Research Article

Molecular mechanisms of myocardial damage in the hypertensive rats and hypertensive rats with metabolic disorders (diabetes mellitus, atherosclerosis)

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Abstract

Introduction: Despite the success which was achieved in the treatment of arterial hypertension, for optimization of the treatment, it is necessary to study the pathogenesis of primary arterial hypertension and target organ damage on the molecular level.

Materials and methods: Our team studied the molecular mechanisms of myocardial damage during arterial hypertension and metabolic disorders. We used the spontaneously hypertensive rats (SHR) as an experimental model, and, additionally, we modeled diabetes mellitus and atherosclerosis in these rats.

Results and discussion: Our study obtained evidence of a much higher level of the energy imbalance in the cardiomyocytes and more intensive production of reactive oxygen species in the SHRs with diabetes mellitus and atherosclerosis compared with the healthy animals and the animals with only hypertension. The indicated defections create an environment for further cellular damage – mitochondrial dysfunction, depletion in the thiol-disulfide system, and formation of highly reactive NO products. At the same time, we have noticed a higher activity of the Hsp70 in the hypertensive groups compared with the normotensive animals. The source of these deviations is in the formation of mitochondrial dysfunction of cardiocytes, the cause of which is oxidative modification of the protein structures of mitochondria under conditions of activation of oxidative stress reactions, insufficiency of mPT pores, and impaired mitochondrial chaperone function. The presented data give reason to believe that mitochondrial dysfunction, which develops against the background of deficient HSP70, is an integral aspect of arterial hypertension, contributes to its aggravation, and triggers a cascade of molecular and biochemical mechanisms of myocardial damage. These mechanisms include disturbances in the L-arginine-NO-synthase-NO system, production of mitochondrial iNOS oxygen radicals, neutralization of the vasorelaxant effect of NO and its transformation into an active participant in nitrous stress due to reduced intermediates of the thiol-disulfide system. The question of cause-and-effect relationships of oxidative stress remains open for discussion.

Conclusion: We envisage that studies in this direction may lead to a better insight into a pathogenetic therapy of essential hypertension, diabetes mellitus, and atherosclerosis.

Graphical abstract:
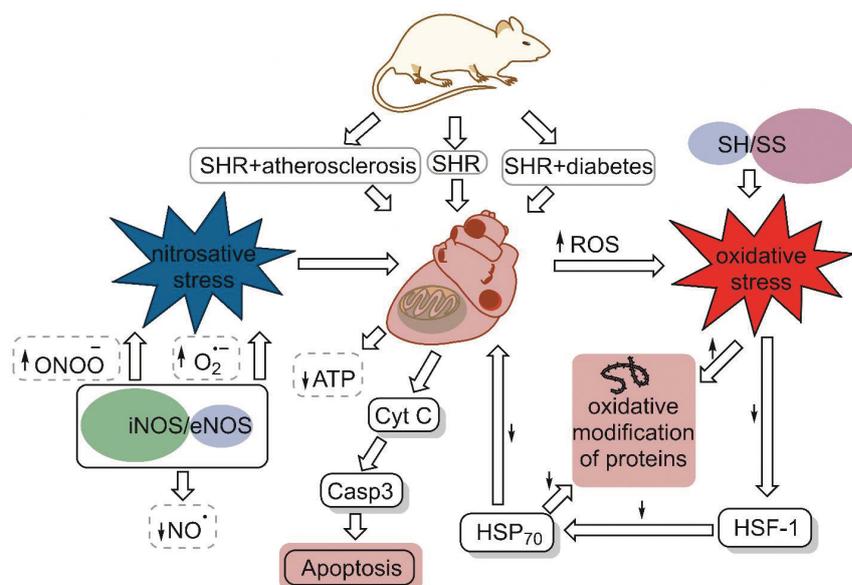
Keywords

arterial hypertension, diabetes mellitus, atherosclerosis, organ damage.

Introduction

Cardiovascular diseases are the world's biggest killers and lead to a significant reduction in the life expectancy of the population. These diseases have remained the leading causes of death over the decades. Arterial hypertension is currently one of the most common diseases ~40% of the world's population (Whelton and Williams 2018; Mills et al. 2020). In this regard, two provisions are decisive. First, the majority of patients with cardiovascular diseases have multiple risk factors (atherosclerosis, diabetes mellitus) that reinforce each other's action. It is necessary to understand how they interact and to identify common pathways in their pathogenesis. Secondly, vascular pathology usually manifests itself late in the development of the disease, when the process of damage to target organs (heart, brain, kidneys) has already been going on for many years. Despite that some success has been achieved in reducing cardiovascular events, it is still not enough. So, for further optimization of the treatment, it is necessary to study the pathogenesis of primary arterial hypertension and target organ damage on the molecular level. Advances in deciphering the mechanisms of the formation of hypertension are closely related to studies of the molecular messenger – NO. The unique chemical nature and a large number of intracellular targets for NO and its physiologically active redox forms leave open the question of how specifically the damaging effect is mediated. A number of studies have shown the participation of NO in the regulation

of blood pressure, the process of formation of endothelial dysfunction, in the formation of mechanisms of cytotoxicity and damage to target organs. However, the role of this system in the development of cytotoxicity has not been studied (de Champlain et al. 2004; Lankin et al. 2005; Pollock 2005). Oxidative stress in arterial hypertension suppresses the production and metabolism of NO, leads to desensitization of adreno- and angiotensin II receptors, changes in the selectivity of ion channels and impaired contractile function of the myocardium. A degree of damage to target organs largely depends on the balance of the oxidative-metabolic function and the activity of the antioxidant system. Therefore, an attempt to rethink the mechanisms triggered by arterial hypertension and leading to dysfunction of target organs, from the slightest metabolic shifts to the formation of gross morphological changes, is to consider these processes from the perspective of oxidative stress. In recent years, works have appeared that show that the formation of arterial hypertension, the transfer of normal regulation of blood pressure towards a progressive disease occurs against the background of a developing energy deficit (Mazur 2019). It is in this connection that elucidation of the role of mitochondrial dysfunction in the development of arterial hypertension, in the mechanisms of damage to target organs is of such great interest today. The damage intensity of the target organs depends on the oxidative function and activity of the antioxidant systems. Therefore, an attempt to rethink the molecular mechanisms triggered by arterial hypertension and leading

to impaired function of target organs is an essential component of the successful management of arterial hypertension.

So, the aim of our work was to study the molecular mechanisms of myocardial damage during arterial hypertension and metabolic disorders (diabetes mellitus and atherosclerosis).

Materials and methods

Animal models

All experimental studies and manipulations were carried out in accordance with the regulation on using the animals in biomedical experiments. The studies were performed on a sufficient number of experimental animals. The protocols of experimental studies were approved by the decision of the Ethical Committee of Zaporizhzhia State Medical University (Minutaes No. 13 of January 31, 2018).

The 10 mature male Wistar-Kyoto rats with a bodyweight of 220–270 g and 65 mature male spontaneously hypertensive rats (SHR) with a bodyweight of 280–300 g were treated in the ordinary laboratory environment (12 h light cycle, T = +22 °C) with free access to water and food. All rats were 8–9 months old. The animals were obtained from the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of the Ukraine. The duration of the quarantine (acclimatization period) for all the animals was 14 days. During the quarantine, every animal was inspected daily (behavior and general condition). Before the beginning of the study, the animals that met the criteria for inclusion in the experiment were divided into groups by using the randomization method. The animals that did not meet the criteria were excluded from the study during the quarantine. Cells with animals were placed in separate rooms.

Rodents were allocated into 4 experimental groups: 1st group – 10 Wistar-Kyoto rats (intact); 2nd group – 10 SHRs (control); 3rd – 30 SHRs with atherosclerosis; and 4th – 25 SHRs with diabetes mellitus (DM). Atherosclerosis was modeled by daily administration of hyperlipidemic oil mixture (cholesterol 40 mg/kg + ergocalciferol 350000 U/kg + tween-80 10 mg/kg) orally for 20 days. While simulating atherosclerosis, 7 animals from 3rd group died. Diabetes mellitus was simulated by a single intraperitoneal administration of streptozotocin in a dose 50 mg/kg after 12 h of starvation. During the first day after administration of streptozotocin, the rats received 20% glucose orally, next day – 10% glucose orally. Four rats from the 4th group died while modeling diabetes mellitus.

For all rats, the systolic blood pressure (BP) was measured on the tale artery by the non-invasive system BP-2000 (Visitech Systems, USA). The first measurement was done a day before the beginning of the experiment and then on the 20th days of the experiment. The BP measurement was carried out in silence, with the exclusion of loud noises and the human voice. The animals were allowed to get accustomed to the restrainer for 5 days before the

procedure. On the day of measurement, the animals were put in individual restrainers, then 10 measurements of BP were performed for each rat. The animals were in the restrainer for no more than 30 min.

On the last day of the experiment after the 16 h of starvation, the rats were anesthetized with 40 mg/kg of pentobarbitone intraperitoneally. Then the animals were devitalized.

We used Sigma-Aldrich (USA) for buying all the necessary chemical compounds.

Biochemical analysis

The heart was washed with cold 0.9% saline solution. The washed heart was cleansed from fat and connective tissue. Blood clots were removed from the internal cavities, and the heart was washed once more with 0.15 M of KCl (T = +4 °C) 1:10. Then we homogenized the heart in a 10-fold volume of medium (sucrose-250, tris-HCl buffer-20, EDTA-1 pH 7.4). A cytoplasmic fraction was isolated by a differential centrifugation method at a Sigma 3–30k reefer centrifuge (Germany) for 10 min at 1000 g, and then the supernatant was re-centrifuged for 10 min at 14000 g. A mitochondrial fraction was isolated by centrifugation for 7 min at 1000 g, and then the supernatant was re-centrifuged for 20 min at 16000 g. For long-term storage, the specimens were frozen at -80 °C.

The markers of oxidative damage of the proteins (aldehyde phenylhydrazone (APG) and ketone phenylhydrazone (KPG)) were determined by the interaction of oxidized amino acid residues with 2,4-dinitrophenylhydrazine (2,4-DNPH) and the formation of APG and KPG, which have an absorption spectrum of 274 nm and 363 nm, respectively.

The state of the antioxidant system was assessed by the activity of glutathione reductase (GR), and thiol content. The activity of GR was determined by the rate of NADPH reducing. The content of thiols was determined spectrophotometrically by their reaction with 5,5-dithio-bis-7-nitrobenzoic acid.

The state of energy metabolism was determined by the levels of the ATP, ADP, AMP and lactate activity. Adenyl nucleotides were determined by thin-layer chromatography. The lactate content was determined by the Hohorst method. As an advanced analysis of the state of myocardial energy supply, the additional parameters were calculated by taking the ratios of adenyl nucleotide's fractions:

- Energy charge: EC = ATP+1/2ADP/ATP+ADP+AMP;
- Energy potential: EP = ATP/ADP;
- Index of phosphorylation: IoF = ATP/ADP+AMP;
- Thermodynamic control of breath: TCoB = ADP/AMP.

We conducted studies of the opening of mitochondrial pores as the signs of mitochondrial dysfunction (violation of the barrier functions of mitochondrial membranes). The opening of the mitochondrial pores was determined spectrophotometrically.

Immunoblotting

Metabolites of NO were determined by the level of nitrite ions in the Griess reaction. The activity of total NOS was determined by the difference between the rates of NADPH oxidation. Nitrotyrosine was determined by a solid-state immunosorbent ELISA Kit.

Also, we analyzed the fractions of iNOS and heat shock proteins (Hsp70) in the heart homogenate. Proteins were separated in 10% polyacrylamide gel, and separation was carried out by electrophoresis at a voltage of 100 V. The proteins from the gel were transferred to a nitrocellulose membrane at a voltage of 100 V and 0.35 A for 1 h. After transfer, the membrane was placed in a buffer 1% bovine serum albumin for 20 h. After the washing in the shaker for 5 min with a solution of 0.1 M PBS, the membrane was placed in an antibody's solution to iNOS (1:500) or Hsp70 (1:500) and incubated for 2 h at room temperature, then was washed in the shaker 4 times for 5 min with 0.1 M of PBS. The membrane was placed in a solution of secondary antibodies (1:1000) and incubated for 2 h, then washed in a shaker 4 times for 5 min with 0.1 M of PBS. The membrane was placed in a solution of ExtrAvidin-Peroxidase + 1% solution of bovine serum albumin (1:1000), incubated for 1 h and washed. For visualization, the membrane was treated with a solution of AEK (1 tablet of 3-amino-9-ethylcarbazole was dissolved in 2.5 ml of DMF containing 47.5 ml of 0.05 M of acetate buffer, pH 5.0, 25 µl 30% H_2O_2). The membrane was incubated in the substrate mixture for 5–10 min, then washed with distilled water several times and dried between sheets of filter paper under a stream of cold air. We performed the assessment of the heart slides in ultraviolet excitation spectrum with the wavelength 390 nm by using a light filter with high emission 38HE (Carl Zeiss), microscope AxioScope (Carl Zeiss), immersion lens F-Fluar 40×/1.30

Oil (Carl Zeiss) and immersion oil Immersol 518F (Carl Zeiss). All the images were obtained with a camera Axio-Cam-ERc 5s (Carl Zeiss). All images were done with the same brightness, exposition and correction settings. Image analysis was performed in ImageJ (NIH, USA). We assessed the density of the immunopositive cells.

Statistical analysis

All statistical calculations were done by STATISTICA for Windows. The evaluation of differences between the groups was done by using one-way ANOVA or ANOVA for repeated measurements with post hoc Bonferroni correction or Kruskal-Wallis criterion with post hoc Dunn correction. A significant difference was considered at $P<0.05$.

Results

Oxidative protein modification is one of the earliest indicators of damage to the functional intracellular macromolecules. An analysis of our experimental data (Tables 1, 2) showed that SHRs with steadily increased blood pressure had highly prooxidant environment in the myocardial mitochondria and eminently enhanced oxidation of the protein molecules in the cytosolic fraction. The markers of the spontaneous and metal-catalyzed oxidative protein modification (APG and KPG) in the mitochondrial and cytosolic fractions were increased most significantly in the myocardium of the SHRs with diabetes mellitus. So, in mitochondrial factions, the levels of APG and KPG were 3.44 and 3.0 times higher in normotensive rats, respectively. The spontaneously hypertensive rats with atherosclerosis also had more intensive formation of APG and KPG in cardiomyocytes than the rats from group 1

Table 1. The indices of oxidative damage of proteins in the myocardial mitochondria

Experimental groups	BP, mm Hg	Spontaneous oxidative protein modification		Metal-catalyzed oxidative protein modification	
		APG, U/g protein	KPG, U/g protein	APG, U/g protein	KPG, U/g protein
SHR with DM (group № 4)	217±1.08[a]	28.84±1.03[a,b,c]	25.54±0.70[a,b,c]	44.31±1.24[a,b,c]	35.78±0.89[a,b,c]
SHR with atherosclerosis (group № 3)	201±1.3[a]	20.49±1.08[a,b]	19.77±0.61[a,b]	37.82±1.3[a,b]	29.76±0.62[a,b]
SHR (group № 2)	198±1.23[a]	9.84±0.33[a]	7.57±0.11[a]	30.81±1[a]	17.89±1.28[a]
Normotensive rat (group № 1)	105±1.1	2.86±0.4	2.5±0.53	7.26±0.84	5.28±0.58

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2; [c] - P<0.05 vs group № 3.

Table 2. The indices of oxidative damage of proteins in the myocardial cytosol

Experimental groups	BP, mm Hg	Spontaneous oxidative protein modification		Metal-catalyzed oxidative protein modification	
		APG, U/g protein	KPG, U/g protein	APG, U/g protein	KPG, U/g protein
SHR with DM (group № 4)	217±1.08[a]	52.56±1.55[a,b,c]	34.12±1.07[a,b,c]	65.01±2.55[a,b,c]	44.20±1.86[a,b,c]
SHR with atherosclerosis (group № 3)	201±1.3[a]	46.67±1.24[a,b]	27.94±1.46[a,b]	58.67±1.23[a,b]	39.14±1.31[a,b]
SHR (group № 2)	198±1.23[a]	37.13±0.65[a]	19.23±0.43[a]	50.45±2.09[a]	30.55±1.65[a]
Normotensive rat (group № 1)	105±1.1	15.72±0.25	8.77±0.24	23.57±0.58	14.24±0.14

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2; [c] - P<0.05 vs group № 3.

(intact rats) and group 2 (control SHRs), (7.1- and 7.8-time increase, respectively, in mitochondria and 2.96- and 3.1-time in cytosol), but the level of these compounds was lover compared with group 4 (SHRs with DM).

There was the most pronounced increase in products of metal-catalyzed OMB – (APG and KPG) –in 4.24 and in 3.4 times in mitochondria and in 2.14 and 2.14 times in the cytosol, respectively, compared with the normotensive animals. In the groups of SHRs with DM and atherosclerosis, the marker levels of OMB in mitochondria and cytosol of the myocardium were higher than in groups 1 and 2. An increase in the markers of metal-catalyzed OMB indicates the depletion of antioxidant reserves in the cytosol and mitochondria of the myocardium and a pronounced "hidden" degree of violation of cytosolic-mitochondrial compensatory shunts of energy production.

Our further studies revealed the significantly increased rates of the spontaneous opening of mitochondrial pores in the cardiomyocytes of the SHRs compared with the healthy animals (Table 3). An indicator, characterizing swelling mitochondria, rises in the rats with spontaneous arterial hypertension, also in SHRs with atherosclerosis and sugar diabetes, in 14.2, 29.2, and 31.63 times, respectively (when compared with the normotensive animals). The most severe mitochondrial dysfunction was observed in the myocardium of the SHRs with DM and atherosclerosis, but without a statistically significant difference between groups 3 and 4.

Table 3. The spontaneous opening of mitochondrial pores of cardiomyocytes

Experimental groups	$\Delta\Psi$
SHR with DM (group № 4)	0.348±0.01[a,b]
SHR with atherosclerosis (group № 3)	0.322±0.009[a,b]
SHR (group № 2)	0.157±0.012[a]
Normotensive rat (group № 1)	0.011±0.001

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2.

Energy deficit was observed in the cytosolic and mitochondrial fractions of the heart in the animals from 2nd, 3rd, and 4th experimental groups, and the deepest disturbances of energy production manifested themselves in the hypertensive rats with additional metabolic disorders – DM and atherosclerosis (Tables 4, 5). The lowest content of ATP in mitochondria was observed in the myocardium of SHRs with atherosclerosis and diabetes mellitus (2.0–2.6 times less than in control). The greatest ATP deficiency in the cytosolic fraction of myocardial homogenate was found in the group of SHRd with diabetes mellitus (2.36 times less than in control).

These data correlate with the discovered mitochondrial dysfunction and oxidative damage of important enzymatic and structural proteins in cardiomyocytes.

An in-depth analysis of the high-energy phosphate content in the mitochondria showed that there was no difference in the energy charge (EC) and the index of phosp-

Table 4. The indices of energy metabolism in myocardial mitochondria

Experimental groups	Lactate, µmol/g tissue	Lactate, µmol/g tissue	ATP, µmol/g tissue	ADP, µmol/g tissue	AMP, µmol/g tissue
SHR with DM (group № 4)	13.92±0.38[a,b]	2.92±0.38[a,b]	1.3±0.17[a,b]	0.73±0.05[a]	2.1±0.02[a,b,c]
SHR with atherosclerosis (group № 3)	12.96±0.32[a,b]	2.36±0.32[a,b]	1.7±0.14[a,b]	0.7±0.04[a]	1.3±0.04[a,b]
SHR (group № 2)	8.2±0.1[a]	3.90±0.09[a,b]	2.32±0.13[a]	0.8±0.04[a]	0.77±0.01[a]
Normotensive rat (group № 1)	4.07±0.17	4.07±0.177	3.49±0.15	0.95±0.01	0.3±0.02

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2; [c] - P<0.05 vs group № 3.

horylation (IoF) from the experimental groups (Table 6). However, the energy potential (EP), which reflects changes in the activity of the respiratory chains, was much lower in all SHR groups compared with the healthy animals. There was the lowest rate of this parameter in the SHRs with DM (2.25 times lower when compared with the normotensive rats).

A similar tendency was observed with thermodynamic control of breath (TCoB), which reflects the dependence of the respiratory chain activity on the phosphorylation intensity. This rate was reduced 1.4 times in the group of the SHRs with diabetes mellitus compared with the normotensive control. The largest decrease in the phosphorylation index, indicating a violation of the relationship between ATP and the pool of ADP-AMP, was observed in the SHRs with atherosclerosis (2.49 times lower than in the control). An analysis of the content of lactic acid in the mitochondria of the heart of the experimental ani-

Table 5. The indices of energy metabolism in myocardial cytosol

Experimental groups	Lactate, µmol/g tissue	ATP, µmol/g tissue	ADP, µmol/g tissue	AMP, µmol/g tissue
SHR with DM (group № 4)	17.26±1.34[a,b]	1.01±0.03[a,b]	0.52±0.01[a,b]	1.31±0.01[a,b]
SHR with atherosclerosis (group № 3)	17.86±1.03[a,b]	1.13±0.1[a,b]	0.5±0.03[a,b]	1.37±0.02[a,b]
SHR (group № 2)	9.18±0.18[a]	1.9±0.06[a]	0.7±0.03[a]	0.41±0.01[a]
Normotensive rat (group № 1)	5.46±0.24	2.36±0.11	0.83±0.02	0.16±0.01

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2.

mals (Tables 4, 5) showed its deficiency in mitochondria of the SHRs with diabetes and atherosclerosis, and the most significant (1.72 times compared with the control) decrease was observed in the SHRs with atherosclerosis.

Table 6. The parameters of state of the myocardial energy supply

Experimental groups	EC	EP	IoF	TCoB
SHR with DM (group № 4)	4.41	1.78	3.8	0.34
SHR with atherosclerosis (group № 3)	3.9	2.42	3.72	0.53
SHR (group № 2)	4.06	2.7	3.67	1.03
Normotensive rat (group № 1)	4.87	3.67	3.97	3.16

In the cytosolic fraction of the myocardial homogenate of the experimental animals, on the contrary, a significant increase in the level of lactate was found, indicating the activation of anaerobic glycolysis. The highest level of lactic acid was observed in the groups of SHRs with diabetes mellitus and atherosclerosis (16 times compared with the control), which, however, did not provide the energy needs of the myocardium.

Our further studies determined violations of the thiol-disulfide system (decreased restored equivalents, increased oxidized intermediates) in the cytosolic and mitochondrial fractions of the heart in the hypertensive animals from all groups. Thus, a decrease in GR activity was revealed in the heart of the SHRs compared with the normotensive group (Tables 7, 8). DM and atherosclerosis resulted in even more pronounced deprivation of this enzyme (in mitochondria – 3.1 and 7.88 times, respectively, and in cytosol – 3 and in 5.72 times, respectively). Decreased GR activity in the cardiomyocytes correlates with a significant deficiency of the reduced thiol compounds. Deprivation of GR activity in the myocardium of SHRs was observed against the background of a pronounced deficiency of reduced thiol compounds. Yes, in mitochondria and cytosol myocardium of SHRs without metabolic disturbances, the content of reduced thiols was 2.17 below the level of the normotensive animals. The greatest deficiency of reduced thiol compounds was observed against the background of a pronounced deprivation of activity GR – in the group of SHRs with atherosclerosis 3.32 and at 3.07 times lower in mitochondria and cytosol than thes level of the normotensive animals, respectively.

Also, we established a reduction in the NO formation in the heart of the SHRs due to a decreased NOS activity in the cytosol and mitochondria (the lowest NOS activity was in mitochondria and cytosol myocardium of the SHRs with atherosclerosis – 3.4 and 4.5 times lower than the values of the normotensive rats) (Tables 9, 10). DM and atherosclerosis led to a higher level of NO deficiency in the cardiomyocytes, as evidenced by a significant decrease in the NO stable metabolites – nitrite ions (the most expressed deficit of nitrite-anion in mitochondria and cytosol myocardium please check with atherosclerosis – 2.88 and 4 times lower than in the normotensive rats). The Western blot showed an increased iNOS expression in the myocardium of the SHRs compared with the normotensive group (Table 11). Also we found an increased content of the nitrosative stress marker (nitrotyrosine) in the animals with spontaneous hypertension on the background of increased expression of iNOS. Moreover, the highest

Table 7. Activity of the thiol-disulfide system in the myocardial mitochondria

Experimental groups	GR, μmol/ min/g protein	Thiols, mmol/g protein
SHR with DM (group № 4)	2.57±0.28[a,b]	163.58±12.66[a]
SHR with atherosclerosis (group № 3)	1.01±0.24[a,b,c]	126.04±8.24[a,b,c]
SHR (group № 2)	3.78±0.21[a]	193.9±7.74[a]
Normotensive rat (group № 1)	7.96±0.34	419.92±16.14

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group № 1; [b] - P<0.05 vs group № 2; [c] - P<0.05 vs group № 4.

Table 8. Activity of the thiol-disulfide system in the myocardial cytosol

Experimental groups	GR, μmol/ min/g protein	Thiols, mmol/g protein
SHR with DM (group № 4)	4.02±0.28[a,b]	200.51±9.66[a,b]
SHR with atherosclerosis (group № 3)	2.12±0.3[a,b,c]	157.27±18.51[a,b,c]
SHR (group № 2)	6.58±0.4[a]	248.91±6.79[a]
Normotensive rat (group № 1)	12.14±0.76	483.23±14.34

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group №1; [b] - P<0.05 vs group №2; [c] - P < 0.05 vs group №4.

Table 9. Activity of the NO system in the myocardial mitochondria

Experimental groups	NOS, nmol/ min/g protein	Nitrotirizines, nmol/g protein	Nitrites, μmol/g tissue
SHR with DM (group № 4)	1.42±0.2[a,b]	65.72±0.13[a,b]	1.84±0.29[a,b]
SHR with atherosclerosis (group № 3)	1.26±0.56[a,b]	71.03±0.14[a,b,c]	1.5±0.27[a,b]
SHR (group № 2)	2.7±0.17[a]	58.56±0.1[a]	2.9±0.35[a]
Normotensive rat (group № 1)	4.36±0.26	9.04±0.33	4.33±0.56

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group №1; [b] - P<0.05 vs group №2; [c] - P<0.05 vs group №4.

Table 10. Activity of the NO system in the myocardial cytosol

Experimental groups	NOS, nmol/ min/g protein	Nitrotirizines, nmol/g protein	Nitrites, μmol/g tissue
SHR with DM (group № 4)	3.48±0.31[a,b]	27.72±0.3[a,b]	4.35±0.8[a,b]
SHR with atherosclerosis (group № 3)	3.02±0.32[a,b]	35.03±0.09[a,b,c]	3.05±0.98[a,b]
SHR (group № 2)	7.15±0.64[a]	20.56±0.1[a]	7.76±0.67[a]
Normotensive rat (group № 1)	13.74±0.75	5.04±0.33	12.23±0.78

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group №1; [b] - P<0.05 vs group №2; [c] - P<0.05 vs group №4.

nitrotyrosine level was in the SHR group with atherosclerosis (7.0 and 7.85 times higher in cytosol and mitochondria compared with those in the normotensive values).

As a final point of our study, we found increased levels of the heat shock protein (Hsp70) in the cytosolic and mitochondrial fractions of the heart in the hypertensive animals from all the groups compared with the intact group (Table 12).

Table 11. Activity of the iNOS in the myocardial mitochondria

Experimental groups	iNOS, U/g protein
SHR with DM (group № 4)	2.4±0.01[a]
SHR with atherosclerosis (group № 3)	3.11±0.06[a,b,c]
SHR (group № 2)	2.28±0.16[a]
Normotensive rat (group № 1)	0.71±0.02

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group №1; [b] - P<0.05 vs group №2; [c] - P<0.05 vs group №4.

Table 12. Level of Hsp70 in the heart

Experimental groups	Hsp70, U/g protein	
	Mitochondrial fraction	Cytosolic fraction
SHR with DM (group № 4)	10.74±0.22[a,b]	12.12±0.27[a]
SHR with atherosclerosis (group № 3)	11.8±0.43[a,b,c]	13.48±0.18[a,b,c]
SHR (group № 2)	9.35±0.37[a]	12.92±0.32[a]
Normotensive rat (group № 1)	6.51±0.26	8.78±0.3

Note: Data are expressed as mean ± standard error. [a] - P<0.05 vs group №1; [b] - P<0.05 vs group №2; [c] - P<0.05 vs group №4.

Discussion

Biochemical studies of the myocardial cytosol and mitochondria in the group of SHRs, especially in the groups of hypertensive rats combined with DM or atherosclerosis, revealed signs of intensive oxidative stress.

The primary "burst" of free radical reactions may be observed due to the excessive release of catecholamines leading to a formation of reactive oxygen species (Dhalla 2018; Zhou et al. 2019). A sympathetic nervous system under chronic stressful conditions, such as hypertension, DM, atherosclerosis, is associated with an increased level of synaptic and circulating catecholamines. It is well known that alterations in hemodynamics or coronary blood flow (e.g., due to artery constriction, stimulation of cardiac contraction) because of high concentrations of catecholamines can induce hypoxic environment in the tissue. When systems of catecholamine biotransformation (catechol-O-methyl transferase, monoamine oxidase) are saturated or damaged in the aggressive environment, high levels of catecholamines become a substrate for intensive oxidation. The catecholamine oxidation results in the formation of aminochromes (highly reactive and toxic compounds). In turn, aminochromes can produce oxyradicals that lead to further myocardial cell damage due to oxidative modification of the functional compounds (e.g., components of the channels, enzymes).

Common metabolic disorders, such as atherosclerosis and DM, are associated with increased production of reactive oxygen species as well, which can be considered a key process, especially under permanent high blood pressure (Stocker and Keaney 2004; Yuan et al. 2019). So, the SHRs with atherosclerosis have a greater intensity of the oxidative stress compared with the hypertensive rats, and, as a result, possible oxidative modification of low and high density lipoproteins, thereby promoting further damage. There is the highest level of oxidative modification in the SHRs with DM. This can be due to more severe energy depletion in the cells and the production of more oxyradicals through redox cycling.

An increased level of the markers of oxidative modification, which we discovered in our study, indicates depletion of the antioxidant reserves in the myocardial cytosol and mitochondria, disturbance in the mitochondrial-cytosolic shunts of energy production, and mitochondrial dysfunction.

Mitochondria are a key to cell survival; they are actively involved in a complex network of intracellular regulation of different metabolic processes – from the exchange of specific metabolites to the release of apoptogenic factors.

Mitochondrial abnormalities, which we discovered in our study, are accompanied by arterial hypertension and are at a much higher level in the SHRs with DM or atherosclerosis. Increased production of reactive oxygen species modifies mitochondrial membrane fluidity, energy production, and, finally, provokes a formation of vicious cycles with further oxidative damage. Additional oxidation of some important functional cell components, such as cardiolipin, can sensitize mitochondria to calcium, which is a strong trigger for the mitochondrial pore opening and apoptosis.

This definitely emphasizes the importance of mitochondria as therapeutic targets in the treatment of hypertension, DM, and atherosclerosis. Our previous studies and the studies of other authors showed that some drugs can reduce mitochondrial injury secondary to hypertension (Plummer et al. 2015; Belenichev et al. 2018; Sorriento et al. 2018; Mazur et al. 2019). The drugs, such as antioxidants, that can specifically target the mitochondria may have significant potential to eliminate mitochondrial dysfunction and prevent end-organ damage due to hypertension, DM, and atherosclerosis.

The processes of disruption of ATP synthesis in the mitochondria are closely related to or, possibly, directly depend on the intensity of oxidative modification of mitochondrial proteins and other important structural molecules and the concentration of calcium inside mitochondria. We assume that the most significant step toward reducing myocardial energy supply during hypertension, and especially on the background of metabolic disorders, is an oxidative violation of permeability of the mitochondrial membranes with the unlimited opening of the mitochondrial pores. This creates an environment for an uncontrolled yield of the numerous matrix proteins, which can even initiate programmed cellular death. Myocardial energy deficit can significantly aggravate hypertension, DM, atherosclerosis and contribute to the end-organ damage (Madamanchi and Runge 2007; Sivitz and Yorek 2010; Eirin et al. 2014).

The revealed reduced NOS activity in the mitochondria and cytosol of the myocardium in the hypertensive rats from all the experimental groups can be associated with the reorientation of the nitroxidergic system during the pathological conditions. The overproduction of reactive oxygen species, calcium overload, and mitochondrial

dysfunction may additionally form highly reactive NO products, such as peroxynitrite. The uncontrolled formation of cytotoxic derivatives of NO leads to nitrosylation of active protein components of channels, receptors, intracellular messengers, etc, and can induce nitrosative stress. All this can definitely increase the severity of cardiovascular and metabolic disturbances (Mozos and Luca 2017; Bruno et al. 2019).

Also on this background, the expression of inducible NOS is enhanced in the mitochondria (especially, in the SHRs with atherosclerosis). The higher level of iNOS expression can be due to a much higher intensity of the inflammation that is present during atherosclerosis. A further increase in iNOS activity and depletion in the thiol-disulfide system of mitochondria can lead to the apoptosis initiation. Under physiological conditions, the components of the thiol-disulfide system contribute to the normal NO deposition and transportation, NO bioavailability and prevention of the formation of peroxynitrite.

Comparing the results of the studies conducted, it can be concluded that the largest changes were observed in SHRs with the most prominent shifts of the myocardial thiol-disulfide system (SHRs with atherosclerosis), and this can easily turn NO from an important cellular messenger into an agent of the nitrosative stress. In this regard, it is very important to verify the specific factors determining an fine border between the cytoprotective or cytotoxic properties of NO at a certain stage of the disease.

Heat shock proteins (Hsp70) are induced in the cells of all living organisms in response to the numerous stress factors, such as hypoxia, ischemia, and metabolic disturbances (Pockley et al. 2003; Pauletto and Rattazzi 2006; Srivastava et al. 2016). These proteins act as intracellular molecules with chaperone function and cell protection, help in protein folding, translocation of other proteins, and removing defective proteins. Also, the pro-inflammatory processes are well correlated with an increased level in Hsp70. We found in our study the highest activity of Hsp70 in the SHRs with atherosclerosis, one of the basic pathogenetic mechanisms of which is inflammation and reactive oxygen species formation.

So, the elevation of Hsp70 may have supposed to be a risk factor for the progression of cardiovascular diseases, including hypertension and atherosclerosis. On the other hand, this can support the concept that Hsp70 might protect against the progression of cardiovascular events.

Conclusion

Our study obtained evidence of the presence in the SHRs with metabolic disorders (diabetes mellitus and atherosclerosis) of a much higher level of the energy imbalance in cardiomyocytes and more intensive production of reactive oxygen species compared with the healthy animals and animals with only hypertension.

Indicated defections create an environment for further cellular damage – mitochondrial dysfunction, depletion in the thiol-disulfide system, and formation of highly reactive NO products. At the same time, we noticed a higher activity of Hsp70 in the hypertensive groups compared with the normotensive animals, which can potentially be a marker of progression of the cardiovascular diseases or can play an important role in cell protection.

However, the question of the causal relationships remains open for discussion. The future studies are required to determine whether these factors play a causative role in the pathogenesis of essential hypertension, diabetes mellitus, atherosclerosis, or they are the consequences of the diseases. We envisage that studies in this direction may lead to get a better insight into a pathogenetic therapy of essential hypertension, diabetes mellitus, and atherosclerosis.

Conflicts of interests

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