in 100% cases resulted in the formation of thoracic aorta SM relaxation reaction. Its amplitude was (70 ± 9) % as compared to (86 ± 6) % in control

rats. Thus, training in the form of swimming did not significantly change the amplitude of endothelium-dependent relaxation of thoracic aorta SM in adult rats.
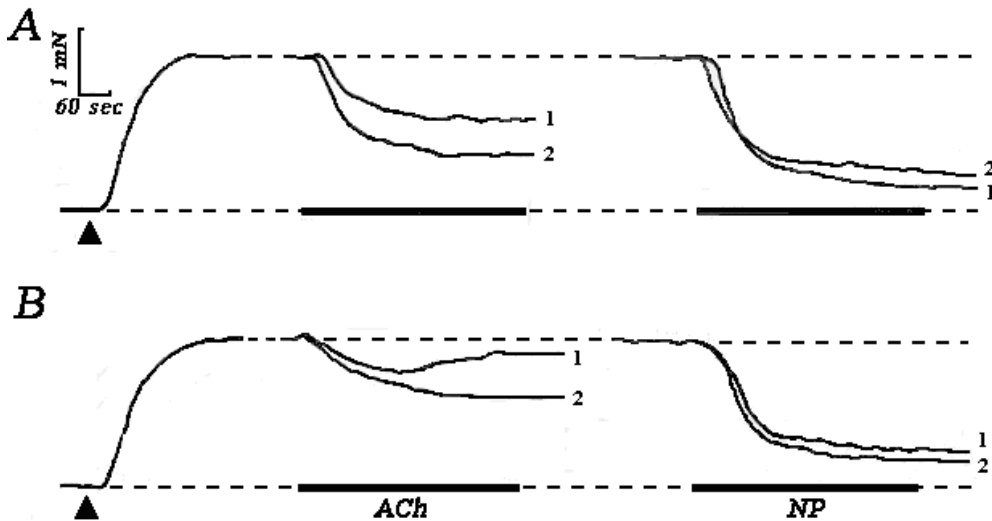
FIG. 5. Effect of training in the form of swimming in adult (12 month, **A**) and aging (24 month, **B**) on acetylcholine (Ach) -and nitroprosside (NP) -mediated contractile reactions of preactivated smooth muscles of thoratic aorta. The dark line under the curves denotes the duration of agonist's action. The dashed line denoted initial (below) and preset (above) level of tonic tension in smooth muscles. The beginning of their activation (noradrenalin, NA, 10^{-6} M) is marked by an arrow. 1 – before training, 2 – after training

Therefore, under the conditions of long-lasting training in the form of swimming in adult rats increased were only those NO-induced reactions of thoracic aorta SM relaxation, which are brought to effect through endothelium.

According to our previous results,[1] in aging rats only partially (in 27% of experiments) was observed standard reactions of thoracic aorta SM relaxation in response to acetylcholine iodide (10^{-6} M) with the amplitude of (17 ± 4) %. In majority of experiments in response to this agonist were observed reversed reactions. On the contrary, endothelium-independent reactions of thoracic aorta SM of such animals were almost unaffected and the amplitude comprised (72 ± 10) %.

Training in the form of swimming in aging rats (n = 15) revealed an interesting tendency. Videlicet, vasodilatation of preactivated thoracic aorta was detected in 40% of experiments in response to acetylcholine iodide. The amplitude of such reactions as compared to the untraining aging rats was twice as great and comprised (35 ± 3) % (Fig. 5). Nevertheless, most reactions (60%) in response to this agonist remained damaging. Endothelium-independent reactions of thoracic aorta SM relation in response to sodium nitroprusside in aging rats after training (n = 12) were observed in all the ex-

periments and their amplitude comprised (73 ± 5) %, in other words did not undergo any considerable changes.

Thus, training in the form of swimming in aging rats promotes partial recovery of the frequency and the amplitude of only the endothelium-mediated reactions of thoracic aorta SM relaxation. Such a potentiating effect of training in aging rats was even greater than in adult rats. Nevertheless, age-related endothelial dysfunction to a great extent remained.

Special attentions demands paradoxical, independent of arginase activity, reciprocal age-dependent changes in urea pools in aorta of training rats – their increase in adult and, vice versa, decrease in aging rats, which, in general, is in good agreement with the changes (considerable and insignificant improvement) in vessels reactions after training. Such correlation dependence exists between vessels' reactions and the content of stable NO metabolites – nitrite- and nitrate-anions, and also OI, which is an integral value that includes changes in pools so of oxidative (nitrite, nitrate), as non-oxidative (urea) arginine metabolism.

Analysis of obtained results suggests that training in the form of swimming in adult and aging rats stimulates so oxidative de novo synthesis of nitric oxide in aorta, as its non-oxidative synthesis in salvage pathway. Yet, the effectivity of such process is age-dependent. According to the other authors,[18,19] after training in the form of swimming in rats eNOS expression was detected in aorta and lungs and acetylcholine-induced vasorelaxation was enhanced in mesenterial unit.

IV. CONCLUSIONS

1) Found age-dependent effects of training: increase in activity of salvage NO synthesizing pathway in aorta and blood plasma, with higher effect in adult than in aging animals; considerable activation of iNOS in aorta of adult and in blood plasma of aging rats; and insignificant changes in cNOS in adult and absence of such changes in aging rats.

2) In aorta and in blood plasma under the condition of training, age-independent, were seen considerable increase in stationary pools of stable NO_2 and H_2O_2 metabolites, and a decrease in stationary pools of stable NO_3 and urea metabolites.

3) After training in the form of swimming in aorta and in blood plasma of adult and especially aging rats was seen a rapid decrease in arginase activity, which promoted L-arginine pools normalization, available for its oxidative metabolism by NO-synthases (mainly cNOS).

4) In conditions of training in adult and especially in aging rats was seen considerable increase in frequency and amplitude of just NO-and endothelium-mediated dilatory reactions of aorta SM, and those, which are conducted without endothelial participation, did not significantly change. This happens due to the increase in the ratio nitroreductase/NOS (this ration is higher in adult rats, than in aging), that is to say supersedence of salvage NO synthesis.

5) Obtained data enable to assume that physical training in the form of swimming

(preconditioning) stimulates NO production in the vessels' endothelium so in adult, as in aging animals, and therefore, can be accounted as an effective agent in non-medicament correction of the age-related endothelial dysfunction.

6) Estimation of stationary pools of stable NO metabolites in the plasma blood component can be an essential new criteria/marker of the training process effectiveness in sportsmen.

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Action of Nitrogen Oxides and Hydrogen Peroxide on Ca^{2+} Transport in Sarcoplasmic Reticulum of Permeabilized Myocytes of Utera

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ABSTRACT: Investigations were conducted on a model of digitonin-permeabilized myocytes from pig uterus using $^{45}Ca^{2+}$. We have established that in the presence of 10 mM of sodium azide, which reliably represses the energy-dependent accumulation of Ca^{2+} in mitochondria, sodium nitroprusside (10 µM – 0.1 mM), nitrite-anion (0.1 µM – 10 µM) and hydrogen peroxide (0.1 µM – 10 µM) stimulated energy-dependent introduction of Ca^{2+} in sarcoplasmic reticulum. When studying processes of passive Ca^{2+} release from this pool, which was preliminary accumulated in the ATP-dependent process, we have discovered that administration of sodium nitroprusside (10 µM), nitrite-anion (10 nM – 0.1 mM) and hydrogen peroxide (10 nM) resulted in the decline of passive Ca^{2+} release, while 0.1 mM of H$_2$O$_2$ resulted in the opposite effect. These findings allow us to assume that the tested chemicals can reduce Ca^{2+} concentration in the myoplasm, and hence, the contractile activity, acting on the level of sarcoplasmic reticulum.

KEY WORDS: nitrogen oxide, hydrogen peroxide, calcium, sarcoplasmic reticulum, utera

I. INTRODUCTION

Premature labor and pregnancy break-down are the main problems of the reproductive medicine in developed countries. Modern therapeutic methods for missbirths treating are mostly inadequate, and the processes of utera contractile function regulation are poorly studied.[10,39] The ability of nitric oxides to relax smooth muscles determines the high interest for the usage of NO donors in obstetric-gynecologic practice. Numerous studies point to the important role of nitric oxides in the mechanisms, which control the utera contractile activity. For example, they are believed to take part in the processes of the long term relaxation at the background of the decreased sensitivity to urethro-contractile agents, which is seen in pregnancies at the background of increased progesterone content in utera tissues (progesterone blockage).[16,26,28,34] Yet, No-mediated relaxation of myometrium, unlike the relaxation of smooth muscles of vessels and gastrointestinal tract, is independent of the increase in the cyclic guanosine-monophosphate (cGMP) content in the tissue. Among the alternative mechanisms of NO action foreseen is the activation of potassium channels of high conductance (HK$^+_{Ca}$) and phosphatases of the light chains of myosin.[10,16] Beside nitric oxides a prospective agent in low physiological concentrations, which relaxes myometrium can be

hydrogen peroxide.[11,38] This substance is metabolically connected with the nitric oxide, in particular, it is able to activate endothelial isoform of NO-synthase, and the latter is the source of H_2O_2 when substrate co-factors are lacking.[36] Hydrogen peroxide in physiological concentrations plays a role of hyperpolarizing factor in smooth muscles, acting directly on the concrete subtypes of calcium channels, increasing cyclic adenosine monophosphate (cAMP) content or influencing oxidative metabolism of arachidonic acid. It should be noted that H_2O_2 is able to stimulate activity of soluble guanylate-cyclase.[20,23] Thus, nitric oxide and hydrogen peroxide can reveal the unilateral activity, induce relaxation of myometrium smooth muscles.[16,38] There is luck of data concerning mechanisms of impact of physiologically considerable concentrations of H_2O_2 on the myometrium. The possibility of nitric oxide hydrogen peroxide production in utera, so in its endothelial tissue, as in myometrium was already shown.[26,34,12]

Calcium plays a unique role of a second messenger in cells and also as a trigger of contraction in smooth muscle cells (SMC). At the bottom of contraction initiation lies increase in cytosolic calcium concentration as a result of its entrance into the myoplasm from extra-and intracellular pools down the concentration gradient. Increase in the Ca^{2+} concentration in myoplasm results in intensification of Ca^{2+}-calmodulin complex formation, and further stimulation of kinases of the myosin light chains and initiation of contraction. The main mechanism of cytosolic free Ca^{2+} increase is the opening due to the peacemaker activity of voltage-gated L-type Ca^{2+} channels, which are also regulated by the intracellular second messengers.[32] When contractile activity is initiated increase in Ca^{2+} concentration in SMC appears rapidly and in good supply by its release from sarcoplasmic reticulum (SR) (calcium-induced Ca^{2+} release through rhyanodine receptor channels).[21,32] Calcium-induced Ca^{2+} release could be the possible, yet it is poorly studied, way for initiation of contractile activity in myometrium SMCs.[8,39] Decrease in cationic content is provided mainly by functioning of Mg^{2+} and Ca^{2+}-ATPases and Na^+-Ca^{2+} exchanger in the plasma membrane (PM), by Mg2+, Ca^{2+}-ATPases SR (SERCA – sarcoendoplasmic reticulum Ca^{2+}-ATPase) and by buffering.[21,24,32] It is assumed that in myometrium SMC SR directly releases Ca^{2+} to PM. This activates localized there system of calcium excretion. Ca^{2+} release from SR activates also K_{Ca} channels, which inhibits PM excitation. It is assumed that in some SMC 60 – 80% of Ca^{2+} after the increase in its concentration is being accumulated in SR[21], yet morphological studies indicate that in SMC SR reaches only 8% of the cell volume.[18] It was established that SR size in myometrium SMCs is significantly increased under treatment with estrogens and during pregnancy. In other words, SR could be more important under functional load.[30] Thus, SR is an important element of calcium signal transduction into SMCs and is an important target of substances, which control utera contractile activity. In the context of the prevalence of cGMP-independent way of nitric oxides and hydrogen peroxide influence on myometrium relaxation, we assume that SR can be a direct target of the mentioned above substances.

With regard to the insignificant size of SR in myometrium SMCs the appropriate model for studying of Ca^{2+} exchange in it would be permeabilized myocytes in which non-specific PM permeability is increased by means of different detergents, in particu-

lar digitonin.[1,7] Such experimental approaches enables to investigate transport processes in context of native morphology of intracellular membrane structures.

The aim of or work was to investigate nitric oxide and hydrogen peroxide influence on energy-dependent and passive (calcium induced and caffeine induced) Ca^{2+} transport in SR on the suspension of digitonin permeabilized rat myometrium SMC using $^{45}Ca^{2+}$.

II. METHODS

Suspension of utera SMCs from non-pregnant rats (n = 5), estrogenized 16 hours before the tissue harvesting was obtained using collagenase and trypsin soybean inhibitor.[25] The general number of cells and the number of alive cells was counted using hemocytometer (Gorjaev's chamber).

When studying influence of nitric oxides and hydrogen peroxide on energy-dependent Ca^{2+} accumulation in SR permeabilized myocytes for 5 minutes accumulated Ca^{2+} ($^{40}Ca^{2+}$ + $^{45}Ca^{2+}$) in the medium of the following content (in mM): KCl – 125, NaCl – 25, ATP – 3, $MgCl_2$ – 3, K_3PO_4 – 2, $CaCl_2$ – 0.1, NaN_3 – 10, tris-HCl – 50, pH 7.4, digitonin – 0.1 mg/ml with or without sodium nitroprusside or hydrogen peroxide in concentration mentioned below. Presence of sodium azide is conditioned by the need of inhibition of energy-dependent Ca^{2+} accumulation in mitochondria,[1,7] capacity of which is much greater than SR. Aliquots of cell preparation were taken and the chemical reaction was stopped by the fast separation of components using filter. Than the net radioactivity was counted.[1,4,7]

When studying influence of nitric oxides and hydrogen peroxide on energy-independent Ca^{2+} accumulation in SR its accumulation in the mentioned above medium without studied substances was stopped in 5 minutes by 10 μM of tapsigargin, hereafter the cell preparation was 5x diluted in the medium of the following content (in mM): KCl – 125, NaCl – 25, caffeine – 2, $CaCl_2$ – 0.1, tris-HCl – 50, pH 7.4. In the diluting medium were present sodium nitroprusside, sodium nitrite and hydrogen peroxide in the mentioned below concentrations. In 1 minute aliquots of the cell preparation were taken and reaction was stopped by the fast separation of components using filter with the further calculation of the net radioactivity.[1,4,7]

III. RESULTS

In our previous work[4] where we used the model of permeabilized with digitonin rat utera myocytes and $45Ca^{2+}$ was shown that passive Ca^{2+} transport from SR (the cation was previously accumulated in the energy-demanding process) is sensitive to 2 mM of caffeine, 0.1 mM lidocaine, and also to exogenous Ca^{2+} (was activated when Ca^{2+} concentration increased from 10^{-7} M to 10^{-4} M and decreased when it comprised 10^{-3} M) and pH (was stimulated by medium alkalization). Moreover, it was significantly inhibited by Mg^{2+} (0.2 – 4) mM. Data analysis enables to assume that our model is adequate for studying processes of passive Ca^{2+} release from SR through the channels of rhy-

anodine receptor under the influence of physiologically active substances. Possibility to reliably test energy-dependent Ca^{2+} accumulation in SR in this model was testified also in other works.[1,7]

cGMP-independent action of NO (its donors) on myometrium SMC is to a great extent connected with the decrease of intracellular Ca^{2+} concentration and relaxation, which could be explained by SERCA activation and inhibition of depot-driven calcium channels of PM. An important target could also be rhyanodine receptor, which has many sulfhydryl groups, which are sensitive to oxidants.[29] Possible also is the other way, when intensification of Ca^{2+} transport through PM or stimulation of its release from SR (sub-plasmalemmal area) result in the local increase of its concentration near the PM and also to K^+_{Ca} channels, membrane hyperpolarisation and excitation shut-off.[24] Possibility of activation of ATP-dependent Ca^{2+} transport from myometrium cells we have demonstrated only under relatively high concentrations of nitrite-anions.[2]

It was established that under 10 mM of sodium azide, which reliably inhibits energy-dependent Ca^{2+} accumulation in mitochondria[7], sodium nitroprusside ($10^{-5} – 10^{-3}$) M and nitrite-anions ($10^{-7} – 10^{-5}$) M stimulated energy-dependent influx of Ca^{2+} into SR of SMC (Fig. 1A,B). At the bottom of cGMP-independent SERCA activation by nitric oxides[5,12] lies redox-regulation of the enzyme through oxidation of functionally important cystrin-647.[40] In the presence in the medium of superoxide-anion peroxinitirte is being formed, which modificates indicated amino acid reside and it is also able to activate SERCA.[6] However, peroxinitrite is able to inhibit transport system by nitrosilation of tyrosine resides in SERCA.[19]
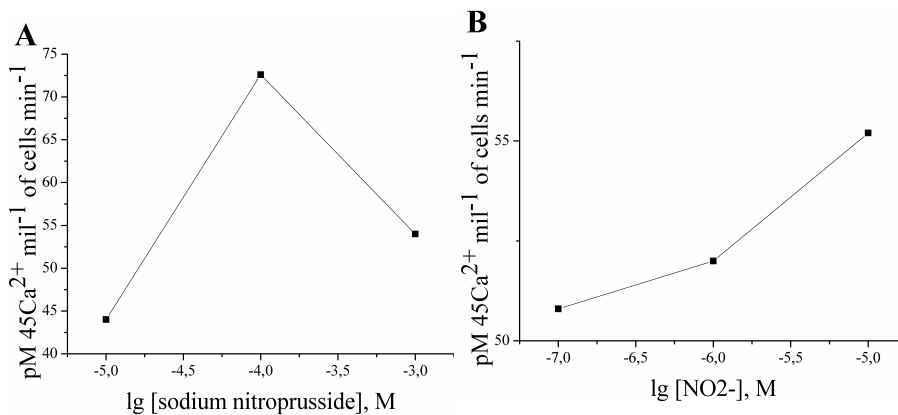
FIG. 1. Effect of sodium nitprosside (**A**) and nitrate-anions (**B**) in increasing concentration on the energy-dependent Ca^{2+} accumulation by the suspension of permeabilized rat utera myocytes at the background of 10 mM sodium azide

It was shown that sodium nitroprusside (10^{-5} M) and nitrite-anions (10^{-8} M – 10^{-4} M) result in inhibition of the passive Ca^{2+} release from SR, which was previously accumulated in the ATP-dependent process (Fig. 2A,B). The other authors have proven that nitrosilation of sulfhydyl groups of rhyanodine receptor by NO is possible in the

heart and the skeletal muscles, and the direction of the effect (activation or inhibition) highly depends on the donor concentration.[29] On the intact arterial myocytes from guinea pigs was shown that sodium nitroprusside inhibited noradrenalin-activated increase in Ca^{2+} concentration in myoplasm. Moreover, the effect was cGMP-independent and was accompanied by the spark activity.[29] In SMC from pig trachea NO inhibits Ca^{2+} release from SR through the channels of rhyanodine receptors.[17]
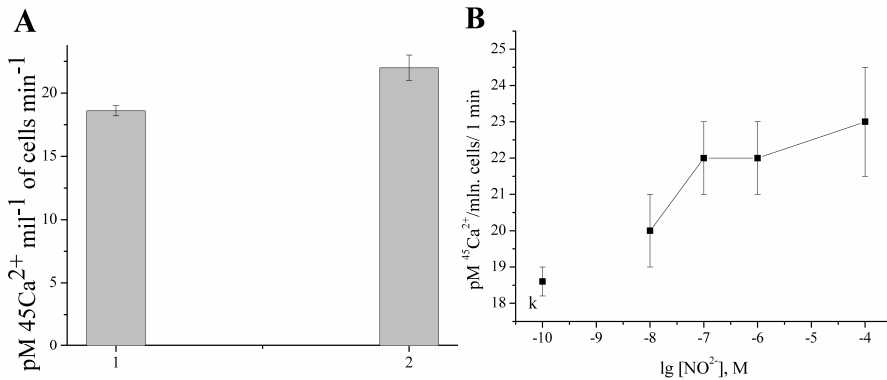
FIG. 2. Effect of 0.01 mM sodium nitroprosside (**A**) and nitrite-anions (**B**) on the passive Ca^{2+} transport from sarcoplasmic reticulum of permeabilized myocytes from rat utera. Preliminary Ca^{2+} accumulation was done in the energy-dependent process at the background of sodium azide (10 mM). Along the y axis – Ca2+ amount remained in the intracellular pools per minute of the passive release; for **A**: 1 – control, 2 – at the presence of sodium nitroprosside; for **B**: k – control (without nitrite-anions). *P<0.05 as compared with the control
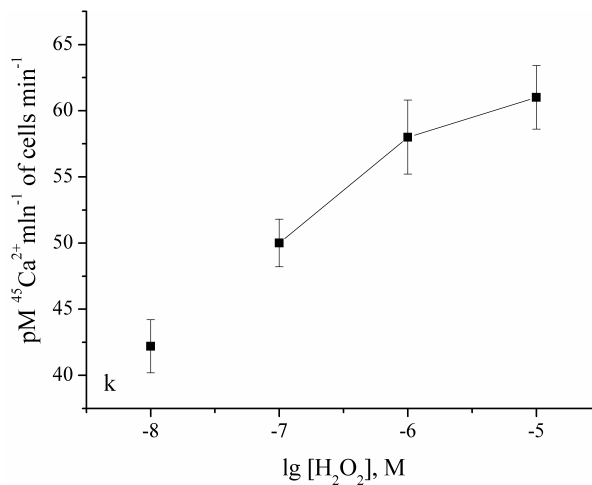
FIG. 3. Energy-dependent Ca2+ accumulation by the suspension of permeabilized myocytes at the background of 10 mM sodium prusside and hydrogen peroxide in an increasing concentration, k - control

It was also shown that hydrogen peroxide stimulates energy-dependent Ca^{2+} influx into SR (Fig. 3). In the investigations of the other authors were shown the opposite effects, namely, processing of SR vesicles with H_2O_2 resulted in the irreversible inhibition of so calciumtransporting, as ATPase activity of SERCA.[31] On the other hand, SERCA inhibition is not the cause of the contractile effect, induced by H_2O_2 (30 µM) in rat aorta.[33] In SMC from aorta of cattle H_2O_2 in micromolar range of concentrations does not induce any significant inhibition of SERCA, yet such an effect induces superoxide-anion.[35] From our point of view, H_2O_2 action considerable depends on the concentration: the low (submicromolar and used by us micromolar) can differ from the higher ones (conditions of the oxidative stress). The latter assumption is confirmed by experiments, in which was studied H_2O_2 impact on the passive Ca2+ release from permeabilized myocytes (Fig. 4). In concentration of 10-8 M hydrogen peroxide inhibited this process; meanwhile in concentration of 10-4 M the opposite effect was observed. Results, obtained by other authors point to the biased effect of H2O2 on the investigated process. Stimulation of Ca2+ release from SR in rat cardiomyocytes was observed under 100 µM of H_2O_2, meanwhile 1 µM did not exert any effect.[14] Hydrogen peroxide in concentration of 10 µM enhanced cation transport through the channels of skeletal muscle rhyanodine receptor, built in the bilipd layer. In the heart of the mentioned effects lies redox modification of channel structures.[27] At the same time even in high concentrations (1 mM) H_2O_2 decreases caffeine-induced Ca2+ from RS in cardiomyocytes from guinea pig.[15] Analogous data were obtained on the treated with saponin rat cardiomyocytes[22] skeletal fibres[9] and permeabilized SMC[37]. Inhibition of caffeine-induced artery contraction by hydrogen peroxide in rabbits is explained by authors through the increase in cGMP synthesis and increase in cyclooxygenase activity.[13]
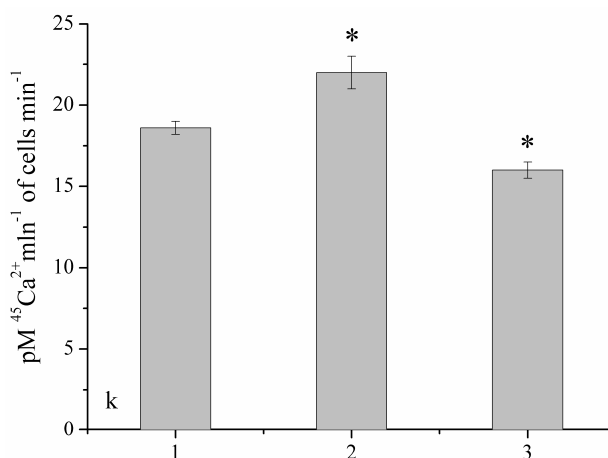
FIG. 4. Efect of H_2O_2 on the passive Ca^{2+} transport from sarcoplasmic reticulum of permeabilized myocytes from rat utera.). Along the y axis – Ca^{2+} amount remained in the intracellular pools per minute of the passive release; 1 – control, 2 – in the presence of 10 nM of hydrogen peroxide, 3 – 0.1 mM of H_2O_2, as compared with the control*P<0.05

Obtained results enable to assume that low concentrations of nitric oxide and hydrogen peroxide can lower Ca^{2+} content in SMC myoplasm, and dependent on this contractile activity of myometrium, acting on the SR level. The concrete mechanisms of action of the mentioned substances demand further investigations.

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Increased Expression of Voltage-Dependent Anion Channel and Adenine Nucleotide Translocase and the Sensitivity of Ca²⁺-Induced Mitochondrial Permeability Transition Opening Pore in Old Rat Heart

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ABSTRACT: We have investigated mRNA and protein expression of voltage-dependent anion channel (VDAC), mRNA adenine nucleotide translocase (ANT) as well as the sensitivity of the mitochondrial permeability transition pore (MPTP) to Ca²⁺ in the adult and the old rat heart. It was shown that in the old rats' hearts VDAC mRNA expression increased by 1.7 ($P < 0.05$) times and mRNA ANT expression increased 1.8 ($P < 0.05$) times in comparison with adult animals. Western Blot analysis has shown that the level of the VDAC protein expression in old rats' hearts also significantly increased as compared with adult animals. In old rats' hearts sensitivity of MPTP opening to calcium ($10^{-7} - 10^{-4}$ M) determined by mitochondria swelling increased two-fold ($P < 0.05$). Therefore, an increased VDAC and ANT expression, as the main structure-functional components of the MPTP, and an increased sensitivity of MPTP opening to Ca²⁺ caused an increase in mitochondrial membranes' permeability in aging. Each of these factors may contribute to alterations in mitochondrial barrier properties and lead to mitochondrial dysfunction.

KEY WORDS: voltage-dependent anion channel expression, adenine nucleotide translocase, mRNA expression, mitochondrial permeability transition pore, heart, aging

I. INTRODUCTION

Aging is a complicated generalized process, which is accompanied by an oxidative stress, which in turn causes in the organism such pathological processes as atherosclerosis, diabetes, neurodegenerative disorders, ischemia-reperfusion damages of the heart and aging, connected with the mitochondrial dysfunction.[14] Mitochondria are the most important organelles of the cell responsible for the oxidative phosphorylation, cell signaling and intracellular calcium homeostasis maintaining.[4] One of the manifestations of functional damages in mitochondria is the change in the barrier properties of membranes of these organelles, namely, due to formation of a non-selective calcium-dependent cyclosporine-sensitive mitochondrial pore (MP) between the outer and the inner mitochondrial membranes, which is the main mechanism in development of the cell apoptosis.[7]

The urgent metabolites exchange between mitochondria and cytoplasm goes down through the outer and the inner mitochondrial membranes and is of a great importance for the normal cell functioning. In mammal mitochondria through the outer membrane metabolites are transported by means of voltage-dependent anion channel (VDAC) [6,23], at the same time through the inner membrane committed about 50 processes of metabolites transport by means of 30 transporters, among which is adeninenucleotidetranslocase (ANT) [21]. VDAC and ANT are the main components of MP and carry out the proper functions.[8,13] It was proven that VDAC is the pore-forming protein porin with the molecular mass of 30 – 35 kDa.[25] Purified VDAC protein can form channels with the pore diameter of 2 – 3 nm and with the electrophysiological properties inherent to MP. VDAC comprise approximately 0.3% of the total mitochondrial protein, which is equivalent to 100 pM of VDAC protein per 1 mg of mitochondrial protein.[7] So, the change in the inner mitochondrial membrane permeability is regulated by different ligands, namely CA^{2+}, ATP, sodium glutamate etc, directly involving VDAC. In particular, VDAC conditions the transport through the outer mitochondrial membrane of adenine nucleotides[22], Ca^{2+}[10], citrate, succinate and phosphate[15]. Nowadays found are three isoforms of VDAC in mammals – VDAC1, VDAC2 and VDAC3, each of which can play different physiological roles.[16] VDAC interacts with ANT on the inner mitochondrial membrane and with kreatinkinase octameter in the intermembrame space.[3] ANT is the ligand for MP forming on the inner mitochondrial membrane. It is known that under physiological conditions ANT transports macroergic molecules ATP and ADP through the mitochondrial membrane.[17] In mice were uncovered two ANT isoforms (ANT1 and ANT2), while in humans – three (ANT1, ANT2 and ANT3), which are differently expressed in different tissues. It was shown that ANT1 isoform gene is expressed predominantly in skeletal muscles, heart and brain; and ANT2 isoform gene-in lungs, kidneys, spleen and liver; and ANT3 isoform gene – in all the tissue types.[9,11] There is a connection between the permeability change of mitochondrial membranes and MP sensitivity to inducers of its opening, especially at aging. Changes in the rates of expression of the main components of MP – VDAC and ANT – can condition changes in mitochondrial membranes permeability.

The aim of our work was to investigate expression levels of mRNA and VDAC protein, ANT mRNA, and also to study MP sensitivity to calcium in the hearts of old and aging rats.

II. METHODS

Experiments were conducted on male Wistar rats 6 (n = 6) or 24 (n = 6) month old, which were kept on the standard diet of vivarium of the Bogomoletz Institute of Physiology in compliance of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 1986).

II.A. RNA Extraction. Reverse Transcription and Polymerase Chain Reaction

RNA was extracted from the heart tissues using the kit "Trizol RNA-rep" ("Isogen",

Russia). The reverse transcription was conducted using the kit "First standard cDNA Synthesis Kit" ("Fermentas", Lithuania), using 2 – 2.5 μg of the total RNA and the Random hexamer primer. Obtained in the result of the reverse transcription DNA (cDNA) was used for polymerase chain reaction (PCR) for amplification of the fragment of the VDAC and ANT gene and glyceraldehyde-3-phosphatedehyderogenase (GAPDH) as the endogenous control. For the quantitive analysis of gene expression were used primers with the following nucleotide sequence:

direct – 5'CATATCAACCTGGGCTGTG-3',
reverse – 5'-TTGGCTGCTATTCCAAAGC-3' for VDAC;
direct – 5'-TTCCCCACCCAAGCTCTCAACT-3',
reverse – dd5'-CGGCTGTCACACTCTGGGCAATCA-3' for ANT;
direct – 5'-GGGTGTGAACCACGAGAAAATATGA-3',
reverse – 5'-AGCACCAGTGGATGCAGGGGATGAT-3' for GAPDH.

Amplification mixture for PCR in the total volume of 25 μL contained 5 μL of cDNA solution, 5 μL of 5x PCR-buffer, 0.2 mM of the aqueous mixture of the four nucleotides, 0.5 units of Taq-polymerase ("AmpliSens", Russia), 30 pM of each of the primers ("Metabion", Germany) and deionized water. Fragments amplification consisted of 37 cycles: denaturation at 94°C (50 sec), primers hybridization at 72°C (1 min). PCR was conducted in the thermocycler "GeneAMPSystem 2700" ("Applied Biosystems", USA). Obtained amplificates were separated in 1.5% agarose gel, which contained ethydium bromide. Visualization and estimation of the amplificates' brightness after horizontal electrophoreses (170 V for 30 min) was conducted using transilluminator and ViTram software ("Biokom", Russia).

II.B. Estimation of VDAC Protein Expression Level

The level of the VDAC protein expression was estimated be means of Western-Blott-analysis. Gel-electrophoresis of the protein suspension from the heart mitochondria was conducted in 12% solution of polyacrylamide gel at the background of sodium dodecyl sulphate using Laemmli method in the Hoefer miniVe chamber ("Amersham", England). For the proteins separation was used electrode buffer of the following content (in mM): trisHCl – 25, glycerine – 192, sodium dodecyl sulphate solution – 0.1%; pH 8.3. Previously the samples were boiled for 3 minutes in the buffer with β mercaptoethanol. The total protein content was estimated using Lowry method.[19] The protein in the amount of 100 μg was placed into the cavity for electrophoresis. After the electrophoretic separation proteins were put onto the PVDF-membrane ("Sigma", USA) by means of semidry transfer Hoefer miniVE Blot Module ("Amersham", England). For this was used a buffer of the following content (in mM) : trisHCl – 25, glycerine – 192, sodium dodecyl sulphate solution – 0.1%, methanol solution-20%; pH 8.3. After the protein transfer the membrane was blocked with the 5% solution of the dried fet-free milk for 18 – 20 hour at 4°C and treated with the primary monoclonal antibodies to VDAC ("Sigma", USA) in dilution 1 : 1000 for 2 hour at 20°C. Afterwards the membrane was washed out in the twin-phosphate buffer (PBS-T) and incubated with the secondary anti-rabbit immunoglobulins G, conjugated with the horseradish peroxi-

dase ("Sigma", USA) in dilution 1 : 2000 in the PBS-T buffer for 1 hour at 20°C. For the visual estimation by means of staining of the proteins transferred from the gel onto the membrane was used substrate-staining for peroxidase 3-amino-9-ethycarbasol. The quantitive analysis of the obtained immunoblots was conducted by means of their scanning and processing using GelPro software.

Mitochondria were extracted using differential centrifuging method.[1] Experiments on MP opening were conducted using spectrophotometric registration of swelling of mitochondria from rat hearts under the background of Ca^{2+} in the incubating medium.[1]

Obtained data were treated with the variance analysis using Excel (MS Office XP) and Origin 6.0 (Microcall Inc., USA)

III. RESULTS AND DISCUSSION

Changes in expression of VDAC and ANT mRNA in the hearts of adult and aging rats are shown on the Fig. 1. It was shown that in the hearts of aging rats the rate of VDAC mRNA expression is increased by 1.7 times, and ANT mRNA – by 1.8 times as compared with the adult animals (Fig. 1A,B).
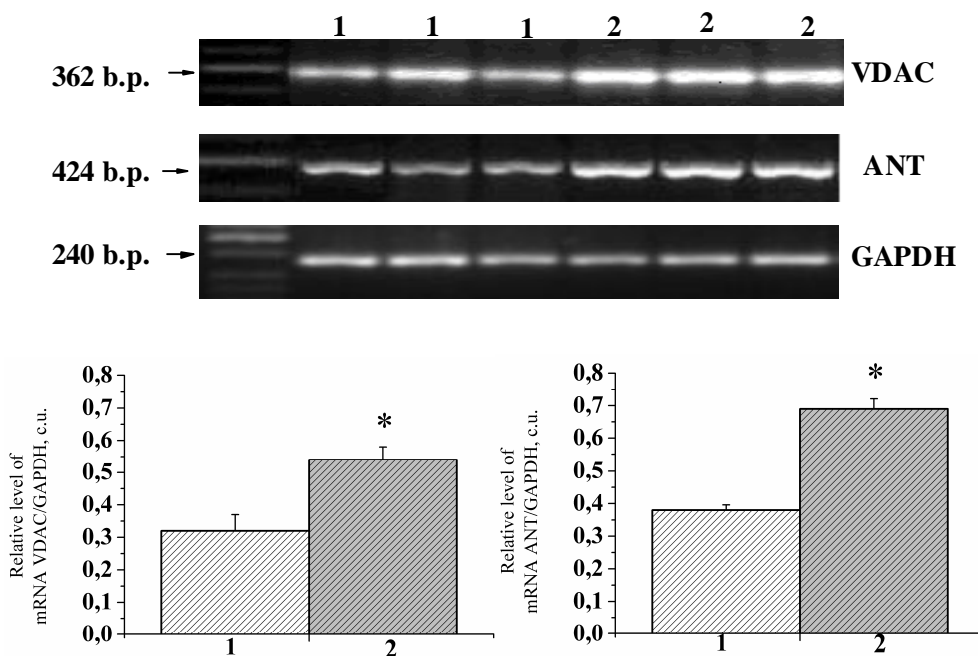
FIG. 1. Expression of the gene of voltage-dependent anion channel (VDAC) (**A**) and adenine nucleotide translocase (ANT) (**B**) in the heart tissues of adult (1) and aging (2) rats (n = 6). *P < 0.05 as compared with the adult rats; glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as the endogenous control; **C** – an example of the electrogramm photo, obtained during polymerase chain reaction; n.p. – nucleotide pair

Thus, according to the obtained results aging is accompanied by a considerable increase in VDAC and ANT mRNAs expression in rats' hearts. Using Western-Blott-analysis we have shown that the VDAC protein expression rate in the hearts of aging rats is also increase as compared with the adult rats (Fig. 2).
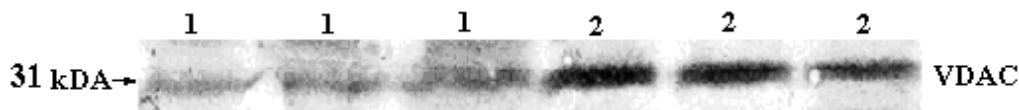
FIG. 2. Expression of the voltage-dependent anion channel (VDAC) protein in heart tissues of adult (1) and aged (2) rats (n = 6)

It is known that there is a connection between VDAC and the proteins of Bcl-2 family. previously in our experiments by means of PCR was shown an increase in mRNA expression of proapoptotic Bax in the hearts of aging rats as compared with the adult ones.[2] By means of the other methods (Western-Blott-analysis and immunohys-tochemistry) was shown an increase in the Bax expression level at aging.[5] The estab-lished by us increase in the expression level of VDAC mRNA, which interacts with Bax, is in a good agreement with the results of estimation of the increased initial level of bax mRNA expression in old rats' hearts.[2] When studying cell lines with mutated mitochondrial DNA, were also estimated increased levels of mitochondrial VDAC expression. The latter and also established by means of confocal microscopy VDAC/Bax co-localization provided an increase in the mitochondrial membrane per-meability, which resulted in cell apoptosis.[27] Moreover, in transformed tumor cells levels of VDAC expression are much higher than in normal cells. Authors conclude that mitochondrial VDAC can play the role of pharmacological targets in respect of anticanerogenic substances.[24]

When studying concentration calcium-dependent ($10^{-7} - 10^{-4}$) M mitochondria swelling we have established an increased MP sensitivity to the inducer of its opening in the hearts of aging rats as compared to the adult rats (Fig. 3) on the basis of the fact that Ca^{2+} in the lowest concentration 10^{-7} M induces more significant mitochondria swelling in the hearts of aging rats that in the hearts of adult rats, and such difference comprises 5%. It should be noted that in the absence of Ca^{2+} was seen an insignificant mitochondria swelling in aging rats' hearts as compared with the adult rat hearts. This fact could point to the increase in mitochondrial membrane permeability with age.

One of the main targets in apoptosis development, in which involved proapop-totic Bax and BAK, is VDAC, which interacts with these proteins, which in turn re-sults in the increase of mitochondrial membrane permeability at aging. Ca^{2+} also causes increase in mitochondrial membrane permeability, yet independently of Bax and BAK. Prevention of permeability changes in mitochondria induced whether by Bax and BAK or by Ca^{2+}, is regulated by antiapoptotic proteins of Bcl-2 family. These proteins reveal a broad spectrum of action from apoptosis inhibition to its induction.

Thus, bax – one of the members of this family – can change VDAC physical properties in such way, that it becomes "open" for release of cytochrome c from mitochondria to cytplasm, and Bcl-2 can maintain VDAC in the "closed state".
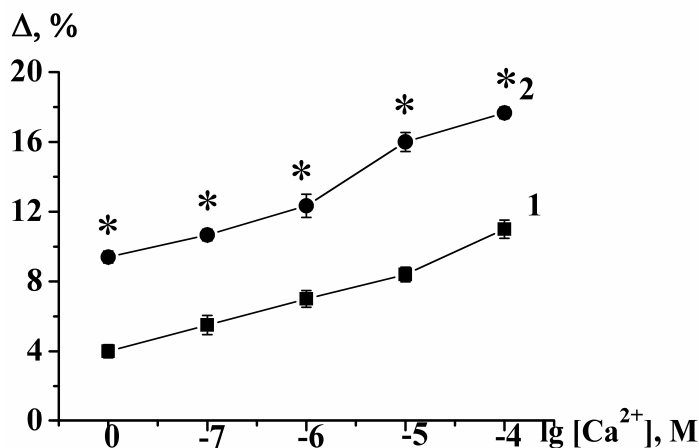
FIG. 3. Concentration dependence of the difference in mitochondria swelling under the presence of calcium in adult (1) and aging (2) rat hearts (n = 5); (Δ – the difference between the mitochondria swelling parameter on the 15th minute of their swelling and the parameter of the initial value of the optical density of this suspension on the 1st minute); *P < 0.05 as compared with the adult rats

Activation of mechanisms of the one or the other forms the cell death can be determined by the number of the open MP. Thus, under the conditions of MP forming in several mitochondria in the cell activates autophagy, when there are more opened MP in mitochondria apoptosis is being initiated, which is probably the consequence of the increased amount of cytochrome c and apoptosis-inducing factor.[12] Thus, in case of a great number of opened MP possible is apoptosis initiation on the contrary to the fact that the minimal number of opened MP is in principle does not influence the process of the cell death.[26]

Our results enable to assume that increased levels of VDAC and ANT expression are connected with increased formation of MP in organelles membranes in the hearts of aging rats, which in turn can lead to the increased permeability of mitochondrial membranes and to the increased sensitivity of MP to Ca^{2+}, which causes mitochondria swelling and as a result – release of apoptotic factors and apoptosis development (Fig. 4). There is an assumption according to which there is a possible dependence between the number of the formed MP and Ca^{2+} concentration in the cytoplasm, which is crucial for regulation of mitochondrial membrane permeability.[20]

So, an increase in levels of expression of VDAC and ANT as the main structure-functional components of MP and the increased sensitivity of MP to Ca^{2+}, induced by increased permeability of mitochondrial membranes in the aging rats' hearts, can be

one of the pathways of mitochondrial barrier functions distortions and can induce cell death as a result of mitochondrial dysfunction.
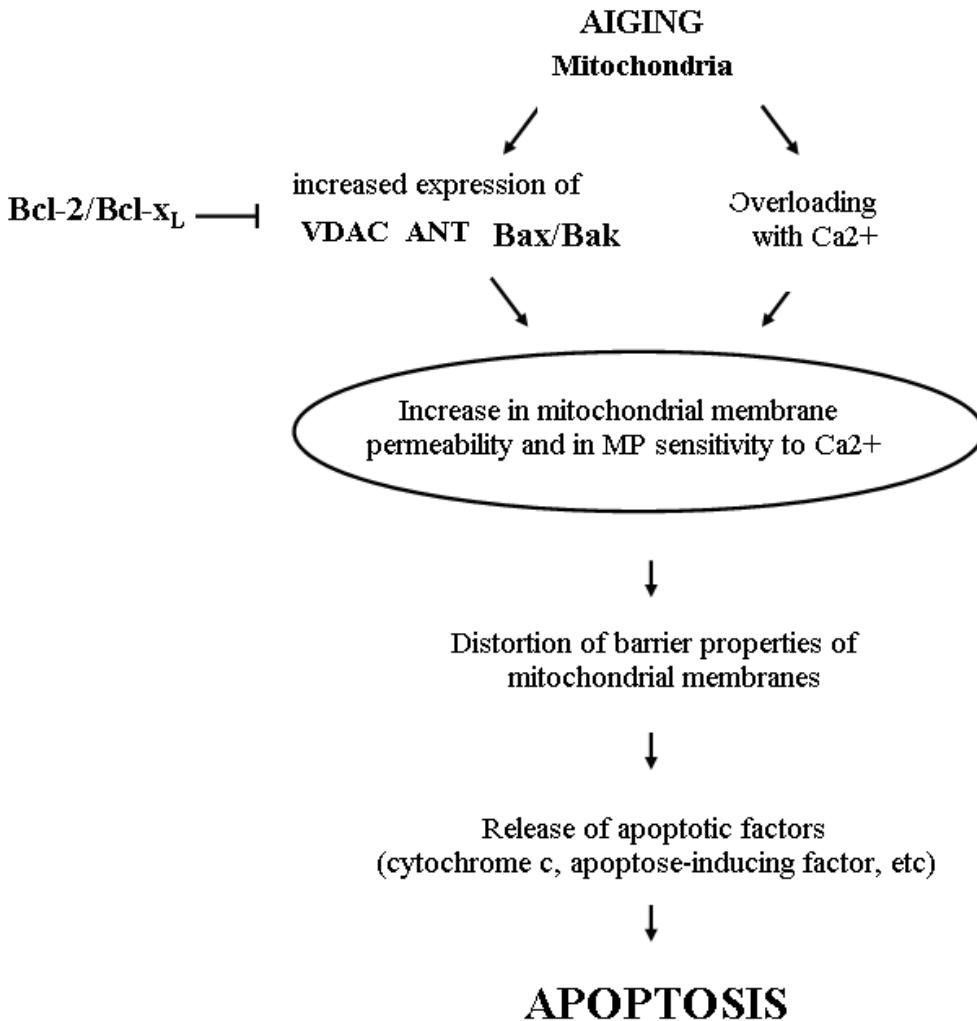
FIG. 4. Diagrammatical representation of the processes, which condition increased sensitivity of mitochondrial pore (MP) to calcium and apoptosis at aging; VDAC – voltage-dependent anion channel; ANT adenine nucleotide translocase

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Influence of Intermittent Hypoxic Training on Hemodynamic Effects of NOS-1 Activation in Medullary Cardiovascular Neurons in Rats

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ABSTRACT: In our experiments, male Wistar rats, weighing 300 ± 40 g, were exposed to intermittent hypoxia in a special chamber by its ventilation with hypoxic mixture containing 12% O_2 in N_2 5 times a day for 15 minutes in every 15 minutes for 10 days. After completing intermittent hypoxic training, in acute experiments on anesthetized with urethane (1700 mg/kg) rats, we studied changes in the SAP induced by a modulation of neuronal NO-synthase (NOS-1) activity in the neurons of the medullary cardiovascular nuclei (nucleus of tractus solitarius, NTS; nucleus ambiguous, AMB, lateral reticular nucleus, (LRN). In control rats housed in normoxic conditions, NOS-1 activation with injections of L-arginine (5.8 – 58 nM) into the medullary nuclei involved in the cardiovascular control induced the SAP lowering in most experiments in a dose-dependent manner. In rats submitted to intermittent hypoxic training, NOS-1 activation with injections of L-arginine (5.8 – 58 nM) into the medullary nuclei under study resulted in hypotensive responses which were more expressed as compared with those responses induced by its injections into the medullary nuclei in rats under normoxia. The data obtained give evidence for some additional activation of neuronal NO-synthase in the neurons of the medullary nuclei following intermittent hypoxic training. Effects of NOS-1 activation were comparable in all the tested nuclei. An effect of NOS-1 activation was quite short-lasting; it was the most pronounced on the first day after completing hypoxic training. In three days after hypoxic training, injections of L-arginine into tested medullary nuclei resulted in the SAP drop that was similar to that in control animals housed in normoxic conditions. On the contrary, inhibition of NOS-1 in the neurons induced by injections of NOS-1-antagonist L-NNA (23 nM) into the medullary cardiovascular nuclei resulted in a comparable increase in the SAP in both control and hypoxically trained rats. Effects of L-arginine injections into the medullary nuclei were blocked by preliminary administrating of a specific NOS-1 inhibitor 7-nitroindazol. On the first day after intermittent hypoxic training we observed the SAP elevation. Although it was statistically insignificant, and we observed the elevated SAP just after completing hypoxic training, these data provide evidence to support the concept that rats submitted to intermittent hypoxia exhibit an increase in sympathetic activity. The SAP elevation might be also induced by the stressor effect of keeping rats in a chamber to provide hypoxic training. There is an impression that intermittent hypoxic training might lead to two simultaneous but opposite directed results: an activation of sympathetic nervous system and an activation of NOS-1 in the medullary neurons.

KEY WORDS: intermittent hypoxic training, neuronal NO-synthase, medullary cardiovascular neurons

I. INTRODUCTION

It is known that nitric oxide (NO) fulfills in the organism the most important signaling functions, revealing a broad spectrum of biological activity. Its participation in the nervous control of the blood circulation is mainly realized through the neuronal systems of dorsomedial and ventrolateral medulla.[10,17,25,26,29] In the medulla of different species NO-synthesizing neurons, involved in the nervous control of blood circulation[30] were identified and even mapped. In nervous cells NO is synthesized through oxidation of the guanidine terminal group of L-arginine. This process is mainly catalyzed by neuronal NO-synthase (nNOS or NOS-1). For this reason argumentative is the assumption that hypoxia influences NOS-1 expression in the cardiovascular neurons and, thus, NO production. There are some data indicating that NO is of great importance for adaptive mechanisms of intermittent hypoxia, among which there is an activation of NO synthesis and limitation of its excessive production.[20,21]

Mechanisms of cardiovascular system adaptation to the intermittent hypoxia have being widely studied.[11,21,28] It is known that the duration, frequency and severity of hypoxia are those critical factors, which determine the direction of its influence on the cardiovascular system – from protective (anti-stressful, antihypertensive) to damaging (lung and systemic hypertension, myocardial infarction, insult). Adaptation to the intermittent hypoxia is thought to be dealt with the cardiovascular protection against the harder and more enduring hypoxia, as well as against other types of stress.[8,32] It was shown that intermittent hypoxic training (IHT) improves metabolic processes in both cardiovascular system and central nervous system resulted from an increase in a number of mitochondria in the brain and in the liver, activation of the respiratory chain 1 and an improvement of the oxidative phosphorylation effectivity.[4,15,18] Analysis of the published data indicates that in the most cases studies of IHT effects on the the cardiovascular system dealt with its peripheral components. In particular, it was shown that adaptation to the intermittent hypoxia activates stress-limiting system of prostaglandins in the heart in particular and in the organism in the whole. Thus, such adaptation can be essential for cardiovascular protection via stress limitation and prevention of stress damages,[3] leading to an increase in the calcium pump activity in the sarcoplasmic reticulum membranes[1]. NO production increased as a result of a chronic hypoxia makes a deposit into the systemic vasoconstriction relief.[24] Protective influence of adaptation to the intermittent hypoxia is widely used to treat and prevent a lot of diseases, and to increase training effectiveness. Role of IHT in the nervous cardiovascular control by medullary NO-synthesizing neurons has not been elucidated yet. Therefore the goal of our work was to study IHT impact on the effects of NOS-1 modulation in the cardiovascular neurons of rat medulla.

II. METHODS

Experiments were carried out on 12 month' Wistar rats, whose weight comprised (300 ± 40) g. Animals were kept in the vivarium and were regularly supplied with fresh

bedding, food and water. The experiments were conducted in accordance with the Guide for Care and Use of Laboratory animals and national bioethics legislation. The control group was formed by the rats (n = 10), which were breathing the room air. The experimental group was formed by the rats (n = 10), which were exposed to IHT in the 10 L glass chamber at the normobaric conditions. The chamber was ventilated with the hypoxic mixture (12% CO_2 in N_2) 5 times for 15 minutes with 15-minute interval everyday during the 10 days. After IHT had been completed, acute experiments on the anesthetized with urethane (1.7 g/kg intraperitoneally) rats were conducted. In those experiments we studied effects of NOS-1 modulation in the medullary neurons involved in the nervous control of the vascular tone and the heart activity. Simultaneously 5 rats were placed in the chamber. The experiments were conducted within 3 days after completing the hypoxic training. An arterial cannula was inserted into the carotid artery to measure the systemic arterial pressure (SAP) and record it using strain gauge of a hemodynamic setup. The cardiac rate was calculated from the pulse arterial pressure. After fixing the animal's head in the stereotaxis frame the dorsal surface of the medulla was exposed, and test agents were injected into the medullary nuclei (nucleus of tractus solitarius, NTS; mucleus ambiguus, AMB; lateral reticular nucleus, LRN) according to the stereotaxis atlas.[23] The substrate for endogenous NO synthesis aminoacid L-arginine (5.8 nmol and 58 nmol) and NOS-1 antagonist L-N^{G}nitro-L-arginine (L-NNA, 23 nmol) were injected into the medullary structures. Specific antagonist of NOS-1 7-nitroindasol (30 mg/kg) was administrated intraperitoneally 30 minutes before the experiment started. The SAP changes were recorded starting from the moment of an agent injections into the medullary nuclei till the SAP completely restored.

Statistical analysis of the obtained data was performed using Student's t -test and the standard computer program. Values P < 0.05 were considered to be statistically significant.

III. RESULTS

III.A. Impact of L-Arginine Injection into the Medullary Nuclei of the Control Rats on the SAT Level

In control rats, which were under normoxic conditions, NOS-1 activation by unilateral injections of the substrate for endogenous NO synthesis L-arginine in the population of the neurons within the nuclei under study resulted in a dose-dependent decrease in the SAP. It should be noted that in some cases (30%) hypertensive responses on L-arginine injections into the medullary nuclei were observed, and their magnitude aso depended on L-arginine dose. Here, we analyzed only hypotensive responses, caused by NOS-1 activation. Following injections of 5.8 nmol of L-arginine into NTS the SAP level decreased by 27.2% (from 101 mmHg ± 2 mmHg to 74 mmHg ± 4 mmHg; P < 0.05), into AMB by 23.8% (from 98 mmHg ± 3 mmHg; P < 0.05), and into LRN – by 18.8% (P < 0.05). Injections of 58 nmol of L-arginine into the medullary nuclei resulted in more significant decrease in the SAP. After injections into NTS the SAP decreased by 41.4%, on average (P < 0.01, n = 10); and its injections in AMB and

LNR induced the SAP lowering by 35.2% (P < 0.01, n = 10) and 32.8% (P < 0.01, n = 8) correspondingly. The peculiarity of hypotensive responses was relatively short latencies (of about 5 sec), the maximum response was observed within 20 – 40 sec, on average and the initial level of the SAP restored within 3 – 6 minutes.
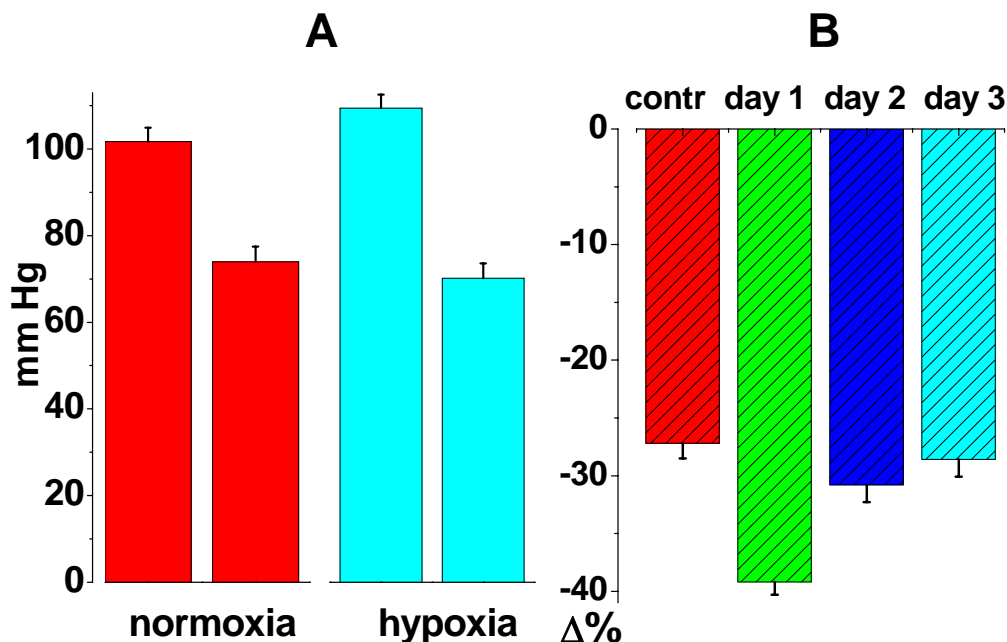
FIG. 1. Intermittent hypoxic training influences the effects of NOS-1 activation in the nucleus of tractus solitarius (NTS) of rats. **A** – changes in the the systemic arterial pressure (SAP, mmHg) induced by injections of 5.8 nmol L-arginine into NTS of the rats under normoxia and on the first day after hypoxic training. **B** – relative changes in the systemic arterial pressure, on L-arginine (5.8 nmol) injections into NTS under normoxia (control), and on the first, second and third day after the hypoxic training had be completed

III.B. IHT Impact on the Effect of NOS-1 Activation in Medullar Cardiovascular Neurons

Effects of NOS-1 activation in cardiovascular nuclei of the dorsomedial medulla were studied within 3 days after IHT had been completed. On the first day, L-arginine injection (5.8 nmol) into NTS resulted in the SAP decrease by 35.8%, on average (from 109 mmHg ± 4 mmHg to 70 mmHg ± 5 mmHg; P < 0.05) as compared to its initial level. If we compare a decrease in the SAP induced by NOS-1 activation in neurons of this nucleus in adapted to the intermittent hypoxia rats (35.8%) and in rats, which were kept in normoxic conditions (27.2%), it comes evident that on the first day after IHT completion hypotensive responses in rats were by 8% more pronounced, on average (Fig. 1A). So, IHT in our experiments must have moderately increased NOS -1 activity in the neurons

of this nucleus. We have noticed that IHT promoted an increase in the initial level of SAP (by 7.6%, on average) just after IHT had been completed (the first day), it reached 109 ± 4 mmHg, on average, and under normoxia it was 102 ± 2 mmHg. In two days L-arginine injection in this nucleus induced 30.8% decrease in the SAP ($P < 0.05$) as compared to its initial level. In other words, although the effect of the intermittent hypoxia decreased on the second day as compared to the first one (35.8%), it still remained significant as compared to the control (27.2%). In three days injections of L-arginine in this nucleus resulted in the SAP lowering, by 28.6% ($P < 0.05$, Fig. 1B), which was similar to that under normoxia. The similarity in the SAP changes was observed on L-arginine injections into all the medullary nuclei under study.
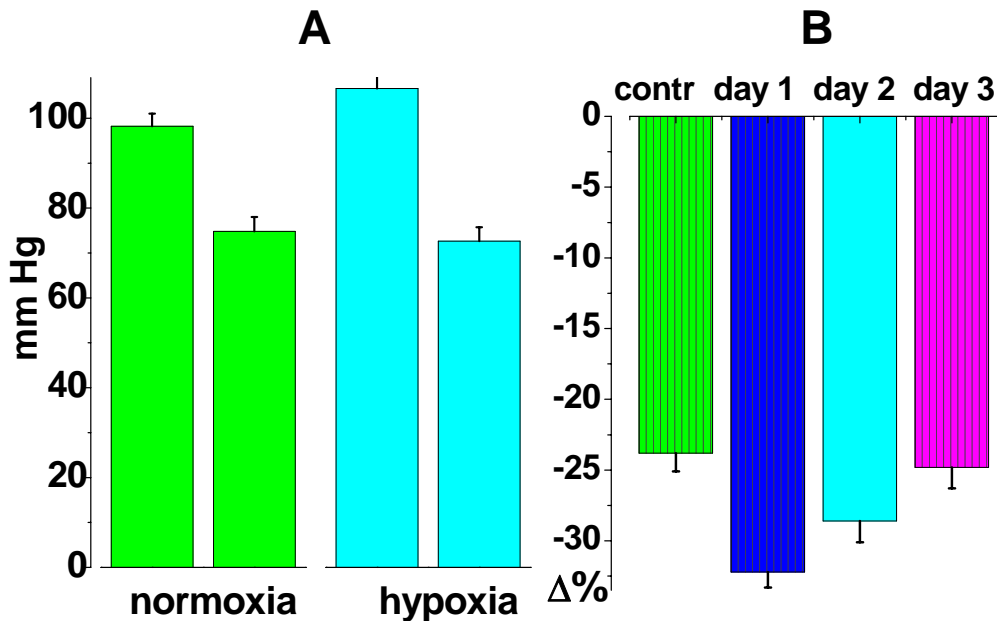
FIG. 2. Effect of intermittent hypoxic training on the systemic arterial pressure shifts following L-arginine injections into the mucleus ambiguus (AMB) of rats. **A** – changes in the the systemic arterial pressure (SAP, mmHg) induced by injections of 5.8 nmol L-arginine into n.ambiguus, AMB) of the rats under normoxia and on the first day after hypoxic training. **B** – relative changes in the systemic arterial pressure, on L-arginine (5.8 nmol) injections into AMB under normoxia (control), and on the first, second and third day after the hypoxic training had be completed

On the first day after IHT had been completed, L-arginine injections into AMB resulted in the SAP drop from 107 mmHg ± 3 mmHg to 73 mmHg ± 3 mmHg (by 31.9%; $P < 0.05$; Fig. 2A). At the same time in control rats the SAP decreased from 98 mmHg ± 3 mmHg to 75 ± 3 mmHg, which comprised 23.8%, ($P < 0.05$). Comparative analysis of the effects of NOS-1 activation indicates that in hypoxically trained rats hypotensive responses induced by L-arginine injections in this nucleus were more ex-

pressed (by 8.1%, on average), and the initial SAP level elevated by 8.6%, on average, against that in the control animals. In two days, the training effect of intermittent hypoxia powered down: on the first day the SAP decrease comprised 31.9%, on average, in two days after the amino acid injection, it comprised 28.6% (P < 0.05). In three days, a decrease in the SAP resulted from NOS-1 activation comprised 24.8% (P < 0.05), on average, and hypotensive responses did not differ much from those in control animals (23.8%, Fig. 2B).
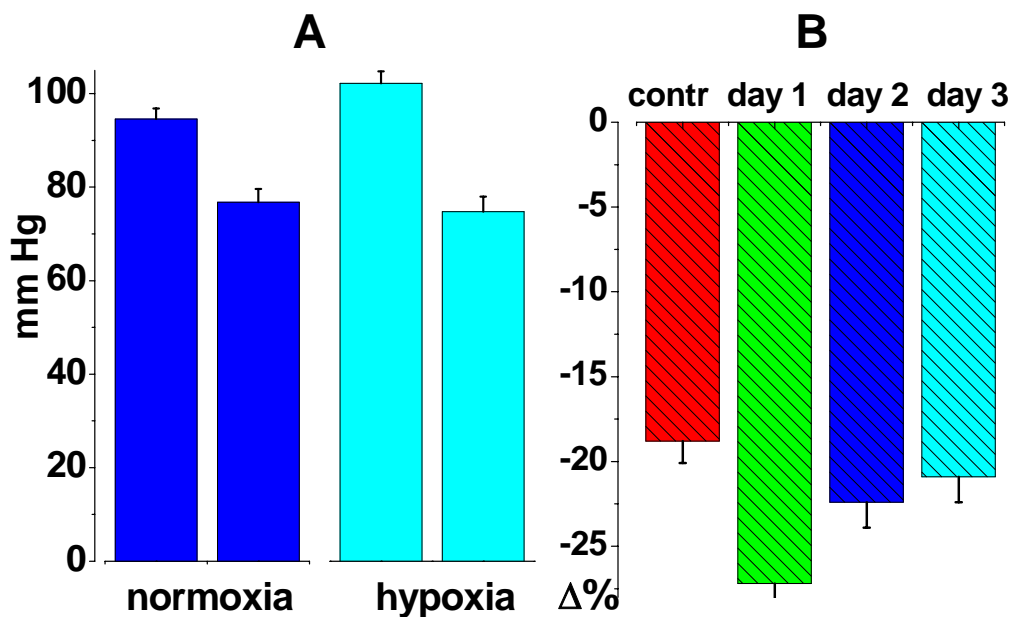
FIG. 3. Intermittent hypoxic training influences the effects of NOS-1 activation in thelateral reticular nucleus (LRN) of rats. **A** – changes in the the systemic arterial pressure (SAP, mmHg) induced by injections of 5.8 nmol L-arginine into LRN of the rats under normoxia and on the first day after hypoxic training. **B** – relative changes in the systemic arterial pressure, on L-arginine (5.8 nmol) injections into LRN under normoxia (control), and on the first, second and third day after the hypoxic training had be completed

L-arginine injections into the lateral reticular nucleus (LRN) of hypoxically trained rats on the first day after IHT completion resulted in the SAP lowering from 102 mmHg ± 3 mmHg to 75 mmHg ± 3 mmHg (26.8%; P < 0.05). The SAP decrease was 8%, on average, greater than that in control rats (18.8%; Fig. 3A). In in control rats the SAP decreased from 95 mmHg ± 2 mmHg to 77 mmHg ± 3 mmHg (P < 0.05). In other words, IHT, on the one hand, promoted an increase in the initial level of SAT, and on the other hand, it enhanced the effect of these amino acid injections in this nucleus (Fig. 3A). An additional NOS-1 activation in the neurons in all the medullary nuclei under study, by IHT appeared to be of a short-term. It powered down on the second day after completion of the hypoxic training, and it almost diminished on the

third day (Fig. 3B). On the third day after IHT completion, L-arginine injections in this nucleus like in other nuclei did not differ much from those in control rats.

In a similar way, injections of 58 nmol of L-arginine into the medullary nuclei under study induced changes in the SAP, but more expressed as compared to those when injecting smaller amount of the amino acid. On the first day after IHT had been completed, an activation of endogenous NOS-1 synthesis induced the SAP decrease by 49.2%, on average ($P < 0.01$) when injecting L-arginine into NTS; by 39.2% ($P < 0.01$) when injecting L-arginine into AMB, and by 38.4% ($P < 0.01$) when injecting L-arginine into LRN. In three days, effect of L-arginine injections into the medullary nuclei was not statistically different from the control values.

We haven't observed any considerable changes in the cardiac rate after L-arginine administration into the medullary nuclei, which was caused by the specificity of the experimental procedure: in our study, in most cases left-side injections were made. In the previous experiments we analyzed the structure of the hemodynamic response to different agents injected in populations of the neurons in the medulla,[5,6,27] and we showed that there is laterality of sympathetic innervations to the heart and vessels. The SAP is known as an integrative parameter of the cardiovascular system, and the neurons responsible for chronotropic responses of the heart seem to be localized mostly within the right half of the medulla while those responsible for vasomotor control are within the left side of it. The left-side injections of inhibitory agents into the medulla induced mainly the SAP drop which seemed to result mostly from a decrease in the peripheral vascular resistance without any considerable changes in the heart component of the response. At the same time the right-side injections resulted in a decrease in the SAP, which was mainly due to the decrease in cardiac rate. In some cases when carrying out a right-side L-arginine injections we observed moderate decrease in cardiac rate.

The data obtained demonstrate that IHT in the chosen regime promoted moderate NOS-1 activation, which is witnessed by increased manifestation of hypotensive responses induced by L-arginine injections into the medulla as compared to those in control rats. Such IHT effect appeared to be of a short duration: the maximum enhancement of the SAP drop was observed only on the first day. In the following days effect of hypoxic training was powered down, and the size of a hypotensive response on L-arginine injections into all medullary nuclei under study in three days was almost similar to that in the control rats.

III.C. Impact of IHT on the Effect of NOS-1 Inhibition in the Medullary Cardiovascular Neurons

In normoxia, injections of NOS-1 inhibitor L-NNA in all the medullary nuclei under study were in most experiments accompanied by an increase in the SAP., After this inhibitor injection into NTS the SAP increased by 25.2% ($P < 0.05$); into AMB – by 25% ($P < 0.05$) and into LRN – by 24.1% ($P < 0.05$; Fig. 4A). Injections of L-arginine into the medullary nuclei after the preliminary injection of a specific NOS-1 antagonist 7-nitroindasol were non-effective.
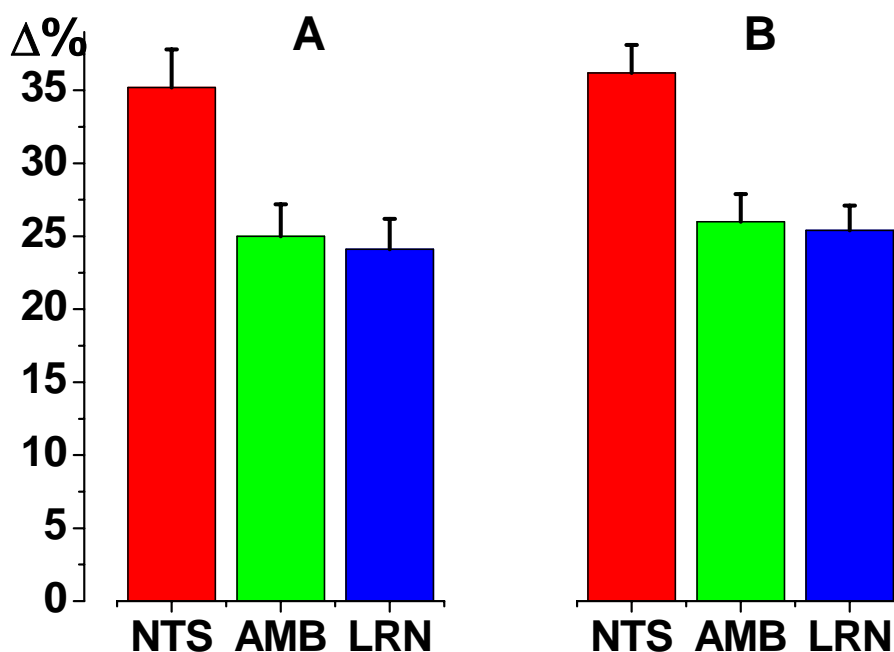
FIG. 4. Changes in the systemic arterial pressure (SAP,mmHg), induced by injections of neuronal NO-synthase inhibitor (NOS-1) into the nucleus of tractus solitarius (NTS), mucleus ambiguus (AMB) and lateral reticular nucleus (LRN) under normoxia (**A**) and after the intermittent hypoxic training (**B**)

After IHT, NOS-1 inhibition by injections of its inhibitor L-NNA into the medullary nuclei was accompanied by the development of hypertensive responses, which were similar to those in rats under normoxic conditions. Thus, after L-NNA injections into NTS the SAP level increased by 36.2%, on average ($P < 0.05$), into AMB – by 26% ($P < 0.05$) and into LRN – by 25.4% ($P < 0.05$). L-arginine injections into all the nuclei under study did not cause any significant changes in the SAP after the preliminary administration of the specific antagonist of NOS-1 7-nitroindosol.

IV. DISCUSSION

In the course of experiments we have established that in rats intermittent hypoxia promoted an increase in the SAP following L-arginine injections into the medulla on the first day after hypoxic training had been completed by 7.6 mmHg (7.6 – 8.6) %, on average ($P < 0.05$) as compared with that in the control. On the second day it was less expressed, and it was similar to that in normoxia on the third day. A tendency for SAT to be increased just after hypoxic training was most likely a consequence of the fact that rats to some extend reacted on the hypoxic interventions as on the stressful impacts. It is believed that the SAP increase induced by the intermittent hypoxia depends on the set of factors, such as nervous and hormonal changes, which influence cardio-

vascular system control on the central and peripheral levels. Thus, there are some data indicating that intermittent hypoxia stimulates peripheral chemo receptors, enhancing sympathetic impacts on the adrenals, heart and vessels, increasing threat the SAP.[7,16] For this reason, after the preliminary denervation of the carotid body there is no any increase in the arterial pressure.[31] The more pronounced SAP increase (by 13.7 mmHg but in the other hypoxic regime) was observed by the other researches.[11] As it turned out, intermittent hypoxia promotes an increase in c-fos expression in NTS and in the ventrolateral medulla,[13] – in those regions of rat medulla, which are involved in the control of the vascular tone and reflector control of the sympathetic activity and which integrates peripheral afferent inputs from chemo receptors with the sympathetic output to target-organs. So, the set of obtained data indicates that intermittent hypoxia can promote moderate activation of central and peripheral apparatus of sympathetic nervous system, which explains some increase in SAT under such conditions. Data analysis points to some differences in SAT changes, which to a great extend are due to the peculiarities of a hypoxic regime, used by different authors.[28] Thus, there are data that indicate that short and hard hypoxia can induce stable hypertension in animals and humans, and adaptation to normo-and hypobaric hypoxic training is able to prevent development of experimental hypertension and even to lower SAT in animals with hypertension.[12,16] It should be noted that in many clinical studies adaptation to intermittent hypoxia is declared as an antihypertensive tool.[29] Yet, some authors[2] tend to think that in rat with spontaneous hypertension adaptation to IHT is always accompanied by the SAT decrease, and this phenomena is hardly ever observed in rats with normal arterial pressure.[16] One has the impression that there are some differences between humans and rats in their reaction on IHT. In our investigation we used rats with normal arterial pressure and we did not observe any SAT decrease after IHT completion. Moreover, right after completion of hypoxic training SAT revealed a tendency to increase. Perhaps this is connected with the fact that the procedure of hypoxic training is more stressive for animals than for humans. That circumstance that IHT in the chosen regime is not accompanied by considerable and stable increase in SAT indicates that hypoxia was relatively soft. On the other hand that circumstance that the moderate SAT increase we observed just on the first day after IHT completion and did not observe on the following days gives a ground to think that, perhaps, an increase in this measurement is to a greater extend a reaction of the animal on the stress during its stay in the chamber, rather than a reaction on hypoxia.

It is assumed that nitric oxide plays an important role in the adaptive mechanisms to hypoxia due to stimulation of its synthesis and limitation of excessive production, which promotes cardioprotection, vasoprotection, neuroprotection and antistressive defence.[19-21] As it is known, NO production is a calcium-dependent process. Yet, there is some data[1] indicating that adaptation to an intermittent hypoxia increases activity of a calcium pump in sarcoplasmic reticulum membranes in cardiomyocytes, which promotes increase in NO production. It was also proven[14] that hypoxia potentiates NO synthesis and increases cytosolic calcium content in the endothelial cells of the pulmonary artery. In our previous investigations it was shown that nNOS activation by

means of L-arginine injections in the population of medullar neurons involved in the nervous control of the blood flow function is accompanied by development of hypotensive reactions as a result of attenuation of descending symphatoactivating impacts to the heart and vessels.[27] In the conducted investigation IHT enhanced hypotensive effects of nNOS activation as compared with those reactions observed in response to administration of similar doses of L-arginine into the medullar nuclei under normoxia. Comparative analysis of hypotensive reaction, which is developed in response to injections of substrates for endogenous NO synthesis in population of cardiovascular neurons has shown that effects of nNOS activation in all the investigated medullar nuclei were qualitatively and quantitatively similar. It should be noted that the most pronounced increase in hypotensive reactions in response to L-arginine injection into the medullar nuclei comparing with those observed by us in rats, which were under normoxia conditions, we have observed only on the first day after IHT completion, whereupon the effect of the intermittent hypoxia powered down. In other words, IHT in regime that we used had a short-time impact on the system of nervous control of blood-flow functions by NO-synthesizing neurons from the medulla. One has the impression that duration and effect of IHT are partially dependent of its regime. Thus, adaptation to hypoxia (12 – 10% O_2, 4-5 hours per day for 40 days) revealed a pronounced hypotensive effect in rats with the spontaneous hypertension.[20,22] In the same manner hypotensive effects were seen in rats and patients after normobaric breathing with the gaseous mixture (9 – 14% O_2, 3 – 8 min with 3-min normoxic intervals, 40 – 60 minutes per day for 20 – 30 days).[9] Results of our investigation indicate that moderate hypoxia (12% O_2) during the short period of time (10 days) is able to activate nitric oxide synthesis by medullar cardiovascular neurons, which can have a positive meaning for people with increased SAT indexes. Analysis of obtained results enables to assume that during IHT we observed two opposite tendencies: on the one hand to some extend took place activation of sympathetic nervous system, which promoted increase in the initial SAT, on the other hand nNOS activity increased, which conditioned SAT decrease.

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Neutrophil Apoptosis and Hypoxia

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ABSTRACT: Neutrophils are the most abundant population of leukocytes, which constitute the defense against pathogens. Released by neutrophils the proteolytic enzymes and reactive oxygen species help in eliminating infections, but also cause extensive tissue damage. Neutrophil apoptosis plays an essential role in cell homeostasis and resolution of inflammation. It is mediated by a complex network of intracellular apoptotic/survival signaling pathways and can be modulated by a variety of extracellular stimuli such as hypoxia. Here, we review recent studies on the mechanisms of neutrophil death and survival accentuating on neutrophil apoptosis under hypoxic conditions. Neutrophils possess components of both extrinsic and intrinsic apoptotic routes. However, in neutrophils this mechanism has special features. The involvement of death receptors, caspases, mitochondria, and Bcl-2 proteins are discussed. Both the transcription factor NF-κB and p38MAPK regulate the neutrophil apoptotic program. Despite that reactive oxygen species (ROS) can directly promote and/or adjust apoptosis, there is no consensus about the role of ROS on neutrophil lifespan. Thus both the types of ROS involved and the site of their generation may be important for neutrophil apoptosis. Finally, hypoxia can activate several signaling pathways. The possible differences between the effects of sustained and intermittent hypoxia are also addressed.

KEY WORDS: neutrophils, apoptosis, hypoxia

I. TAKE-HOME MESSAGES

- Neutrophil apoptosis is a central process for homoeostasis and successful resolution of inflammation, but in neutrophils it has special features because neutrophils are committed to cell death.

- Similar to other cells neutrophil apoptosis possesses components of extrinsic death receptor and intrinsic mitochondrial apoptotic pathways in which NF-κB and p38MAPK controlled proteins such as Bcl-2 family members and caspases are involved.

- ROS generation is involved in neutrophil apoptosis of activated or infected cells

but is not absolutely required as a mediator of neutrophil apoptosis under physiological conditions.

- In contrast to other cells, in which hypoxia induces apoptosis, in neutrophils hypoxia causes a profound inhibition of apoptosis both in vitro and in vivo. The survival effect of intermittent hypoxia was much more prominent then sustained hypoxia.

II. AN OVERVIEW OF NEUTROPHIL APOPTOSIS

Neutrophils are the most common type of leukocytes in the circulation, which constitute the first line of defense against pathogens. They are bone marrow derived, terminally differentiated, short lived (8 – 20 hrs) inflammatory cells that are released to the circulation continuously. Senescent neutrophils are cleared from the blood by liver, spleen and bone marrow in direct contact with flowing blood.[1] Neutrophils can exist in the circulation in one of three functional states: quiescent, primed or activated.[2] When quiescent neutrophils encounter a stimulus they are left in a primed state. Upon encountering a second stimulus, they proceed to activation, releasing reactive oxygen species (ROS), proteolytic enzymes and inflammatory mediators, which are implicated in clearance of infections.[2] However, an uncontrolled release of formidable array of toxic substances may inflict damage to surrounding tissues and propagate inflammation. Neutrophil apoptosis (NA) is a fundamental mechanism involved in maintaining a normal level of neutrophils and ensuring the rapid resolution of inflammation.[3,4] NA triggers the phagocytosis of apoptotic neutrophils by macrophages and is vital for limiting of tissue damage in vivo.[3] If neutrophil viability is prolonged, destruction of surrounding cells will take place. When this process, is initiated in the vasculature it is implicated in cardiovascular diseases. Importantly, mature neutrophils can undergo apoptosis even without requiring any apparent inductive stimuli. It suggests that the apoptotic program may already have been initiated in circulating neutrophils.[5]

NA is mediated by a complex network of intracellular death/survival signaling pathways and can be modulated by a variety of extracellular stimuli such as cytokines and hypoxia. NA can be initiated by the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway. The last one may play a pivotal role in the control of spontaneous NA.[6,7] The caspase cascade represents the main mechanism which is activated by both pathways. Caspases are synthesized as inactive zymogens and are activated by proteolysis, leading to enzyme cleavage and nuclear DNA fragmentation. Caspase-8 is the initiator caspase triggered by death receptors, whereas initiator caspase-9 cleavage is the signature of the mitochondrial pathway. Caspase-3, an effector caspase, is activated by the caspases-8 and -9.[8] Figure 1 illustrates the sequence of events of NA. The data describing NA pathways are summarized in a number of recent reviews.[3-6,9,10]
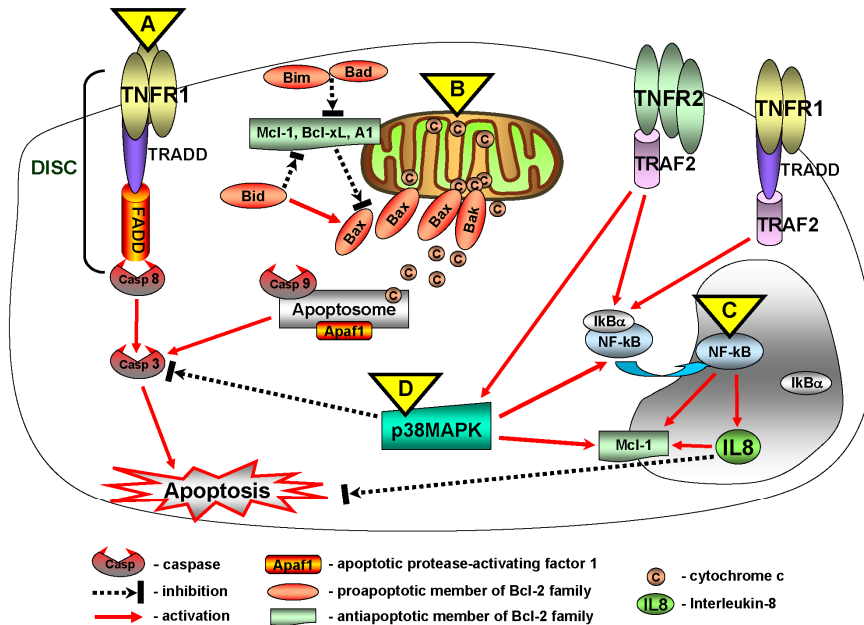
FIG. 1. (**A**) The extrinsic pathway of NA is initiated upon ligation of death receptor TNFR1, which induces formation of a death-inducing signaling complex (DISC) consisting of the death receptor, TNF receptor associated death domain-containing proteins (TRADD), Fas-associated death domain (FADD), and an initiator caspase-8, which activates the terminal effector of apoptosis caspase-3. (**B**) The intrinsic pathway involves mitochondria. Under normal condition neutrophils express high levels of the pro-apoptotic molecules of Bcl-2 family (Bax, Bak, Bim, Bid and Bad) and low levels of anti-apoptotic members (A1, Bcl-xL, Mcl-1). During apoptosis cytosolic Bax and Bak are translocated into the outer membrane of mitochondria and induced cytochrome c release. In the cytoplasm, cytochrome c complexes with apoptotic protease-activating factor 1 (Apaf-1) and pro-caspase-9 to form a protein complex the 'apoptosome', which is involved in caspase-3 activation. (**C**) Neutrophil survival pathway induced by NF-κB activation. In the cytoplasm NF-κB is held by inhibitory proteins IkBα. The release of IkBα from the NF-κB complex allows active NF-κB translocate into the nucleus. TNFR1 and TNFR2 are involved in NF-κB activation by recruitment of TNF receptor associated factor 2 (TRAF2). NF-κB regulates the synthesis of IL-8 and activates the anti-apoptotic Mcl-1 proteins. (**D**) Involvement of the p38MAPK in NA. p38MAPK may induce NF-κB activation, activate the anti-apoptotic Mcl-1 proteins and can directly phosphorylate and inhibit caspase-3 activity, thereby hinder NA

III. THE DEATH RECEPTOR PATHWAY

In the extrinsic pathway, ligation of a death receptor such as tumor necrotic factor receptor 1 (TNFR1) or CD95 induces the formation of a death-inducing signaling

complex (DISC). DISC consists of the death receptor, TNF receptor associated death domain-containing proteins (TRADD), Fas-associated death domain (FADD) adaptor protein, and an initial caspases (A in Fig. 1). Clustering of death receptors following ligation promotes aggregation of pro-caspase-8 molecules within the DISC, inducing their autoproteolysis and generation of active caspase-8, which activates the down-stream caspase-3, that are the terminal effectors of apoptosis.[5,6,9] Importantly, in hu-man neutrophils DISC may form spontaneously.[5] TNFR1 signaling is also known to promote neutrophil survival through the nuclear transcription factor (NF-κB) activa-tion, which can be induced by recruitment of TNF receptor associated factor 2 (TRAF2). In both signals the TRADD may act as a platform adaptor that recruits TRAF2 or FADD and thus activate distinct signaling cascades including activation of NF-κB-induced survival pathway or caspase-dependent pro-apoptotic route. In con-trast to TNFR1, TNFR2 does not contain a TRADD motif[4,11] and recruits TRAF2 in NF-κB activation directly. Additionally, TNFR2 may promote survival by mitogen-activated protein kinases (MAPK) activation.

IV. THE MITOCHONDRIAL PATHWAY

Mitochondria are the site of oxidative phosphorylation in the cells and classically de-fined as organelles highly specialized in ATP generation.[12] It is now generally as-sumed that alteration of mitochondrial function is an early feature of NA.[4-7] In viable cells, these organelles are organized as a diffuse tubular network that clusters during apoptosis. Critically, the mitochondrial route of apoptosis connects caspases and Bcl-2 proteins pathways (B in Fig. 1).

As is summarized by,[4,6,9] Bcl-2 is the prototype for a family of mammalian genes and the proteins they produce. They govern mitochondrial outer membrane permeabi-lization and can be either pro-apoptotic (Bax, Bak, Bim, Bid, Bad) or anti-apoptotic (Mcl-1, Bcl-X_L, A1/Bfl-1). In most cell types, the expression and activity of protective Bcl-2 members is higher than pro-apoptotic members. In contrast, mature neutrophils constitutively express pro-apoptotic proteins, whereas the expression of anti-apoptotic Bcl-2 members is very low or undetectable in resting cells.[3,13] However, anti-apoptotic proteins are highly and transiently expressed when neutrophils are exposed to survival factors, such as heme, IL-8, GM-CSF or hypoxia. The balance between pro- and anti-apoptotic members determines the fate of the cells.[14] Under physiological conditions, the mitochondrial membrane is polarized and has a membrane potential, the mainte-nance of which keeps proteins such as cytochrome c and ROS within the confines of the mitochondria. Pro-apoptotic Bcl-2 proteins exert their effects by activation of an inner mitochondrial permeability transition pore and by induction of apoptogenic fac-tor cytochrome c release. In the cytosol cytochrome c is involved in the assembly of a multimolecular complex known as "apoptosome", which consists of cytochrome c, apoptotic protease-activating factor 1 (Apart 1), and caspase-9 (Fig. 1B). In the pres-ence of ATP this complex induces the proteolitic cleavage and activation of pro-caspase-3 that triggers a downstream cascade of caspase-3 activity.

Bax is the best known pro-apoptotic soluble protein. In freshly isolated neutrophils Bax is found in the cytoplasm in a phosphorylated closed state, heterodimerized to Mcl-1. Under apoptosis Mcl-1 levels are markedly decreased by proteasome-mediated degradation. Waning levels of Mcl-1 release Bax from the heterocomplex Bax:Mcl-1 and allow Bax to translocate to the mitochondria where it is thought to form oligomers and exercise its pro-apoptotic function.[15,16] Whereas the activated Bax and Bak would act as ion channels and adaptor proteins and mediate the release of cytochrome c, the anti-apoptotic Bcl-2 would block NA through inhibition of Bax and/or Bak, by promoting the stability of mitochondrial outer membrane and/or impairing insertion of pro-apoptotic proteins. Additionally, the second groups of pro-apoptotic proteins Bad and Bid can modulate negatively the anti-apoptotic Bcl-2 proteins and positively the pro-apoptotic ones.

Mcl-1 is represented a key anti-apoptotic protein. It is only member of anti-apoptotic Bcl-2 family that has been reliably and reproducibly measured at both the mRNA and protein level in human neutrophils[17] It's well documented that spontaneous apoptosis is accompanied by degradation of Mcl-1, but not other anti-apoptotic molecules.[18] Anti-apoptotic Mcl-1 transcripts are extremely unstable (near 3 hours half-life).[19] Moreover, Mcl-1 is a subject to rapid turnover.[20] Such rapid changes in Mcl-1 function permit neutrophils to switch cell fate very rapidly from survival to death in response to external signals. Importantly, Mcl-1 is up-regulated in response to survival stimuli, thereby having a marked effect on NA.[21]

V. INVOLVEMENT OF NF-κB AND P38MAPK IN NA

Both the transcription factor NF-κB[3,12,22,23] and p38MAPK[12,24,25] regulate the NA program (C and D in Fig. 1).

NF-κB comprises a family of transcription factors that act as regulators of genes involved in NA and its regulation is highly cell specific and redox sensitive. NF-κB is normally found in the cytoplasm held by inhibitory proteins called IkBα and is activated by various stimuli, which converge at the IKK (IkB kinase) complex. IKK phosphorylates IkBα leading to its ubiquitination, followed by proteosomal degradation. The release of IkBα from the NF-κB complex allows active NF-κB translocation into the nucleus and bind to consensus sites in the DNA of responsive genes. NF-κB activity in neutrophils is regulated by mechanisms clearly different from those in other cells. The most important difference is that the newly synthesized IkBα can enter the nucleus, remove NF-κB from gene promoters and transport it back to the cytoplasm. Thus, nuclear accumulation of IkBα is associated with inhibition of NF-κB activity and the induction of NA.[26,27] Using different NF-κB inhibitors it was shown that inhibition of NF-κB is a powerful inducer of NA,[22] in contrast activators of NF-κB provides a strong survival signal.[23] NF-κB controls the expression of survival genes such as the Bcl-2 family members and regulates the synthesis of IL-8.[28] known as one of the most important survival proteins.[29] Anti-IL-8:IL-8 complex suppresses spontaneous NA. The survival effect is correlated with a decline in caspase-3 and caspase-9 activ-

ity, increase in anti-apoptotic protein (Bcl-X_L) and decreased pro-apoptotic proteins (Bax, Bak) expressions.[30]

The p38MAPK activation is part of a general stress response that mediates survival in neutrophils.[24,31] Given the observation that p38MAPK is implicated in the activation of NF-κB[32] it is conceivable that this might lead to expression of survival genes of the Bcl-2 family and IL-8. Moreover, p38MAPK can directly phosphorylate and inhibit the activities of caspases-8 and caspase-3 and thereby hinder neutrophil apoptosis.[24]

VI. ROS AS INTRACELLULAR MEDIATORS OF NEUTROPHIL APOPTOSIS

During the last decade, ROS molecules (superoxide anion-O_2*-, hydrogen peroxide-H_2O_2, and the hydroxyl radicals-OH $^-$) moved from a category of merely unwanted side products of oxidative metabolism to important messenger molecules. Among all cell types neutrophil possess the most powerful system of ROS.[33] ROS are generated in cells as a consequence of normal mitochondrial oxidative metabolism and also as part of the respiratory burst, that participate in microbial killing.[34] The mitochondria serve as the primary source in the quiescent state whereas in activated neutrophils the primary ROS generated by the nicotinamide adenine dinucleotide phosphate oxidase (NADPHox) system.[6,7,35,36] The latter is a multi-enzymatic complex responsible for the generation of high amounts of O_2*-through the reduction of molecular oxygen. In resting neutrophils, about 95% of the inactive NADPHox is found in the membranes of subcellular granules and vesicles, and the rest resides in the plasma membrane or distributed among cytosol. The cell activation results in phosphorylation of NADPHox cytosolic subunit and translocation of the granule pool to plasma or phagosomal membrane. The activation of the granule pool of NADPHox induces intracellular ROS production, while the stimulation of the membrane-bound oxidase mainly generates extracellular release of ROS. Importantly, intracellular generation, but not extracellular release of ROS, leads to NA.[29]

Among the ROS activated molecular targets are the caspases, the phosphoinositol PI3K/Akt pathway molecules and NF-κB.[13] Moreover, ROS can mediate death receptor clustering[37] and rapidly (during minutes) activate p38MAPK systems.[34] As was discussed by,[38] ROS may be involved in NA by various ways as direct oxidation of DNA or/and modification of proteins and enzymes. Additionally, lipid peroxidation by ROS may contribute to membrane rupture, eliciting release of the contents of intracellular compartments.[39] Finally, H_2O_2 could be an intermediate in the intracellular signaling mechanism of NA, and its oxidized products, such as OH- (the most toxic of the oxygen intermediates resulting in DNA damage), may be crucial for NA.[38] The functional role of ROS in NA is controversial and the precise signal transduction pathways are not fully understood. However, most reports affirm that ROS directly cause NA.[35] Increased production of H_2O_2 was noted in neutrophils cultured for 4 hours in the absence of any external stimulus.[40] The neutrophil incubation with H_2O_2 resulted in concentration-dependent increase in the rate of NA.[41] Both ionizing and ultraviolet radiation are capable of inducing NA, and both generate ROS. Catalase, which decreases

the intracellular H_2O_2 levels in cultured neutrophils, inhibits NA[38,42] and increases IL-8 expression.[36] Similarly, prolonged survival of neutrophils was detected in patients with chronic granulomatous disease with hereditary defect in ROS production,[42] which was associated with enhanced IL-8 levels.[43] In contrast, it was shown that ROS is also associated with activation of survival signaling routes, in which NF-κB activation could be involved.[12] Critically, the type of ROS molecules involved could be important for NA. For instance, increased intracellular levels of superoxide in neutrophils lead to activation of NF-κB, whereas exposure of neutrophils to hydrogen peroxide inhibits nuclear translocation of NF-κB.[44] All these data, however, do not imply that ROS are absolutely required as mediators of NA, especially under physiologic conditions[45] and the apparent contradictory ROS effects on NA could be as a result of the activation status of cells.[13] Thus, several groups have demonstrated that ROS generation does not affect the rate of spontaneous[45,46] and Fas/APO-1 triggered NA[37] or underlie the pro-apoptotic effect of TNF-α, but promote apoptosis in PMA-activated neutrophils.[45] H_2O_2 does not affect nuclear translocation of NF-κB in resting cells, but decrease it in LPS or TNF stimulated neutrophils.[28,40] Moreover, the types of activating stimuli (different cytokines, infection and phagocytosis, PMA or LPS activation and hypoxia) may be crucial for ROS effects on NA. For example, NADPHox-derived intracellular ROS that is generated during phagocytosis induces NA via caspase activation, whereas treatment of the same neutrophils with fMLP results in oxidative burst that is almost entirely extracellular, and apoptosis in these cells is slightly reduced.[39]

Finally, NA could be partially related to the different levels and types of cellular antioxidant defenses. Thus, the toxic potential of ROS can be limited by intracellular powerful antioxidant, such as glutathione.[5,37] Apparently, changes in redox status are the earliest event in NA. The intracellular antioxidant defenses of neutrophils may rapidly degrade H_2O_2, thus preventing the formation of by-products such as HO^-.

VII. HYPOXIA-INDUCED NEUTROPHIL SURVIVAL

Hypoxia, i.e. decreased availability of oxygen occurs under a variety of physiologic and pathologic conditions. Hypoxia activates a number of genes which are important in the cellular adaptation to low oxygen environment. Generally hypoxia induces apoptosis in different cell types. However, in contrast to other cells in neutrophils hypoxia causes a profound concentration-dependent and reversible inhibition of apoptosis in vitro.[41,47] Also in vivo work[48] demonstrated prolonged neutrophil survival in healthy subjects exposed to acute hypoxemia.

The hypoxic survival effect was associated with marked stabilization of hypoxia-inducible factor (HIF-1),[22,47] a master regulator of oxygen homeostasis that controls more than 70 target genes including erythropoietin, VEGF, and proteins associated with glucose and energy metabolism.[49] The ability of hypoxia to increase NF-κB p65 transcript abundance and activity, the ablation of hypoxic survival by the NF-κB inhibitors (gliotoxin and parthenolide), and the inhibition of hypoxic induction of NF-κB in HIF-1α knockout murine neutrophils suggests HIF-1α-dependent regulation of the

NF-κB pathway in NA.[22] Additionally, it was documented that hypoxia activates p38MAPK, leading to Mcl-1 activation and a subsequent delay in NA.[21] Similar to many anti-apoptotic stimuli, long exposure to hypoxia decreases ROS generation in neutrophils.[41] Interestingly, short hypoxemia in vivo appears to effects the primed state of the neutrophils for ROS production without significant effect on the stimulated/activated state.[48]

VIII. EFFECTS OF INTERMITTENT HYPOXIA ON NA

While some diseases involve episodes of sustained hypoxia (SH), diseases like vascularized tumors or Obstructive Sleep Apnea Syndrome (OSAS) are associated with intermittent hypoxic (IH) events. OSAS, in particularly, is characterized by intermittent and recurrent pauses in respiration during sleep. The various signaling pathways, caspase-mediated with IH and caspase-independent with SH, were described for PC-12 cells.[50] Moreover, IH leads to HIF-1α accumulation that persists significantly during re-oxygenation. In contrast HIF-1α levels in PC-12 cells exposed to SH were markedly reduced immediately after re-oxygenation.[49] It was also showed that in endothelial cells, IH induced a modification in HIF-1α phosphorylation pattern with progressive increase in HIF-1α phosphorilated form during hypoxic period, which could lead to cell survival and adaptation to hypoxia.[51] In contrast, Ryan et al. using HeLa cells found that HIF-1α is more sensitive to activation by SH than IH and that NF-κB is more sensitive to activation by IH than SH.[52] Using endothelial cell models they also found that IH activates NF-κB at least in part via p38 MAPK activation.[53] However what kind of response is true for neutrophils is unknown.

We compared the effects of IH and SH on NA using a unique computer-controlled incubation chamber which is attached to an external O_2-CO_2 computer-driven controller (BioSpherix OxyCycler C42 system, Redfield, NY). Chamber O_2, N_2, and CO_2 levels were continuously monitored and adjusted according to the desired programmed profile. Additionally a fiber-optic dissolved oxygen electrode was immersed below medium level to accomplish identical specific experimental profiles and to monitor dissolved oxygen concentrations. Using several IH cycles (3-6 and 10 cycles) and oxygen profiles ranging from 5 to 0.1% O_2 we established that the effects of IH were dose- and time-dependent. Importantly, NA was already significantly decreased after three cycles of IH at 5% oxygen concentrations as compared with normoxia, indicative of a relatively fast neutrophil activation over a period of 3 hours. Moreover, under all IH conditions NA was significantly lower compared to SH both in whole blood and in purified neutrophil cultures.[54] This trend was seen in each subject individually, but values were slightly higher in purified neutrophils compared with whole blood, due to neutrophil purification. Also in patients with OSAS NA was significantly attenuated.[54] This was verified by flow cytometry, morphological features of apoptosis as nuclear and chromatin condensation, and a significant reduction in caspase-3 activity. Critically, the percentage of apoptotic neutrophils was negatively correlated with the severity of hypoxia.[54] Whether SH and IH trigger a common signaling pathway in NA is currently unclear. We found that similar to

SH the anti-apoptotic effects of IH are mediated via p38MAPK signaling pathway, since the survival effects of hypoxia are lost with inhibition of p38MAPK (unpublished observations). We also determined that NF-κB activity is required for IH survival (article in preparation). Thus, treatment of neutrophils with structurally and mechanistically discrete NF-κB inhibitors gliotoxin, parthenolide, and IMD-0354 under IH resulted in significant increase of NA. Such increased NA was caspase-3 dependent and was accompanied with decreased IL-8 expression. NF-κB activity was found increase in nuclear fractions of neutrophils treated with IH in vitro. Similar, IH activates NF-κB in neutrophils of OSAS patients.[55]

Does ROS also mediate IH-induced decrease in NA? Similar to SH,[41] we found that cytoplasmatic ROS generation was decreased by 90-92% in neutrophils exposed to IH as compared to neutrophils maintained in normoxia. Interestingly, the same levels of basal ROS production were detected in resting neutrophils of both OSAS patients and control subjects.[56] In contrast, after PMA stimulation significant increases in ROS generation were detected in OSAS patients compared to control.[56] This suggests that IH may induce neutrophil priming for ROS production after challenge, which is critical for the clearance of infections but may be dangerous to surrounding tissues. Onset of apoptosis in neutrophils is much more complex than the simple mechanisms we have presented here and the role of ROS molecules and oxidative stress needs to be further elucidated in NA.

IX. ACKNOWLEDGMENTS

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