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Benzimidazole Derivative, 2-ETH Prevents Poststressor Disorders of Coronary Vascular Tone and Myocardial Contractility

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Summary

Introduction. Due to unpredictability of stressors exposure, prevention of stress-induced coronary vascular tone disorders with new classes of drugs with pleiotropic action is quite relevant. At the present time, besides traditionally used vasoactive substances, benzimidazole derivatives with antioxidant and anti-inflammatory activity are considered. *The goal* of the present study is to test the hypothesis that infusion of the 2-ethyl-thiobenzimidazole hydrobromide (2-ETH) before stress is able to effectively prevent stress-induced disorders of coronary vascular tone and myocardial contractility disorders and clarify mechanisms of such 2-ETH action. *Materials and methods*. Coronary vascular tone and myocardial contractility were investigated using Langendorff isolated rat heart model. The BKCa-channel inhibitor, tetraethylammonium (TEA) was added to the Krebs-Henseleit solution to the final concentration of 1 mM. Concentration of the stable products of NO degradation (NO2-/NO3-) was determined spectrophotometrically; activation of lipid peroxidation was assessed in the myocardium using a spectrophotometric method; concentration of iNOS, eNOS, and IL-1β was detected with enzyme-linked immunosorbent assay; concentration of C-reactive protein was estimated with immunoturbidimetric method. *Results*. 2-ETH pretreatment prevented stress-induced decrease in BKCa-channels and eNOS activity, limited iNOS activity in coronary vessels and restored myocardial contractility. These 2-ETH' effects together with its ability to prevent alterations of stress-sensitive organs and limit oxidative, nitrozative stress and systemic inflammation argue why 2-ETH should be considered as a useful tool for pathogenetic prevention of stress-induced pathology. *Conclusions*. 2-ETH should be considered as a useful tool for pathogenetic prevention of stress-induced pathology.

Keywords: 2-ethyl-thiobenzimidazole hydrobromide, lipid peroxidation, coronary vessels' tone, NO-synthase, BK_{Ca} -channels

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Производное бензимидазола 2-ЭТГ предупреждает постстрессорные нарушения тонуса коронарных сосудов и сократительной способности миокарда

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Резюме

Введение. Непредсказуемость воздействия стрессоров диктует необходимость поиска новых классов препаратов с плейотропным действием, позволяющим предупреждать нарушения тонуса коронарных сосудов, обусловленные стрессом. В настоящее время, помимо традиционно используемых вазоактивных веществ, начинают активно изучаться производные бензимидазола, обладающие антиоксидантной и противовоспалительной активностью. *Цель* исследования – проверка гипотезы о способности 2-этилтиобензимидазола гидробромида (2-ЭТГ), вводимого до начала моделирования стресса, эффективно предупреждать стресс-индуцированные нарушения тонуса коронарных сосудов и сократительной активности миокарда, а также уточнение механизмов такого двойного действия 2-ЭТГ. *Материалы и методы*. Тонус коронарных сосудов и сократительную способность миокарда крыс изучали на препарате изолированного сердца по методу Лангендорфа. Ингибитор ВК_{са}-каналов, тетраэтиламмоний (ТЕА), добавляли к раствору Кребса – Хензелейта в конечной концентрации 1 мМ. Концентрацию стабильных продуктов деградации NO (NO₂⁻/NO₃⁻) и интенсивность перекисного окисления липидов определяли в миокарде левого желудочка с использованием спектрофотометрического метода; концентрацию iNOS, eNOS и IL-1β определяли в сыворотке крови с помощью метода иммуноферментного анализа; концентрацию C-реактивного белка в сыворотке крови животных оценивали иммунотурбидиметрическим методом. *Результаты*. Предварительное использование 2-ЭТГ предупреждает постстрессорное снижение функциональной активной активность от стремение функциональной активность от стремение функционального от

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ОРИГИНАЛЬНЫЕ СТАТЬИ (экспериментальные исследования) / ORIGINAL ARTICLES (experimental investigations)

ности BK_{Ca} -каналов и снижение активности eNOS, ограничивает повышение активности iNOS в коронарных сосудах и восстанавливает сократительную способность миокарда. Эти эффекты 2-ЭТГ совместно с его способностью предотвращать изменения стресс-чувствительных органов и ограничивать окислительный и нитрозилирующий стресс, а также системное воспаление свидетельствуют о том, что 2-ЭТГ следует рассматривать в качестве эффективного средства для патогенетически обоснованной профилактики стресс-индуцированной патологии. Выводы. 2-ЭТГ следует рассматривать в качестве эффективного средства для патогенетически обоснованной профилактики стресс-индуцированной патологии.

Ключевые слова: 2-этилтиобензимидазола гидробромид, перекисное окисление липидов, тонус коронарных сосудов, NO-синтаза, BK_{c_a} -каналы

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Introduction

It is well known that even single but intensive stressor exposure could lead to acute coronary blood flow disorder in intact vessels or progression of inflammation in the vascular wall in case of an atherosclerotic lesion [1, 2]. Endothelial dysfunction, which in particular is characterized by disorders of NO and ROS hyperproduction. as well as ion channels disorders are thought to be some of the major mechanisms of stress-induced vascular tone disturbances [3, 4]. Due to unpredictability of stressors exposure, prevention of stress-induced coronary vessels' tone with new classes of drugs with pleiotropic action is too relevant. At the present time, besides traditionally used vasoactive substances, benzimidazole derivatives with antioxidant and anti-inflammatory activity are in the scope of view [5]. The goal of the present research is to testify the hypothesis that preliminary infusion of the 2-ethyl-thiobenzimidazole hydrobromide (2-ETH) before stress is able to effectively prevent from stressinduced disorders of coronary vessels' tone and myocardial contractility disorders and clarify mechanisms of such 2-ETH action.

Materials and Methods

Experimental animals. The study was performed on mongrel female white rats weighing 180–240 g. Rats were provided standard rat chow and tap water *ad libitum*. Experiments were performed in compliance with requirements of the Council for International Organizations of Medical Sciences (CIOMS) and the International Council for Laboratory Animal Science (ICLAS) as described in «International Guiding Principles for Biomedical Research Involving Animals» (Geneva, 1990). The study protocol was approved by the Committee for Bioethics and Humane at the Vitebsk State Medical University (19.01.2018).

Four groups of eight rats were studied: Group 1, Control Rats, which received saline, i.p., for three days; Group 2, 2-ETH Rats, which were injected with 2-ethylthiobenzimidazole hydrobromide, i.p., 3 mg/kg for three days; Group 3, Stress Rats, which were exposed to restraint stress; Group 4, Stress+2-ETH Rats, which were exposed to stress following i.p. 2-ETH pretreatment.

Restraint stress. Restraint stress was induced by fixing rats on their back for 6 h. Then the rats were allowed to rest for 1.5 h, and were sacrificed by decapitation under urethan (0.1 g/100 g body weight, i. p.) anesthesia and blood was collected.

Blood and tissue collection. Blood serum was stored frozen in Eppendorf tubes at -20 °C. After unfreezing, serum concentrations of interleukin 1 β , C-reactive protein (CRP), endothelial NO-synthase (eNOS) and inducible

NO-synthase (iNOS) were measured. The heart was excised, immediately immersed into cold Krebs buffer solution. Left ventricle was isolated, wrapped in foil and stored at -80 °C. After unfreezing, the myocardial tissue was homogenized, and tissue concentrations of stable products of NO metabolism (NO₂⁻/NO₃⁻), conjugated dienes, and malonic dialdehyde were measured. Stress-responsive organs (adrenal glands and spleen) were isolated and their relative wet weight was detected immediately (g/100 g of body weight).

Experiments on Langendorff isolated heart. Coronary vascular tone and myocardial contractility. Prior to thoracotomy in anesthetized rats heparin was injected i. p. (500 IU/kg). Coronary vascular tone was measured in Langendorff isolated hearts as described earlier using a IH-SR 844/1 system (HSE-HA, Germany) equipped with Isotec Pressure Transducers for measurement of aortic and developed intraventricular pressures. Isolated hearts were perfused at a constant coronary flow of 10 ml/min with Krebs-Henseleit buffer solution aerated with carbogen (95 $\% O_2 + 5 \% CO_2$). Computer recording and processing of the measured parameters were performed using the software ACAD (HSE, Germany). The coronary vessels were perfused retrogradely from the aorta, and thus coronary perfusion pressure (CPP) was equal to a ortic pressure. Hearts were paced at a constant rate of 240 per minute by a C-type 224 stimulator (HSE-HA, Germany). After a 15-min stabilization period, developed intraventricular pressure (DIVP) was recorded from a constant-volume latex balloon inserted into the left ventricle. Computer recording and processing of the measured parameters were performed using the software ACAD (HSE-HA, Germany).

Evaluation of BK_{Ca} channel contribution to coronary tone regulation. Each experiment was performed in two stages. During the first stage, the hearts were perfused with Krebs-Henseleit solution. During the second stage, the BK_{Ca} -channel inhibitor, tetraethylammonium (TEA) was added to the Krebs-Henseleit solution to the final concentration of 1 mM. The BK_{Ca} -channel contribution to regulation of the vascular tone was estimated by the magnitude of TEA-induced vasoconstriction, i.e., by the increase in CPP expressed as percentage of the baseline value.

Measurement of serum iNOS and eNOS activities and concentrations. NO synthases activities of blood serum were measured spectrophotometrically at 340 nm wavelength by changes in NADPH concentrations in a medium consisting of 0.1 M Tris-HCI buffer (pH 7.4), 1mM NADPH, and 1 mM L-arginine. For measuring eNOS activity, 10 mM CaCl₂ was added to the medium, and for measuring iNOS activity, 1 mM EDTA was added. NO synthase activities were expressed in nmol/g

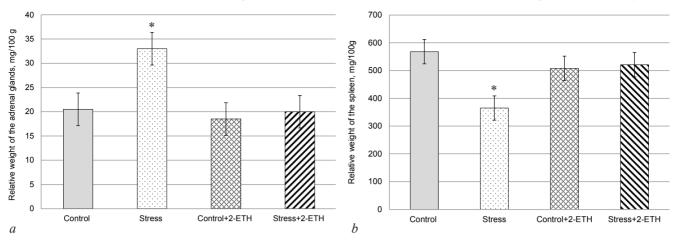


Fig. 1. The relative weight of the adrenal glands (a) and spleen (s) in mg/100 g of body weight of the animals after 6-h restraint stress: * – significantly different from control, p<0.05

protein per minute. Serum protein was measured by the biuret reaction using commercial kits (Cormay, Poland).

Serum concentrations of iNOS and eNOS were measured using ELISA test kits and reagents (for iNOS, Uscn, Life Science Inc. China, Lot L130827587 and for eNOS, Cloud-Clone Corp. USA, USCN, Life Science Inc., China, Lot L141013209). The concentrations of iNOS and eNOS were measured photometrically with universal photometer at λ =450 nm and expressed in ng/ml and pg/ml, respectively. The sensitivity of the assay, or Lower Limit of detection was defined as the lowest protein concentration that could be differentiated from zero. Detection range was 1.56 nmol/L – 100 nmol/L.

Measurement of serum interleukin 1β . Serum concentration of IL-1β was measured by ELISA using test kits (Thermo Scientific, USA, Lot LD145322) and expressed as pg/ml. The assay sensitivity was \leq 1 pg/mL and the intraand inter-assay coefficients of variation were both \leq 10 %.

Measurement of serum C-reactive protein. Since C-reactive protein expresses inflammation intensity and thus indirectly indicates the risk of vascular events, we measured serum concentrations of C-reactive protein with a diagnostic kit C-Reactive Protein (CRP) (BioSystems, Spain) according to the manufacturer's instruction. C-reactive protein concentrations were expressed as mg/l. The assay sensitivity was 0.05 mg/l, and the intra- and inter-assay coefficients of variation were both<5 %.

Measurement of tissue NO metabolites. Concentrations of NO stable metabolites, nitrite and nitrate, were measured in homogenized left ventricle. Nitrate was reduced to nitrite using zinc powder in alkaline medium in the presence of ZnSO₄ ammonium complex. A sample aliquot was mixed with an equal volume of the Griess reagent, and the total content of nitrite and nitrate was measured spectrophotometrically at 540 nm [6].

Evaluation of lipid peroxidation activity. Activation of lipid peroxidation was evaluated by accumulation of conjugated dienes and malonic dialdehyde in the myocardium using a spectrophotometric method. The content of conjugated dienes was calculated using the molar extinction coefficient at 233 nm for conjugated acids [7]. The content of malonic dialdehyde was calculated using the molar extinction coefficient for trimethine complex [8].

Statistical analysis. Data were analyzed statistically with STATISTICA 10.0 and MS Excel software. Quantitative

data are presented as mean \pm SEM. The Mann–Whitney U test was used to determine significance of differences between independent samples. Statistical hypotheses were tested at the critical significance level of 5 % (p<0.05).

Results and Discussion

Six-hour restraint stress induced changes in the weight of stress-responsive organs. Thus, the relative wet weight of the adrenal glands after stress increased by 65 % (p<0.05 vs. Control Rats), and the weight of the spleen decreased by 35 % (p<0.05 vs. Control Rats, Fig. 1A and 1B). Pretreatment with 2-ETH alone did not affects the relative wet weight of both adrenal glands and spleen in groups Control+2-ETH and Stress+2-ETH compared with control data.

In Control Rats, CPP and DIVP measured at the flow rate of 10 ml/min were 82 ± 2.2 mm Hg and 79 ± 2.4 mm Hg, accordingly. Addition of the BK_{Ca} blocker, TEA, to the coronary perfusate caused pronounced vasoconstriction, as reflected by a 95 % increase in CPP (p<0.05 vs. Control Rats; Fig. 2), while DIVP remained unchanged. Without TEA treatment, values for CPP and DIVP of Control+2-ETH Rats were similar to those of Control Rats. In Control+2-ETH Rats, supplementation of perfusate with TEA induced a 113 % increase in CPP (p<0.05 vs. Control+TEA Rats) up to 176±3.4 mm Hg (Fig. 2) while DIVP remained unchanged. Therefore, the intraperitoneal administration of 2-ETH resulted in increased BK_{Ca}-channels functional activity in coronary smooth muscle cells of control isolated hearts.

In isolated hearts of animals exposed to stress without TEA, CPP and DIVP were decreased by 23 % and 21 %, respectively (p<0.05 vs. Control Rats; Fig. 2). Therefore, restraint stress caused decrease of coronary tone and impaired myocardial contractility. Isolated hearts from rats exposed to 6-h restraint stress perfusion with TEA caused a 68 % increase in CPP up to 105±8.0 mm Hg, which was 27 % less than produced by TEA in Control Rats (p<0.05, Fig. 2). Thus, 6-h restraint stress reduced the effect of TEA on CPP, which could be due to the decreased functional activity of coronary BK_{Ca}-channels in coronary circulation. However, TEA did not influence the magnitude of DIVP in the Stress group. Perhaps, TEA in selected concentration blocked mainly BK_{C2}-channels located primarily in coronary smooth muscle cells rather than in mitochondria of cardiomyocytes [4].

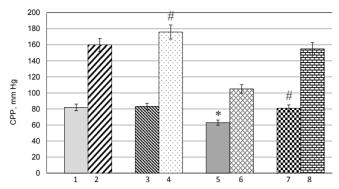


Fig. 2. Effect of intracoronary tetraethylammonium (TEA) on changes in coronary perfusion pressure: Abscissa – experimental groups,
1 – Control; 2 – Control+TEA; 3 – Control+2-ETH; 4 – Control+2-ETH+TEA; 5 – Stress; 6 – Stress+TEA; 7 – Stress+2-ETH;
8 – Stress+2-ETH+TEA; Ordinate – coronary perfusion pressure (CPP), mm Hg. * – significantly different from control, p<0.05; # – significantly different from group of «Control+TEA», p<0.05

Restraint stress produced in 2-ETH pretreated rats was not associated with any statistically significant changes in coronary vascular tone or myocardial contractile function; CPP and DIVP values were comparable with the control. In the Stress+2-ETH group, the intracoronary administration of TEA induced an 89 % rise in CPP up to 154±5.4 mm Hg (no difference from Control+TEA group, Fig. 2), whereas DIVP after TEA infusion was unchanged. Therefore, the prior activation of BK_{Ca} -channels with 2-ETH prevented the stress-induced decrease in coronary vascular tone via activation of BK_{Ca} -channels in coronary smooth muscle cells and restored impaired myocardial contractility.

In the blood serum from stress-exposed rats, the iNOS activity was increased 3.25 times and the eNOS activity was decreased 2.0 times compared with the control (table). In the Control+2-ETH group the iNOS and eNOS activities were 2.6 and 1.6 times increased, respectively, compared with the control. In the Stress+2-ETH group the iNOS activity was raised only 2.3 times and the eNOS activity was elevated 1.4, respectively, compared with the control values (p<0.05, table).

In the Stress group, the serum concentration of eNOS decreased 4.6 times and the serum level of iNOS increased 9.7 times compared with the control (p<0.05, Table). In Control+2-ETH and Stress+2-EHT groups, eNOS and iNOS levels did not differ from the control.

Concentration of NO_2^-/NO_3^- in the homogenized left ventricle increased by 41.7 % in the Stress group compared to the control (p<0.05, table). Concentrations of NO_2^-/NO_3^- in homogenate were similar in the Control+2-ETH, Stress+2-ETH and the Control groups.

Concentrations of conjugated dienes and malonic dialdehyde in myocardium after stress were 3.4 and 3.7 times, respectively, higher compared with control values (p<0.05, table) while these concentrations did not differ from the control in the Control+2-ETH and Stress+2-ETH groups.

In the Stress group, the concentration of C-reactive protein increased twofold compared to the control whereas it did not differ from the control in the Control+2-ETH and Stress+2-ETH groups. The serum concentration of IL-1 β after 6-h restraint stress was 22.3±2.3 pg/ml (ν s. 0.6±0.02 pg/ml in the control, p<0.001, table). The serum concentration of IL-1 β in the Control+2-ETH and Stress+2-ETH groups was similar to the control.

This study had shown that 6-h restraint stress reduces basal coronary vessels' tone, impairs myocardial contractility, produces lipid peroxidation and promotes acute phase response. Basal tone of coronary arteries is an integral value, which in isolated heart depends on the structural properties of vascular wall and the balance between locally produced vasoconstrictors and vasodilators [9]. On the one hand, TEA-dependent functional activity of BK_{cs}-channels in coronary vascular smooth muscle cells after stress was impaired. This fact could be explained by oxidative and nitrozative stress, which is characterized by superoxide radical and peroxynitrite accumulation [10]. This notion is supported impliedly by our results demonstrating augmentation of lipid peroxidation with accumulation of conjugated dienes and malonic dialdehyde and NO hyperproduction with accumulation of its metabolites in the myocardium of stressed rats. Moreover, 6-h restraint stress produces inflammation, which is proved by statistically significant increase of IL-1β and C-reactive protein concentrations as a hallmark of the acute phase response. The CRP, in turn, is able to activate iNOS [11, 12]. It was shown that C-reactive protein activates monocytes [12], which produces proinflammatory cytokines with subsequent iNOS activation and potentiation of inflammatory response. This knowledge partially explains high incidence of cardiovascular «catastrophes» after severe acute stress. It is reasonable to assume the following sequence of events which were caused impairment of BK_C-channels functional activity in the vascular smooth muscle cells of coronary vessels after 6-h restraint stress [13, 14]: oxidative and nitrozative stress \rightarrow cell injury \rightarrow synthesis of proinflammatory cytokines (IL-1 β and TNF- α) \rightarrow activation of transcription factor NF-K $\beta \rightarrow$ accumulation of iNOS enzyme in coronary vessels and systemic iNOS activation \rightarrow NO hyperproduction \rightarrow and/or peroxynitrite formation \rightarrow oxidation and/or nitrozylation of modifiable-sensitive SH-groups of BK_{Ca}-channels molecules \rightarrow impairment of BK_{C3}-channels functional activity. So-called «poststress channelopathy» potentially may lead to coronary constriction and myocardial ischemia which are common events following distress. On the other hand, restraint stress produces decrease in systemic eNOS activity, and, in contrast, elevation of systemic iNOS activity and expression of the enzyme in coronary endothelial cells with accumulation of NO metabolites in the myocardium following 6-h stress. Hence, astonishing of BK_{Ca}-channels and eNOS functional activity after stress «compensatory» promotes activation of iNOS as a «reserve echelon» to prevent coronary vasoconstriction. However, excessive coronary vasodilation persists in parallel with impaired myocardial contractility, which could lead to a «hyperperfusion phenomenon» [15] in the isolated hearts after stress with development of myocardial edema and diastolic dysfunction with progression of myocardial contractility disorders [16]. That is why such excessive stress-induced coronary vasodilation has limited biological significance.

It was shown that 2-ETH pretreatment prior to 6-h restraint stress completely abolishes stress-induced impairment of coronary vessels tone. It was detected that 2-ETH enchances abnormally low eNOS expression in the myocardium and increases systemic activity of the

Effect of the 2-ETH pretreatment on NOS activity and serum concentrations of NOS, IL-1β, and C-reactive protein, and myocardial tissue concentrations of NO2-/NO3-

			and lij	and lipid peroxidation products	products				
Group	Serum eNOS activity, nmol/ g protein/min	Serum iNOS activity, nmol/g protein/min	Serum eNOS concentration, pg/ml	Serum iNOS concentration, ng/ml	Myocardial concentration of NO2-/NO3-, μΜ	Myocardial concentration of conjugated diens, nM/g lipid	Myocardial concentration of malonic dialdehyde, nM/g protein	Serum CRP, concentration, mg/l	Serum IL-1β concentration, pg/ml
Control (n=8)	106.0±9.5	40.0±11.0	51.0±5.5	3.0±0.2	24.0±0.8	109.0±15.4	79.0±6.5	0.162±0.03	0.6±0.02
Stress (n=8)	51.0±7.6*	130.0±15.8*	11.1±1.8*	29.0±10.4*	34.0±4.8*	376.0±98.1*	294.0±64.7*	0.317±0.05*	22.3±2.3*
Control+2-ETH (n=8)	165.0±46.2*#	103.0±21.5*#	47.0±18.1#	4.0±0.08#	24.0±0.7#	108.0±4.0#	78.0±8.7#	0.185 ± 0.03 #	0.3±0.02#
Stress+2-ETH (n=8)	149.0±21.7*#	92.3±10.3*#	39.0±5.9#	3.0±0.2#	23.0±0.5#	101.0±10.5#	81.0±4.5#	0.158±0.02#	6.3±0.2#
N otes: * - Significant difference from Control, p<0.05; # - significant difference from Stress, p<0.05.	difference from Control	l, p<0.05; # – signi	ficant difference fi	rom Stress, p<0.0	5.				

enzyme after restraint stress, but prevents iNOS accumulation in the endothelial cells of coronary vessels. Perhaps, 2-ETH activates BK_{ca} -channels in vascular smooth muscle cells of coronary vessels, which was proved by complete restoration of TEA-induced coronary constrictor response. Such positive effects of 2-ETH could be explained partially by its ability to limit systemic low grade inflammation due to its antioxidant activity as a reactive oxygen species scavenger [17] with subsequent reduction of damage-associated molecular patterns formation, which are able to initiate inflammation. Moreover, different benzimidazole derivatives inhibit inducible cyclooxygenase (COX-2) [18] and suppress inflammasome NLPR3 activity [19], a main source of proinflammatory cytokines with subsequent decrease in the iNOS activity and activation of eNOS. This notion is supported with our data demonstrating that 2-ETH i. p. injection before stress entirely eliminates systemic low grade inflammation and prevents lipid peroxidation and NO₂-/NO₃- accumulation in the heart of stressed rats. Limitation of oxidative and nitrozative stress as well as prevention of acute phase response following 6-h restraint stress after 2-ETH pretreatment, perhaps, also restored BK_{Ca}-channels functional activity and myocardial contractility. Besides these mechanisms, it is well known that some benzimidazole derivatives sensitize myofibrils for Ca²⁺ [20] and cause positive inotropic action via phosphodiesterase inhibition in guinea-pig heart [21]. Thus, 2-ETH abolishes stress-induced coronary vessels' tone and myocardial contractility disturbances after 6-h restraint stress and adjusts coronary perfusion to myocardial contractility.

However, analysis of 2-ETH effects in the Control group lays oneself to ask: «Why did 2-ETH in contrast to Stress group activate iNOS in the serum and increase expression of the enzyme in the myocardium while didn't affect serum NO₃-/NO₃-concentration?» Perhaps, 2-ETH could enhance iNOS gene activity [22]. Different benzimidazole derivatives modify cellular redox state by augmentation of GSH synthesis [23], whereas iNOS is redox-sensitive enzyme [24]. Moreover, 2-ETH pretreatment in the Control group could stimulate aggresomes formation [25]. Aggresomes are cytosolic multiprotein structures containing iNOS, which produce NO in significantly less concentrations compared to protein-unbound enzyme. Formation of aggresomes is thought to be an important mechanism of limitation of iN-OS-derived NO hyperproduction [25, 26]. 2-ETH-induced moderate iNOS activation in the Control group creates conditions for development of a robust adaptive response, which reflects normal interrelations of local coronary tone regulatory systems [27].

Thus, our results had demonstrated that 2-ETH pretreatment prevents decrease in BK_{Ca}-channels and eNOS activity, limits iNOS activity in coronary vessels and restores myocardial contractility following 6-h restraint stress. These 2-ETH' effects together with its ability to prevent alterations of stress-sensitive organs and limit oxidative, nitrozative stress and systemic inflammation argue why 2-ETH should be considered as a useful tool for pathogenetic prevention of stress-induced pathology.

Conflict of interest

The authors declare no conflict of interest.

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