

# Molecular and Biochemical Mechanisms of Mitochondrial Dysfunction in Spontaneously Hypertensive Rats

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## Abstract

**Objective:** In conditions of hypertension, ATP synthesis can be violated that can lead to the development of energy deficit of cardiomyocytes. One of the reasons of such energy deficit could be mitochondrial dysfunction. Aim of this work was study of molecular and biochemical mechanisms of mitochondrial dysfunction in spontaneously hypertensive rats. **Materials and Methods:** Experiment was carried out on normotensive albino male rats and spontaneously hypertensive male rats. Animals were kept in the vivarium during 20 days. On Day 20 of the experiment

we measured blood pressure of rats by plethysmography. In the myocardium of normotensive rats and animals with spontaneous hypertension we defined the degree of opening of mitochondrial oscula, the content of adenine nucleotides in the mitochondrial and cytosolic fractions of rat myocardium and energy metabolism parameters (energy charge, energy potential, phosphorylation rate and thermodynamic breath control). **Results:** We observed the opening of mitochondrial oscula in the myocardium of spontaneously hypertensive rats. In mitochondrial fraction of spontaneously hypertensive rats heart homogenate we defined a significant growth of ADP, reduction of “energy potential” and a tendency to lower the content of ATP, “energy charge” and “phosphorylation rate”. In spontaneously hypertensive rats heart homogenate we found the decreasing of heat shock protein (HSP 70) content in the both mitochondrial and cytosolic fractions. **Conclusions:** Thus, we determined the formation of cardiomyocyte mitochondrial dysfunction in spontaneously hypertensive rats. HSP 70 deficit, which was found in cytosolic and mitochondrial fractions of spontaneously hypertensive rats myocardium, was one of the causes of mitochondrial dysfunction.

**Keywords:** Hypertension, heat shock proteins, mitochondrial dysfunction

## Introduction

Hypertension occupies leading role in the structure of morbidity and mortality of the population in both developing and developed countries. [1-3]. One-third of the world adult population suffers from this disease and its prevalence with age only increases [4]. Hypertension can lead to target organ damage, and to development of many cardiovascular diseases (stroke, heart failure and chronic kidney disease) [1, 5, 6]. Despite the updating of diagnosis and pharmacotherapy of the disease, the need for antihypertensive therapy to treat damaged organs and, especially myocardium, requires continuous and deep researches aimed at clarifying molecular and biochemical mechanisms of the disease pathogenesis and, first of all, indicators reflecting the morphology and characterizing the level of myocardium energy supply [7, 8].

ATP amount in heart, that used per minute of contraction, is much higher than the ATP pool, so maintaining of high ATP content is critically important for normal cardiac performance [9-11]. In conditions of hypertension, ATP synthesis can be violated that can lead to the development of energy deficit of cardiomyocytes [12]. So that mitochondrial dysfunction, which can accompany hypertension, can be a reason of energy deficit of cardiomyocytes [12]. Energy deficit may be associated with mitochondrial dysfunction, which in its turn can be caused by abnormal content of heat-shock proteins (HSPs). This group of stress-induced proteins, mainly HSP 70, are actively synthesized when cells are exposed to oxygen free radicals and nitric oxide [13, 14]. HSP 70 regulatory effect provides protection to cells from many different stressors, and abnormal content of this protein can lead to development of biochemical violations.

Based on the foregoing the aim of this work was study of molecular and biochemical mechanisms of mitochondrial dysfunction in spontaneously hypertensive (SH) rats. Since hypertension disturbs not only function, but also myocardial morphology and metabolism, it was important to identify possible changes in energy production and the level of heat-shock proteins in cardiomyocytes [15].

## Materials and Methods

### Animals

The experiment was carried out on eight normotensive albino male rats weighing 220-270 g and ten male spontaneously hypertensive (SH) rats weighing 220-300 g. All animals were on standard food ration of vivarium, with natural alteration of day and night during 20 days. Rats were received from nursery of «Institute of Pharmacology and Toxicology, Academy of Medical Sciences of Ukraine». All experimental procedures and operative interventions were done in accordance with WMA Statement on Animal Use in Biomedical Research.

### Experimental procedure

On 20th day of the experiment we measured blood pressure of all rats by plethysmography using Transoni K Animal Research Flowmeter T-106 Series (USA). On 21th day, the animals were decapitated under thiopental anesthesia and their hearts were removed to conduct biochemical tests. The heart was rinsed in chilled saline and homogenized in liquid nitrogen in a mortar. Accurately weighed portion was placed into 0.1514 KCl (final dilution 1:10). Cytoplasmic fraction was isolated by differential centrifugation on Sigma 3-30 K refrigeration centrifuge (Germany) at a temperature (4°C) in a 10-fold volume of medium containing 250 of sucrose (mmol), 20 of Tris-HCl-buffer and EDTA-1 (pH 7.4). To purify mitochondrial fragments, they were previously exposed to centrifugation at 1000 g for 7 minutes, and then the supernatant was repeatedly centrifuged at 16 000 g for 20 minutes.

### Biochemical analysis

**Adenine nucleotides content.** Protein-free mitochondria extract of cardiac tissue was exposed to quantitative determination of adenine nucleotides (ATP, ADP, AMP) content by thin layer chromatography using Silufol plates. After separation in a mobile phase consisting of a dioxane, isopropanol, water and ammonia (4: 2: 4: 1), nucleotides were identified under ultraviolet light (260 nm) based on eluates absorption. The results were calculated on the basis of the calibration curve and expressed in  $\mu\text{moles/g}$  of tissue [16].

**In-depth analysis of energy supply.** To deeply analyze the level of myocardium energy supply, we estimated additional parameters of energy balance with due account for adenine nucleotides fraction ratio [17], namely:

- energy charge (EC) =  $(ATP + 1/2ADP)/(ATP + ADP + AMP)$ , which displays the degree of filling of the ATP-ADP-AMP system by macroergic bonds;.
- energy potential (EP) =  $ATP/ADP$ , which indicates about state of mitochondrial electron transport chain;
- phosphorylation rate (PR) =  $ATP/ADP + AMP$ ; which display the ratio between ATP and ADP- AMP pool;
- thermodynamic breath control (TBC) =  $ADP/AMP$ , which displays dependence of activity of mitochondrial respiratory chain from concentration of some components of adenine-nucleotide system and intensity of phosphorylation in general [18].

**Studying of giant mitochondrial oscula opening.** Since disruption of barrier functions of mitochondrial membranes is a sign of mitochondrial dysfunction, we've studied the process of opening of giant mitochondrial oscula (MO) in mitochondria isolated from trial animals' heart tissue. For this purpose, hearts of animals were washed with chilled 0.15 M KCl solution at 4°C. Washed hearts were purified from fat, connective tissue and vessels. Then blood clots were removed from the interior heart cavities. After this hearts were washed again with 0.15 M KCl solution at 4°C. Then heart tissue was thoroughly comminuted and homogenized in 1000 % w/vol of the medium consisting of: sucrose – 250 mM, Tris-HCl-buffer – 20 mM (pH 7.4) and EDTA – 1 mM. Mitochondria were isolated at 4°C by differential centrifugation in the refrigerated centrifuge Sigma 3-30k (Germany). For cleaning the mitochondrial fraction from large cell fragments primary centrifugation was conducted for 7 minutes at 1000 g, and then supernatant was centrifuged for 20 minutes at 17000g. The supernatant was decanted and stored at -80°C. The pellet of mitochondria was resuspended in the medium, containing bovine serum albumin (0.5 mg/ml) and then precipitated by centrifugation for 10 minutes at 17,000 g. The mitochondria were suspended in the isolation medium, suspension contained 40-60 mg protein/ml. To record the opening of MO, to incubation mixture, which consisted of 120 µM of KCl, 0.5 mM of  $KH_2PO_4$ , 2 mM of glutamate, 1 mM of malate and 20 mM of Tris-HCl-buffer (pH 7.4) was added 1 mg of mitochondria suspension. Mitochondrial membranes barrier function changes were determined spectrophotometrically as a decrease in absorbance at 540 nm caused by mitochondria swelling. The process was induced by introduction of 50 µM of  $Ca^{2+}$  into non-mitochondrial medium after  $Ca^{2+}$ -recharge ( $\Delta E$ ) in the study samples, which characterized the intensity of the process.

**HSP 70 content.** Concentration of HSP 70 in the cytoplasmic and mitochondrial fractions of heart homogenate was determined by Western blotting. The proteins were separated in 10% polyacrylamide gel electrophoresis. Separation of protein fractions was performed by electrophoresis at a voltage of 100 V (for gel sealing) until the sample reached the gel interface. Then, with a voltage of 200 V until the sample reached the end of the gel.

The proteins were transferred from polyacrylamide gel to the nitrocellulose membrane by electroelution during 45 minutes. After transfer the membrane was placed in blocking buffer containing 1% solution of bovine serum

albumin (SIGMA, USA, cat. A2153) for 20 h. Then membrane was washed for 5 minutes by 0.1 M phosphate buffer, placed in a solution of a primary antibodies against HSP 70 (1: 500), (Santa Cruz Biotechnology, cat. sc-24) and incubated for 2 hours at room temperature. Then membrane was washed for four times by 0.1 M phosphate buffer, placed in a solution of secondary antibodies (1:1000) (SIGMA, USA, Cat. №051M4885) and incubated for 2 hours. After washing by 0.1 M phosphate buffer, membrane was placed in a solution of ExtrAvidin-peroxidase (SIGMA, USA, Cat. №051M4885) in 1% solution of bovine serum albumin (1:1000) and incubated for 1 hour. For visualization membrane was processed with a solution, which contained: 1 tablet of 3-amino-9-ethylcarbazole (Sigma, USA, cat. a6926), 2.5 ml of DMF, 47.5 ml of 0.05 M acetate buffer (pH = 5.0) and 25  $\mu$ l of 30 % H<sub>2</sub>O<sub>2</sub>. Then membrane was washed with distilled water for several times and dried between sheets of filter paper under a stream of cold air. Detection of HSP 70 was performed with use of densitometry in the program Adobe Photoshop [19, 20].

### Statistical analysis

Results of the experiment were processed using STATISTICA® 6.0 licensed software for Windows (Stat Soft Inc NAXXR712D833214FANS). Individual statistical procedures and algorithms were implemented in the form of specially written macros. Results were represented as sample mean  $\pm$  standard error of the mean. Normality of distribution was assessed by Shapiro-Wilk test (W). Significant differences between trial groups were evaluated by means of Student t-test and Mann-Whitney U-test. For all types of analysis, the differences were deemed to be statistically significant at  $p \leq 0.05$  [21].

## Results

Carried out studies showed of increasing of spontaneous MO opening and swelling rates in mitochondria of SH rats in comparison with normotensive animals. Thus, average index  $\Delta E$  of test group was increased in 7 times, that indicated about high intensity of the mitochondria swelling in the cardiomyocytes of SH rats (Table 1).

We also noted such tendencies during comparison of adenine nucleotides content in cytosolic and mitochondrial fractions of test group heart homogenate (Tables 2 and 3). In both fractions was defined significant decreasing of ATP content in test group: on 33.3 % in cytosolic fraction and 23.3 % in mitochondrial fraction in comparison with control group. In the same time, AMP content in both fractions increased on 57.3 % and 33.3 % respectively. ADP content in both fractions was higher in SH rats heart homogenate in comparison with normotensive animals. But increasing of ADP content in mitochondrial fraction was more significant (45.4 % against 30.6 % in cytosolic fraction).

The energy supply indexes in both groups were different (Table 4). Thus, EC, EP and PR indexes of test group were decreased on 14.86 %, 58.27 %, and

57.42 % respectively in comparison with control group. TBC index was slightly increased (on 4.77 %).

Biochemical studies showed also significant decrease of HSP 70 content in both fractions of heart homogenate of SH rats (Figure 1 and 2, Table 5). It should be noted, that HSP 70 content decreased in mitochondrial fraction of test groups in 8 times and this index was more pronounced than index of HSP 70 content decreasing in cytosolic fraction.

## Discussion

Changes in the mitochondrial structure are key reason for cell degeneration. During the experiment we assessed the functional state of SH rats cardiomyocytes mitochondria, that were actively involved in a complex connection of intracellular regulation of metabolic processes. In pathological processes (hypertension, ischemic heart disease, heart failure) that are accompanied by oxidative stress, mitochondria can be damaged by metabolites, which were formed by the influence of reactive oxygen species (ROS) [22]. Also ROS may cause release of apoptotic factors initiating cell death.

Changes in the permeability of mitochondrial membranes (resulting from dysfunction) could cause formation of a number of mitochondrial oscula and could play central role in the production of reactive oxygen species (ROS). All these could lead to initiation of programmed mitochondrial death and subsequent development of apoptosis [23]. Development of mitochondrial dysfunction lead to disruption of reuptake of neurotransmitters, generation and conduction of impulse, protein synthesis, translation and transcription processes [24]. Hydroxyl radical could cause the opening of mitochondrial oscula with the expression and releasing of proapoptotic proteins to the cytosol [25]. Oscula are opened due to the oxidation of thiol groups of cysteine-dependent protein of inner mitochondrial membrane that prevents its penetration into non-specific channel [26]. In the group of SH rats we noted increasing rate of spontaneous MO opening and swelling of cardiomyocytes mitochondria, which testified of their disruption (Table 1).

It should be noted, that mitochondria suspension of SH rats demonstrated increase absorbency, which also testified about increasing of mitochondria swelling and MO opening. MO closing/opening mechanism acts as functional unity, thereby transforming and strengthening signals coming upon activation of inosine-1,4,5-triphosphate-coupled receptors and mediating regulation of relationship between functional segment and energetic processes of the cell. In SH rats group we found increasing of index that characterizes the swelling of mitochondria, which in its turn should lead to an energy deficit [20, 27].

Changes in adeninyl nucleotides content in mitochondrial and cytosolic fractions of test group were insignificant (ATP tended to decrease, while AMP – to increase in both fractions) in comparison with control group. ADP content was significantly increased only in the mitochondrial fraction. We also noted the downward tendency in energy metabolism indicators. Thus, EP fell significantly,

but TBC did not change much. Such changes were obviously due to the ability of SH rats myocardium to maintain ATP level by activating anaerobic glycolysis and not due to the influence of oxidative phosphorylation onto the process (Tables 2, 3).

Abnormal intracellular permeability of mitochondrial membranes, which leads to the formation of MO, assumes the emergence of a number of matrix proteins [26]. So, we observed decrease of HSP 70 content in the cytosolic and mitochondrial fractions of SH rats heart homogenate in comparison with normotensive animals (Table 4). Such decreasing of HSP 70 content testified about mitochondrial dysfunction.

It is known that heat-shock proteins are induced in response to many stress factors [28, 29], including hypoxia that can accompany hypertension. Major HSP 70 protective functions are associated with their chaperone action [30], i.e. with the ability to recognize damaged or newly synthesized polypeptides, correct their structure by ATP-mediated manner, and remove unrecoverable proteins by proteasome apparatus. In recent years, there were some works about regulation of HSP 70 action on mitochondrial dysfunction, evolving in ischemic brain damage [26]. In our previous studies, we also defined *in vitro* that HSP 70 was capable to prevent fragmentation of citrate synthase, glutathione-S-transferase, glutathione reductase, superoxide dismutase, lactate dehydrogenase, malate dehydrogenase from oxidative damage and regulate thiol disulfide balance [31]. Furthermore, one of the main HSP 70 functions is induction and extension of stable life of HIF-1a, which includes further adaptive response in the cell [32]. We found that HSP 70 extended HIF-1a action and independently regulated expression of mitochondrial NAD-malate dehydrogenase, thereby supporting long active compensatory mechanism generating ATP-malate-aspartate shuttle mechanism [19]. HSP 70 deficiency is one of the reasons of emergence of mitochondrial dysfunction with all its consequences for the life of the cell. HSP 70 deficiency in SH rats myocardium might be associated with mitochondrial dysfunction [26, 33, 34], disruption of prooxidant-antioxidant homeostasis and increasing of number of markers of oxidative modification of proteins, which we had found in the previous studies. We assumed that increasing of production of reactive oxygen species, cytotoxic forms of nitric oxide, which caused modification (reversible and irreversible) of macromolecules, including HSP 70, could reduce expression of gene and could lead to decreasing of protein synthesis.

Thus, we defined the formation of cardiomyocyte mitochondrial dysfunction in spontaneously hypertensive rats, which was caused by an oxidative modification of mitochondrial protein structure induced by an activated oxidative stress, which we had revealed in the previous studies [22, 35]. At the same time, we found decreasing of ATP production in the SH rats myocardium homogenate. But this decrease was not significant that could be explained by activation of anaerobic glycolysis to compensate decreasing of oxidative phosphorylation level in condition of mitochondrial dysfunction. One of the reasons, that caused such dysfunction was HSP 70 deficit, which we found in SH rats cardiomyocytes.

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**Table 1:** Mitochondrial oscula opening rate of SH and normotensive rats

Group	BP, mm of Mercury	$\Delta E$ , eV
Test group (SH rats, n=10)	150	0.146±0.012
Control group (normotensive rats, n=8)	126	0.018±0.001

SH rats – spontaneously hypertensive rats. BP – average group blood pressure.  $\Delta E$  results are represented in eV as sample mean  $\pm$  standard error of the mean. P of test group results  $\leq 0.05$  in comparison with control group

**Table 2:** Adenine nucleotides content in cytosolic fraction of heart homogenate of SH and normotensive rats

Group	ATP $\mu\text{mol/tissue}$	ADP $\mu\text{mol/tissue}$	AMP $\mu\text{mol/tissue}$
Test group (SH rats, n=10)	2.326 $\pm$ 0.098	0.692 $\pm$ 0.036	0.368 $\pm$ 0.013
Control group (normotensive rats, n=8)	3.490 $\pm$ 0.156	0.530 $\pm$ 0.025	0.234 $\pm$ 0.014

SH rats – spontaneously hypertensive rats. All results are represented as sample mean  $\pm$  standard error of the mean. P of test group results  $\leq 0.05$  in comparison with control group

**Table 3:** Adenine nucleotides in mitochondrial fractions of heart homogenate of SH and normotensive rats

Group	ATP $\mu\text{mol/tissue}$	ADP $\mu\text{mol/tissue}$	AMP $\mu\text{mol/tissue}$
Test group (SH rats, n=10)	1.816 $\pm$ 0.066	0.8 $\pm$ 0.04	0.276 $\pm$ 0.015
Control group (normotensive rats, n=8)	2.369 $\pm$ 0.0118	0.55 $\pm$ 0.016	0.207 $\pm$ 0.02

SH rats – spontaneously hypertensive rats. All results are represented as sample mean  $\pm$  standard error of the mean. P of test group results  $\leq 0.05$  in comparison with control group

**Table 4:** Energy balance in myocardium mitochondria of SH and normotensive rats

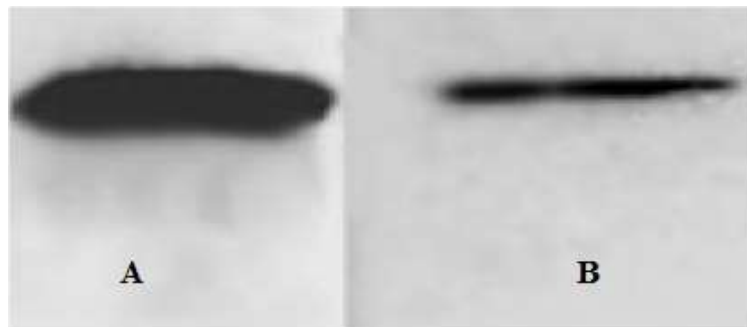
Group	EC	EP	PR	TBC
Test group (SH rats, n=10)	2.762 $\pm$ 0.052	1.794 $\pm$ 0.135	1.331 $\pm$ 0.102	2.94 $\pm$ 0.169
Control group (normotensive rats, n=8)	3.244 $\pm$ 0.13	4.299 $\pm$ 0.144	3.126 $\pm$ 0.128	2.806 $\pm$ 0.256

SH rats – spontaneously hypertensive rats. EC – energy charge, EP – energy potential, PR – phosphorylation rate, TBC – thermodynamic breath control. All results are represented as sample mean  $\pm$  standard error of the mean. P of test group results  $\leq 0.05$  in comparison with control group

**Table 5:** HSP 70 content of heart homogenate of SH and normotensive rats

Trial animals	HSP 70, u/g of protein	
	Cytosolic fraction	Mitochondrial fraction
Test group (SH rats, n=10)	2.326±0.32	0.758±0.304
Control group (normotensive rats, n=8)	8.782±0.304	6.515±0.264

SH rats – spontaneously hypertensive rats. HSP 70 content was expressed in conventional units per gram of protein. All results are represented as sample mean  $\pm$  standard error of the mean. P of test group results  $\leq 0.05$  in comparison with control group

**Figure 1:** Results of heat shock protein HSP70 immunodetection in the cytosolic fraction of the rats heart homogenate. A – normotensive rats; B – SH rats**Figure 2:** Results of heat shock protein HSP70 immunodetection in the mitochondrial fraction of the rats heart homogenate. A – normotensive rats; B – SH rats

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