Modulators of K_{ATP} channels in the prevention of oxidative stress and antioxidant capacity improvement in the rat heart with different resistance to hypoxia upon cobalt treatment

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Abstract

Introduction: The main goal of the study was to investigate the effect of K_{ATP} channel modulators on development of oxidative stress in the heart of rats showing different resistance to hypoxia. **Material and Methods:** The study has been performed on rats showing high- (HR) or low-resistance (LR) to hypoxia under modulators of ATP-sensitive potassium (K_{ATP}) channel opener pinacidil (0.06 mg/kg) and blocker glibenclamide (1 mg/kg) upon cobalt (Co) treatment (30 mg of cobalt chloride/kg b.w., 3 h). Changes in the oxidative stress parameters of the heart tissue, such as lipid peroxidation (LPO), level of oxidatively modified protein (OMP), and antioxidant defence system (superoxide dismutase - SOD, catalase -CAT, glutathione peroxidase - GPx, glutathione reductase - GR) as well as total antioxidant activity (TAA) were analysed. **Results:** Co treatment caused a significant decrease in SOD and CAT activity in the heart of LR rats and GPx activity in HR rats. It also led to a decrease in OMP level in the heart of rats with HR in comparison with controls. **Conclusion:** The obtained results suggest that individual resistance to hypoxia plays a crucial role in Co actions and provides evidence that the effects of K_{ATP} channel opener pinacidil in the heart are mediated through different pathways of the antioxidative system, depending on the individual resistance to hypoxia. Pinacidil exerts a protective effect on the heart tissue by preventing the LPO decrease and significantly reducing OMP levels, as well as increasing TTA in rats with LR.

Keywords: rats, heart, cobalt, antioxidant defence, lipid peroxidation, hypoxia, oxidative stress.

Introduction

Heavy metals are well-known environmental pollutants exerting particularly dangerous effects on human health (6). Because of their wide usage in many industrial branches they are present everywhere in the air, water, and soils (24). Inadequate nutrition may also promote heavy metals toxicity. Exposure to hard metal dust, observed in some occupations, is linked to an increased risk of lung cancer, as it has been proven to induce genotoxicity *in vitro* and *in vivo* (20). Possibly, the genotoxicity mechanisms involve production of reactive oxygen species (ROS) and/or inhibition of DNA repair. In the case of cobalt (Co) toxicology studies have documented both

adverse as well as putative beneficial effects (26). Co is used for the production of alloys and hard metal (cemented carbide), diamond polishing, drying agents, pigments, and catalysts (5). The element influences many metabolic processes causing great damage to many organs in a dose-dependent manner, and some research described its involvement in myocardium injury (11).

Occupational exposure to Co may result in adverse health effects manifested in different organs and tissues. A notable toxic effect of Co intake, leading to severe and often lethal cardiomyopathy, has been observed in the 1960s among heavy beer drinkers after local breweries started to add cobalt chloride or sulfate to beer as a foam stabiliser (20). In

the lethal cases, post-mortem analysis showed marked accumulation of Co in the myocardium. Co cardiomyopathy has also been seen following industrial exposure to Co (6). Linna *et al.* (18) described echocardiographic changes indicating altered left ventricular diastolic function, but without major cardiac dysfunction, among workers in an industrial plant.

However, Co may also have beneficial effects. For example, this trace element takes part in the synthesis of B₁₂ vitamin. Co is a relatively rare transition metal with properties similar to iron, chromium, and nickel. Co stimulates erythropoietin production and increases erythropoiesis, leading to increased oxygen-carrying capacity of the blood, which is helpful under conditions of ischaemia and tissue hypoxia. Moreover, preconditioning with cobalt salts akin to hypoxic preconditioning promotes tissue adaptation to hypoxia.

Myocardial ischaemia has become the leading cause of mortality in western countries. From biological perspective, myocardial necrosis is time-dependent and occurs when the action of the endogenous mechanisms of response to ischaemia are finally overwhelmed. A novel assay, described by Bar-Or *et al.* (1), for the detection of myocardial ischaemia involving the addition of CoCl₂ to plasma or serum has recently been proposed as the first sensitive marker for the diagnosis of myocardial ischaemia (ischaemic insult). During acute ischaemic conditions, the metal binding capacity of albumin for such transition metals as copper, nickel, and Co is reduced, generating a metabolic variant of the protein, commonly known as ischaemia modified albumin (IMA).

The precise mechanism for IMA generation is unknown yet, although it appears that ROS, produced during ischaemia, might generate highly reactive hydroxyl free radicals, resulting in site-specific modification to the N-terminus of the albumin moiety. Generation of IMA, as described by Xi *et al.* (30), might be interpreted as an efficient endogenous mechanism of response to ischaemia, preventing myocardial damage or limiting the extent of myocyte necrosis.

Recent studies have provided insight into the interference of Co with the oxygen sensors in the hypoxia response pathway present in almost all animal cells, contributing to the understanding of the possible carcinogenic effects of Co (6). Most of the studies on Co supplementation are focused on ischaemia-reperfusion injury (21). There are some data on the efficacy of Co in facilitating preconditioning to hypobaric hypoxia and related ailments. Co preconditioning has been shown to attenuate hypoxia-induced oxidative stress in rat brain. Cobalt chloride supplementation at low concentration improved cardiac contractile functions in hypoxia-reoxygenation in rats (8). Shrivastava

et al. (25) have shown that Co preconditioning improves hypoxic tolerance of rats exposed to severe hypobaric hypoxia.

Different toxic and pharmacological agents may cause opposite changes in metabolism pathways (oxidative at first) in animal tissues with genetically determined differences in the sensitivity to hypoxia. These changes are tissue-specific and depend on metabolic processes, redox properties, and pro-oxidant/antioxidant ratio in tissues. So far, the reason for the physiological reactions to hypoxia and hypoxia modulator agents on oxidative metabolism remain not fully understood. It also remains unclear why not all humans and animals react in the same way to the identical factors.

ATP sensitive potassium (K_{ATP}) channels have been postulated to be involved in myocardial protection against ischaemic and reperfusion damage (4). It has been speculated that the opening of these channels during ischaemia and early reperfusion would produce ADP shortening, reducing Ca²⁺ influx and consequently preventing Ca²⁺ overload.

Our previous studies have shown that protection induced by K_{ATP} channel openers was mediated by α -ketoglutarate oxidation and inhibition of lipid peroxidation during stress conditions (27). Our results showed that pharmacological treatment with K_{ATP} channel openers induced protective effect during stress conditions and dystrophy in the liver and myocardium of rats and guinea pigs (28). We reported the relationship between protection induced by K_{ATP} channel openers and oxidation of NAD-generating substrates (α -ketoglutarate) during experimental myocardium dystrophy. Our previous investigations have shown that the pharmacological treatment with pinacidil, an opener of the K_{ATP} channels, induces protection during myocardium dystrophy in the liver of guinea pigs (28).

The aim of the present study was to, firstly, investigate the effect of Co toxicity, and secondly the combined effect of K_{ATP} channel modulators pinacidil and glibenclamide with Co treatment on the total antioxidant activity (TAA), oxidised modified proteins level, and antioxidant enzyme activity.

Material and Methods

Animals and experimental design. The experiments were conducted with the Guidelines of the European Union Council and the current laws in Poland, according to the Ethical Commission. To eliminate diurnal rhythm changes, all examinations started at 10 and ended at 12 am.

Male white rats (180-220 g) were used in the study. The rats were housed at a constant temperature of 20 $\pm$ 2°C. The animals had free access to feed and water throughout the experiments. The animals were divided into two groups: rats with low resistance and

high resistance to hypoxia. Resistance to hypoxia was evaluated as survival time (min) in the altitude chamber 11 000 m above sea level. Survival time was measured after achieving the altitude. Cessation of breathing served as the criterion for resistance to hypoxia.

Drugs and solutions. The following compounds were used: pinacidil - K_{ATP} channel opener, glibenclamide, K_{ATP} channel blocker, KCL, K_2CO_3 , KH_2PO_4 , HEPES, EDTA, and 2-tiobarbituric acid (Sigma). All the compounds were freshly prepared. Pinacidil and glibenclamide were dissolved in saline buffered with NaOH. Cobalt chloride was suspended in distilled water. All other reagents were of analytical reagent grade.

Experimental groups. The rats were randomly assigned into four groups: untreated control group (I) - rats with low resistance - LR ($n = 8$) and high resistance - HR ($n = 8$) to hypoxia; Co group (II) - rats with LR ($n = 8$) and HR to hypoxia ($n = 8$) that received 30 mg of cobalt chloride/kg b.w. for 3 h; Co group (III) - rats with LR ($n = 8$) and HR to hypoxia ($n = 8$) that were intraperitoneally administered K_{ATP} channel opener pinacidil (0.06 mg/kg i.p.); Co group (IV) - rats with LR ($n = 8$) and HR to hypoxia ($n = 8$) that were intraperitoneally administered K_{ATP} channel blocker glibenclamide (1 mg/kg i.p.). Thirty minutes after pinacidil and glibenclamide administration, animals from groups III and IV additionally received 30 mg of cobalt chloride/kg b.w. for 3 h. In 3 h after cobalt treatment, the animals were decapitated. Before the experiment the control group was injected with 1 mL of saline.

Tissue collection. After the decapitation, the heart was excised, weighed, washed in ice-cold buffer, and minced. The minced tissue was rinsed with cold isolation buffer to remove blood and homogenised on ice in a glass Potter-Elvehjem homogenising vessel with a motor-driven Teflon pestle. The isolation buffer consisted of 120 mM KCl, 2 mM K_2CO_3 , 10 mM HEPES, and 1 mM EDTA; pH (7.2) of the buffer was adjusted with KOH.

Bradford method with bovine serum albumin as a standard was used for quantification of protein concentration (3). Absorbance was recorded at 595 nm wavelength. All enzymatic assays were carried out in duplicates, at $22 \pm 0.5^\circ C$, using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by the addition of the tissue supernatant.

Total antioxidant activity (TAA) assay. The TAA in the tissue homogenate was estimated spectrophotometrically at 532 nm wavelength following the method with Tween 80 oxidation by measuring TBARS level (9). Tissue inhibits Fe^{2+} /ascorbate-induced oxidation of Tween 80 resulting in a decrease in TBARS level. Absorbance of the blank was determined as 100%. The TAA in

sample (%) was calculated in reference to the absorbance of the blank.

Thiobarbituric acid reactive substance (TBARS) assay. TBARS was measured by the method of Kamyshnikov (14). The absorbance of supernatant was read at 540 nm wavelength. The TBARS was calculated by using $1.56 \times 10^5 \text{ mM}^{-1} \times \text{cm}^{-1}$ as extinction coefficient. TBARS level was expressed in μmol of MDA/mg of protein.

Carbonyl protein derivatives assay. Oxidative protein modification rate was estimated by the reaction of the resultant carbonyl derivatives of amino acids with 2,4-dinitrophenyl hydrazine (DNFH) as described by Levine *et al.* (17) as modified by Dubinina *et al.* (7). Carbonyl groups were determined spectrophotometrically at 370 nm (aldehyde derivatives (AD), OMP₃₇₀) and 430 nm (ketonic derivatives (KD), OMP₄₃₀) wavelengths, and expressed in nmol/mL of protein.

Superoxide dismutase activity assay. Superoxide dismutase (SOD, E.C. 1.15.1.1) activity in supernatant was determined according to Kostiuk *et al.* (16). SOD activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0). Absorbance at 406 nm wavelength was measured immediately and after 20 min. Activity was expressed in units of SOD/mg of protein.

Catalase activity assay. Catalase (CAT, E.C. 1.11.1.6) activity was determined by the method of Koroliuk *et al.* (15). H_2O_2 decrease in the reaction mixture was measured spectrophotometrically. The absorbance of the obtained solution was measured at 410 nm wavelength and was compared with the values of blank sample. One unit of catalase activity was defined as the amount of enzyme required for decomposition of 1 μmol of H_2O_2 /min/mg of protein.

Glutathione reductase activity assay. Glutathione reductase (GR, E.C. 1.6.4.2) activity was measured according to the method described by Glatzle *et al.* (10). The enzymatic activity was assayed spectrophotometrically by measuring NADPH consumption. Quantification was based on the molar extinction coefficient of $6.22 \text{ mM}^{-1} \times \text{cm}^{-1}$ of NADPH. The blank without NADPH was used and the GR activity was expressed as nmol of NADPH/min/mg of protein.

Glutathione peroxidase activity assay. Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by the detection of non-enzymatic utilisation of reduced glutathione (GSH) as the reacting substrate at an absorbance of 412 nm wavelength after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Moin (22). The rate of GSH reduction was measured spectrophotometrically at 412 nm. GPx activity was expressed as nmol of GSH/min/mg of protein.

Statistical analysis. Results are expressed as mean $\pm$ S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors tests ($P > 0.05$). Variance homogeneity was checked using the Levene's test. The significance of differences in the TAA, TBARS as lipoperoxide level, SOD, CAT, GR, and GPx, as well as the variances in oxidative modified protein content between control and Co group, Co group and modulators of K_{ATP} channels depending on individual resistance to hypoxia were all examined using one-way analysis of variance (ANOVA) with Bonferonni's post-test. The differences were considered significant at $P < 0.05$. In addition, the relationship between the data of all individuals was evaluated using Pearson's correlation analysis. All statistical calculations were performed with Statistica 8.0 software (StatSoft Inc., Poland), using separate data related to each individual.

Results

LPO in the heart of both animal groups (with low and high resistance to hypoxia) was similar upon Co treatment (Fig. 1). Pinacidil administration prior to Co treatment led to LPO level decrease by 58.7% ($P = 0.049$) vs. Co group in rats with LR. In these conditions ($F = 3.91$, $P = 0.018$) glibenclamide administration caused a decrease in LPO by 67.15% ($P = 0.020$). Under glibenclamide administration in rats with HR ($F = 7.36$, $P = 0.001$), a similar decline in the level of LPO was noted (two times, $P = 0.001$) in comparison to the Co group.

TAA in the heart (Fig. 2) of both groups upon Co treatment was similar independently of individual resistance to hypoxia. Pinacidil, as K_{ATP} channels opener, caused a statistically significant increase in TAA level in both groups vs. Co treatment. In rats with LR ($F = 4.38$, $P = 0.011$) TAA was increased by 112.7% ($P = 0.014$) and in HR rats by 37.4% ($P = 0.008$). Glibenclamide effect prior to Co treatment was determined on the basis of hypoxia resistance. In both groups TAA was higher in comparison to Co group, but in rats with LR, TAA increased by 97% ($P = 0.042$) while in HR rats by 38.1% ($P = 0.028$).

OMP level was not significantly changed after Co treatment in rats with LR. For HR rats ($F = 10.81$, $P = 0.000$) only OMP KD ($P = 0.008$) value decreased twice in comparison to control, and this result was statistically significant. Our results demonstrated (Fig. 3) the impact of individual resistance to hypoxia on the processes of proteins oxidation in the heart upon Co treatment. For rats with LR treatment with pinacidil resulted in lowering the OMP level: OMP AD ($F = 11.24$, $P = 0.000$) and OMP KD ($F = 10.84$,

$P = 0.000$). In rats with LR, OMP AD level decreased by 68.6% ($P = 0.000$) and OMP KD by 75.9% ($P = 0.001$) vs. Co group. In HR rats a similar decrease in OMP level was demonstrated only in the case of OMP AD, which decreased by 61.9%, $P = 0.004$. Administration of glibenclamide to rats with HR and LR initiated processes of oxidative protein modifications similar to pinacidil. Statistical changes were not demonstrated in both animal groups in comparison with Co group.

Our results showed a significant decrease in LPO process and oxidatively modified protein content with TAA level simultaneously. These observations correlated with the results of antioxidant enzyme activity upon treatment with modulators of K_{ATP} channels and cobalt.

SOD and CAT activity decreased statistically (Table 1) after Co treatment only in rats with LR: SOD ($F = 4.27$, $P = 0.013$) by 34.8% ($P = 0.036$) and CAT ($F = 19.48$, $P = 0.000$) by 51.2% ($P = 0.000$) in comparison to control. It was also shown that K_{ATP} channel modulators did not influence SOD and CAT activity in LR rats. Pinacidil and glibenclamide caused a statistically significant decrease in the activity of antioxidant enzymes in rats with HR compared with Co group.

Administration of K_{ATP} channel modulators caused a statistically significant decrease in GR activity ($F = 9.35$, $P = 0.000$) in comparison with Co group of rats with LR. Pinacidil caused a decrease in GR activity by 70% ($P = 0.002$) and glibenclamide by 64% ($P = 0.002$) in LR rats vs. Co group. Conversely, pinacidil caused an increase in GR activity by 99% ($P = 0.040$) in HR rats.

GPx activity was lower in HR rats only after Co treatment (by 45.4%, $P = 0.002$). Pinacidil increased GPx activity in both animal groups but the effect was higher in LR rats. In LR rats, K_{ATP} channel opener increased GPx activity two times ($P = 0.005$) and in HR rats by 58.4% ($P = 0.035$) in comparison to Co group. At the same time, GPx activity in HR rats exposed to glibenclamide also increased.

The results of the correlation analysis between lipid peroxidation, oxidatively modified proteins level, and the activity of antioxidant enzymes in the heart of rats with low and high resistance to hypoxia during influence of pinacidil or glibenclamide, and Co treatment, are shown in Tables 2 and 3. The data indicate that Co modifies antioxidant enzyme activity and causes changes in the SOD, CAT, GPx, and GR activity, proving the enzyme-substrate interactions. Modulators of K_{ATP} channel activity participate in significant interactions of the antioxidant enzymes and demonstrate enzymatic activity correction in redox reaction depending on congenital resistance to hypoxia.

Fig. 1. Lipid peroxidation level measured by quantifying the TBARS level in the heart ($\mu\text{mol MDA}/\text{mg protein}^{-1}$) of rats with different resistance to hypoxia under pinacidil or glibenclamide effect and cobalt treatment. Values are expressed as the mean $\pm$ SEM, $n = 8$

LR – rats with low resistance to hypoxia; HR – rats with high resistance to hypoxia

* – $P < 0.05$ compared with control

** – $P < 0.05$ compared with Co treatment

Fig. 2. Percentage of total antioxidant activity (TAA) in the heart of rats with different resistance to hypoxia under pinacidil or glibenclamide treatment and cobalt administration. Values are expressed as the mean $\pm$ SEM, $n = 8$

LR – rats with low resistance to hypoxia; HR – rats with high resistance to hypoxia

* – $P < 0.05$ compared with control

** – $P < 0.05$ compared with Co treatment

Table 1. Antioxidant enzyme activities in the heart of rats with different resistance to hypoxia under pinacidil or glibenclamide effect and cobalt treatment

Parameters		Control	Co	Pin+CoCl ₂	Glib+CoCl ₂
SOD, U/mg protein	LR	431.18 $\pm$ 40.07	281.44 $\pm$ 25.71*	414.02 $\pm$ 43.08	303.99 $\pm$ 35.64
	HR	425.27 $\pm$ 35.02	497.17 $\pm$ 51.47	481.54 $\pm$ 67.61	244.0 $\pm$ 25.83**
CAT, $\mu\text{mol}/\text{min}\cdot\text{mg protein}$	LR	12.55 $\pm$ 0.57	6.12 $\pm$ 0.40*	6.30 $\pm$ 0.70	5.81 $\pm$ 1.09
	HR	9.35 $\pm$ 0.56	10.66 $\pm$ 0.47	5.98 $\pm$ 0.48**	4.18 $\pm$ 0.41**
GR, nmol	LR	4.06 $\pm$ 0.67	4.51 $\pm$ 0.66	1.37 $\pm$ 0.16**	1.61 $\pm$ 0.48**
NADPH ₂ /min·mg protein	HR	3.08 $\pm$ 0.49	2.69 $\pm$ 0.30	5.35 $\pm$ 1.17**	1.63 $\pm$ 0.26
GPx, nmol GSH ·min ⁻¹ ·mg ⁻¹	LR	15.77 $\pm$ 1.15	11.63 $\pm$ 1.12	21.50 $\pm$ 3.09**	13.29 $\pm$ 1.51
	HR	14.98 $\pm$ 1.11	8.18 $\pm$ 1.0*	12.96 $\pm$ 1.23**	15.57 $\pm$ 1.29**

Values are expressed as the mean $\pm$ SEM, $n = 8$

LR – rats with low resistance to hypoxia; HR – rats with high resistance to hypoxia

* – $P < 0.05$ compared with control

** – $P < 0.05$ compared with Co treatment

Fig. 3. Oxidatively modified protein (OMP) level (aldehyde derivatives, AD - A; ketonic derivatives, KD - B) in the heart (E/mg protein) of rats with different resistance to hypoxia under pinacidil or glibenclamide effect and cobalt treatment. Values are expressed as the mean $\pm$ SEM, n = 8
 LR – rats with low resistance to hypoxia; HR – rats with high resistance to hypoxia
 * – P < 0.05 compared with control
 ** – P < 0.05 compared with Co treatment

Table 2. Correlation analysis between the level of lipid peroxidation, oxidatively modified proteins, and antioxidant enzyme activity in the heart of rats with low resistance to hypoxia upon cobalt treatment

Parameters	Correlation coefficient, P	Parameters	Correlation coefficient, P
Control		CoCl ₂	
MDA-CAT	-0.737, P = 0.036	MDA-CAT	-0.707, P = 0.049
TAA-OMP AD	-0.714, P = 0.046	TAA-OMP AD	-0.819, P = 0.013
TAA- GR	0.713, P = 0.047	TAA- GPO	-0.867, P = 0.005
OMP AD-CAT	-0.714, P = 0.031	OMP AD- GR	-0.884, P = 0.003
OMP KD- GR	-0.718, P = 0.044	OMP AD- GPO	0.778, P = 0.023

Table 3. Correlation analysis between the levels of lipid peroxidation, oxidatively modified proteins, and antioxidant enzyme activity in the heart of rats with high resistance to hypoxia upon cobalt treatment

Parameters	Correlative coefficient, P	Parameters	Correlation coefficient, P
Control		CoCl ₂	
OMP AD-SOD	-0.791, P = 0.019	TAA-OMP AD	0.920, P = 0.001
OMP AD- OMP430	0.914, P = 0.0015	TAA- SOD	-0.837, P = 0.01
OMP KD-SOD	-0.811, P = 0.015	TAA- CAT	0.836, P = 0.004
		TAA- GR	-0.839, P = 0.009
		TAA- GPO	0.834, P = 0.005
		OMP AD- SOD	-0.777, P = 0.023
		OMP370-GPO	0.726, P = 0.041
		SOD-CAT	-0.842, P = 0.010
		SOD- GPO	-0.799, P = 0.017
		GPO-GR	-0.862, P = 0.006

Fig. 4. Correlation between the TAA level and GPO and OMP AD levels (A) OMP AD and GR and GPO (B) in the heart of LR rats upon Co treatment

Fig. 5. Correlation between the TAA level and SOD and OMP AD levels (A), TAA and GR and CAT (B) in the heart of HR rats at Co treatment

Discussion

The aim of the current study was to evaluate the effects of K_{ATP} channel modulators pinacidil and glibenclamide administered intraperitoneally to rats in combination with Co on the antioxidant defence system of the heart, and to verify whether the outcome is dependent on subject's individual resistance to hypoxia. Free radicals and related molecules are classified as ROS for their ability to cause oxidative changes within the cell. A wide variety of ROS are produced in the course of normal metabolism in biological systems and they have several important physiological functions, but their accumulation beyond the needs of the cell can potentially damage lipids, proteins, and nucleic acids. Oxidative stress is a condition under which cellular antioxidant defences are insufficient to keep the ROS level below a toxic threshold (24, 29).

Oxidative stress is a normal cellular process involved in numerous aspects of cellular signalling (7,

23). The stress develops as a consequence of an imbalance between ROS production and manifestation, and the ability of biological systems to readily detoxify the reactive intermediates or to repair the resulting damage. Elevated cellular oxidative stress has been observed following exposure to heavy metals and connected injuries (29).

The cells possess an intricate network of defence mechanisms to neutralise excessive ROS accumulation, including antioxidant compounds (*e.g.*, GSH, selenium, zinc, vitamins E, C, and A) and antioxidant enzymes (*e.g.*, SOD, CAT, GR, and GP). Therefore, under physiological conditions, cells are able to cope with the ROS flux.

Scientific literature screening for metabolic changes in animals and humans caused by hypoxia reveals a general problem. The study groups in general use different time-dosage regimens to perform the investigations mostly in animals that lead to different levels of oxidative stress in the subjects. Different levels of oxidative stress as well as different time-

dosage regimens can have adverse effects on oxidative metabolism.

The results of this study provide evidence that treatment with Co administered to rats at a dose of 30 mg/kg b.w. induces effects in the heart tissue which are not related to LPO level and TAA value, and are not connected with the resistance of rats to hypoxia. We have not obtained any statistically significant differences in LPO and TAA levels. However, it is worth noting that Co treatment caused a decrease in the level of OMP in the heart of HR rats in comparison to controls.

In the present study we have observed differences in the investigated oxidative stress parameters and activity of antioxidant enzymes upon Co treatment, and these changes depended on the level of resistance to hypoxia (low or high resistance). GPx activity decreased only in rats with HR after Co treatment. We demonstrated a statistically significant decrease in SOD and CAT activity only in the heart of LR animals after administration of Co. These two types of animals: HR and LR were previously shown to exhibit two different functional and metabolic patterns (2, 27, 28). They are associated with typical differences in the activity of the central nervous system and neuro-humoral regulation, stress-activating and stress-limiting systems, oxygen-transporting function of the blood and the state of membranes or receptors. Moreover, these parameters are coupled with energy exchange and functional activity of the respiratory change in animal tissues.

Lukyanova and Kirova (19) have demonstrated that hypoxic preconditioning contributes to the development of immediate resistance, which is particularly pronounced in LR animals. These animals do not exhibit signs of oxidative stress in tissues during the early post-hypoxic period. By contrast, in HR animals hypoxic preconditioning is followed by activation of free radicals production in tissues. Therefore, activation of these processes in the early period of adaptation does not play a role in the induction of immediate adaptive mechanisms.

Bondarenko *et al.* (2) studied the behavioural reactions of rats with different resistance to hypoxia during different types of the functioning of their dopaminergic systems. They demonstrated that the locomotor activity in the open field test did not correlate with rat's resistance to acute hypobaric hypoxia. However, there was a correlation between this resistance and rat's behaviour during acute stress. Immobility was characteristic for rats with low and particularly medium resistance to hypoxia. This reaction can be abolished by antidepressants, while highly resistant rats were mainly hyperactive. The resistance to hypoxia was associated with extreme parameters of dopaminergic neuron functioning. In low-resistant rats locomotor stereotype was maximal, while perioral stereotype was the minimal; highly resistant rats were characterised by an opposite pattern,

and medium-resistant rats occupied an intermediate position.

Jones and Bergeron (12) reported that prior exposure of newborn rats to 8% hypoxia or CoCl_2 (60 mg/kg b.w.) protects the brain against ischaemic injury, but the mechanisms were found to be different in both cases. These authors reported that hypoxic preconditioning is connected with the modulation of glucose transport and glycolysis by hypoxia and may contribute to the development of hypoxia-induced tolerance (12). In contrast, preconditioning with cobalt chloride did not produce any change in HIF-1 target gene expression. It was suggested that different molecular mechanisms may be involved in the induction of tolerance by hypoxia and CoCl_2 in the brain. However, some investigations (8) showed that the cytoprotective mechanisms elicited by cobalt chloride were mediated by expression of HIF-1 α and its target genes. The differences could be dose-dependent, and attributed to the mode of treatment, as well as the period of CoCl_2 administration to the animals.

Kalpana *et al.* (13) reported that chemical preconditioning of rats with cobalt for 7 d (12 mg Co/kg b.w., orally) before exposing them to hypoxia significantly attenuated the vascular leakage induced by hypoxia. This was attributed to lower VEGF and ROS/NO levels, reduced NF κ B activity, and increased oxygen availability *via* Hif-1 signalling mechanisms. Furthermore, higher levels of anti-inflammatory mediators, namely TGF β , IL-6, HO-1, and MT in Co-preconditioned animals might also be responsible for attenuating the vascular leakage induced by hypoxia. Recently, Peters *et al.* (23) demonstrated that Co supplementation inhibited VEGF production induced by hypoxia in human ECs.

At lower oxygen conditions, HIF-1 α undergoes a stabilisation process and induces activation of genetic sequences that promote efficient adaptations to hypoxia. On the basis of the evidence provided by Xi *et al.* (30), the *in vivo* generation of IMA might be interpreted as an efficient endogenous mechanism of response to ischaemia, preventing myocardial damage or limiting the extent of myocyte necrosis.

Ingested Co is mainly translocated to the kidneys, whereas the liver has been reported to play an active role in rapid removing of Co ions from the blood. In the liver, Co has a long term biological half-life that causes a variety of toxic responses by hepatic cells. Oxidative stress has been proposed as the most important mechanism of Co toxic action in many organs of the body, including the heart, liver, spleen, and kidneys. Co accumulation is similar in fed and ATP-depleted cells. It is known that Co binds with the globin moiety of haemoglobin. The results imply that during long-term Co exposure *in vivo*, the element will be uptaken practically irreversibly by the red cells during their 120 d life span (20).

There is evidence that soluble Co cations exert

a genotoxic and carcinogenic activity (1, 20, 26) *in vitro* and *in vivo* in experimental systems, but there is no evidence in humans. The *in vitro* studies demonstrated that Co nanoparticles induced DNA strand breaks, micronuclei formation, chromosomal aberrations (aneuploidy, polyploidy, and tetraploidy), and morphological transformation of mammalian cell lines. Co nanoparticles exhibit higher genotoxicity than Co fine particles and ions. The nanoparticles also cause inflammation and oxidative stress. Investigators suggest that enhanced oxidative stress, inflammatory response, and abnormal apoptosis may play a major role in the carcinogenicity of cobalt-based nanoparticle-induced carcinogenesis (20).

Co is currently used as doping element because it is involved in the stimulation of erythropoiesis through erythropoietin (EPO) or its recombinant form (30). Recent studies have suggested that cobalt chloride can be used as doping element not only for humans but also for animals, in particular for horses. Cobalt chloride is a well-established hypoxia mimetic and inducer of hypoxia-like responses. It can cause gene modulation at the hypoxia-inducible factor pathway to stimulate EPO transcription and increase its levels in blood (12). Low (therapeutic) doses of cobalt chloride may enhance oxygen carrying capacity. Supraphysiological doses of cobalt chloride may result in systemic cytotoxicity, myocardial dysfunction, and acute inflammatory responses in the myocardium leading to cardiac arrest and death (6, 8, 11).

Experimental data (21) indicate some evidence of a genotoxic potential for Co *in vitro* in human lymphocytes, but there is no evidence available on its carcinogenic potential. There is evidence that hard metal particles exert a genotoxic and carcinogenic activity *in vitro*.

The results of correlation analysis performed in the present study provide evidence that Co effect on the heart tissue of animals with different resistance to hypoxia was mediated by different mechanisms of antioxidant defence (Tables 2 and 3). In control group of LR rats, correlation coefficient was lower in relation to LPO processes, estimated MDA level and CAT activity upon Co treatment. In addition a decrease in CAT activity was observed in rats with LR after Co treatment. Similar statistical relationships were demonstrated for TAA level and OMP AD value (from $r = -0.714$ to $r = -0.819$) after Co treatment of LR rats in comparison to controls. We found statistical difference in the formation of TAA level in LR animals, which was mediated by GR activity in controls. Upon Co treatment such relation depended more on GR, another enzyme of glutathione antioxidant defence (Fig. 4). On the contrary, in HR rats negative correlations were found ($r = 0.713$ versus $r = -0.867$). For control rats with HR, correlation analysis demonstrated statistically dependent relationship between the level of oxidatively modified proteins and SOD activity. In HR animals treated with Co, the role

of all analysed antioxidant enzymes, such as SOD, CAT, GR, and GPO in TAA formation decreased (Table 3 and Fig. 5). It is worth noting that in Co treated HR rats, a negative correlation was found between the glutathione-dependent enzymes such as GPO-GR ($r = -0.862$) and SOD-GPO ($r = -0.799$) (Table 3). The mechanisms of Co toxicity in different animal groups are not clear, but some of Co effects could be related to its high affinity to sulfhydryl groups, which may cause inhibition of crucial enzymes, e.g. of mitochondrial respiration (18).

K_{ATP} channel modulators during ischaemia constitute an important adaptive mechanism protecting the heart against stunning and infarction, as well as participating in preconditioning protection. K_{ATP} channels are normally closed by high ATP concentration and open during ischaemia when ATP generation diminishes. K_{ATP} channels increase the outward K⁺ current and reduce action potential duration. opening these channels attenuates myocardial stunning by reducing calcium influx (4).

The results of our study showed that after Co treatment, K_{ATP} channel modulator impact was significant. Therefore, pinacidil administration decreased the level of LPO in rats with LR vs. Co-treated group. Glibenclamide administration (K_{ATP} channel blocker) also caused a decline in LPO level. In rats with HR, a similar decrease (twofold) in LPO level in the heart was observed after glibenclamide administration. Simultaneously with LPO decrease, a significant increase in TAA level was noted in both groups of animals in comparison to the Co-treated groups after administration of K_{ATP} channel modulators. Our results suggest that the effect of glibenclamide in Co-treated rats depend on the individual resistance to hypoxia. TAA increased by 97% in LR animals and by 38.1% in HR rats in comparison with Co-treated animals.

The results of this study provide evidence that in LR group pinacidil causes a statistically significant decrease in OMP AD and OMP KD level when compared with the Co-treatment. In HR rats a similar decrease in the level of OMP AD was demonstrated upon administration of pinacidil. Glibenclamide treatment followed by Co administration in HR and LR animals decreased the concentration of oxidatively modified proteins as opposed to pinacidil effects. Our results demonstrated a significant decrease in LPO process, oxidatively modified protein content simultaneously with TAA level, and antioxidant enzyme activities under the influence of K_{ATP} channel modulators. Glibenclamide administration caused a significant decrease in the level of both antioxidants in HR rats compared with Co treatment. Pinacidil caused a decline in the activity of GR in both groups (HR and LR), while glibenclamide only in LR group vs. Co treatment. Conversely, in HR animals under pinacidil administration, an increase in GR activity was observed when compared to Co treatment. In LR and

HR rats K_{ATP} channels opener caused an increase in GPx activity, which depended on the level of resistance to hypoxia. At the same time, glibenclamide caused elevated GPx activity only in rats with HR vs. Co treatment.

The results of correlation analysis provided evidence that Co effect on the heart of animals with different resistance to hypoxia was mediated by different mechanisms of antioxidant defence. For LR rats upon pinacidil administration, the significant relation between the OMP KD and GPO ($r = -0.713$, $P = 0.000$) was demonstrated. Positive correlations were found for rats with HR under pinacidil administration between OMP KD and GPO ($r = 0.838$, $P = 0.005$) and SOD-CAT ($r = 0.836$, $P = 0.01$). The results obtained demonstrated that different metabolic pathways of the free radicals elimination are involved.

Our previous studies have established that pharmacological treatment with the K_{ATP} channel opener pinacidil causes the elevation of the mitochondrial respiration rate with a decrease in the efficiency of phosphorylation for FAD-generating substrates (succinate oxidation), but it was more effective in increasing the α -ketoglutarate oxidation with increments of the respiratory control ratio in the liver of guinea pigs (28). It is very important that guinea pigs have more effective hypoxia-dependent metabolic pathways in comparison with rats. FAD-generated substrate oxidation inhibited the efficiency of phosphorylation for α -ketoglutarate oxidation in mitochondria of pinacidil-treated guinea pigs. The results of oxidative phosphorylation are manifested by an increased rate of respiration and decreased phosphorylation efficiency for succinate oxidation. HR to hypoxia and other stress conditions are related to acetylcholine metabolism and domination of α -ketoglutarate oxidation as opposed to LR to hypoxia that is mediated by adrenaline and succinate oxidation in mitochondria (27).

Finally, it should be noted that in the present study cobalt treatment caused a significant decrease in the activity of superoxide dismutase and catalase in the heart tissue of animals with low resistance to hypoxia, and a significantly lower activity of glutathione peroxidase in the high resistance group. In the heart of animals highly resistant to hypoxia, Co treatment led to a decrease in the oxidatively modified protein level in comparison with control. Pinacidil had protective effect on heart tissue by preventing the lipid peroxidation and significantly reducing the level of oxidatively modified protein (as aldehyde and ketone derivatives simultaneously). It also caused an increase in TAA. The effects of pinacidil on the activity of antioxidant enzymes were not always compensated by glibenclamide influence.

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