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Role of Reactive Oxygen Species in the Sensitivity of Rat Hypertrophied Myocardium to Ischemia

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Abstract—The relationship between hydroxyl radical (OH˙) generation in the zone of ischemia/reperfusion and the size of infarction formed was investigated in 18-22-week-old anaesthetized male SHRSP and Wistar rats using a myocardial microdialysis technique. The marker of OH˙ generation, 2,3-dihydroxybenzoic acid (2,3-DHBA), was analyzed in dialyzates by high performance liquid chromatography with electrochemical detection. Myocardial ischemia was induced by ligation of the descending branch of the left main coronary artery for 30 min. The mean value of basal 2,3-DHBA level in the dialyzate samples from SHRSP (243 ± 21 pg for 30 min) was significantly higher than that from Wistar rats (91 ± 4 pg for 30 min, $p < 0.0002$); it positively correlated with left ventricular hypertrophy ($r = 0.806$; $p < 0.05$). During reperfusion total 2,3-DHBA output was 1.8-fold higher in SHRSP than in Wistar rats (659 ± 60 pg versus 364 ± 66 pg for 60 min, respectively, $p < 0.0002$). At the same time, 2,3-DHBA increase above the basal level was the same in Wistar and SHRSP rats (181 ± 25 and 172 ± 36 pg for 60 min, respectively). The infarct size in SHRSP (45.4 ± 4.3%) was significantly higher ($p < 0.05$) than in Wistar rats (32.8 ± 3.3%). There was a significant positive correlation between basal level of 2,3-DHBA and total reperfusion 2,3-DHBA content in SHRSP ($r = 0.752$; $p < 0.05$). Thus, data obtained clearly indicate that the hypertrophied myocardium of SHRSP was less tolerant to ischemia/reperfusion than that of Wistar rats due to chronically increased OH˙ production and enhanced total OH˙ output during reperfusion. Greater myocardial damage in SHRSP than in Wistar rats following the equal increase in OH˙ production above the basal level suggests the existence of deficit of the antioxidant defense in the hypertrophied myocardium.

Key words: oxidative stress, hydroxyl radical, myocardial hypertrophy, myocardial ischemia, microdialysis, high-performance liquid chromatography, salicylate

Imbalance between the rate of generation of free radicals and antioxidant systems accompanies many chronic pathological conditions including cardiovascular diseases: atherosclerosis, hypertonic and ischemic heart diseases, etc. Reduced activities of antioxidant enzymes (glutathione peroxidase and superoxide dismutase) and increased products of lipid peroxidation (LPO) in blood indirectly support this notion [1]. Excess of free radicals is known to exert damaging effects on various biological structures. For example, prolonged ischemia of tissues promotes increased production of reactive oxygen species (ROS) and development of oxidative stress leading to irreversible cell damage, necrosis, and apoptosis [1, 2].

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However, short-term subtoxic increase in ROS level may induce the formation of protective mechanisms attenuating negative effects of subsequent acute oxidative stress. For example, ischemic preconditioning (i.e., one or several episodes of ischemia–reperfusion) may increase cell resistance to long-term ischemia [3], and ROS are considered as triggers of this phenomenon [4, 5].

Tissue level of free radicals *in vivo* under experimental conditions can be determined by using spin traps and electron paramagnetic resonance or by analyzing hydroxylated products of aromatic compounds using high performance liquid chromatography (HPLC) with electrochemical detection [6, 7]. Employing myocardial microdialysis with salicylic acid followed by monitoring of level of 2,3-dihydroxybenzoic acid (2,3-DHBA), a product of the reaction between salicylate and hydroxyl radical

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(OH˙), we detected increased basal level of hydroxyl radical in the hypertrophied myocardium of SHRSP *in vivo* [8]. The reasonable question arises whether this increased basal level of ROS is toxic. Does it induce or deplete protective resources of hypertrophied myocardium? Singal et al. demonstrated that pressure-induced hypertrophy of rat myocardium resulted in higher resistance to hypoxia and to action of ROS *ex vivo*; this was attributed to higher resources of the antioxidant enzymes superoxide dismutase and glutathione peroxidase [9-11]. Later this group also reported reduced level of myocardial antioxidant enzymes at post infarction [12] and pressure-induced [13] myocardial hypertrophy. Hearts of SHR [14] and SHRSP [15] with induced ischemia/reperfusion differ from the hearts of WKY by more pronounced impairment of contractile function. The level of prooxidant enzymes in hypertrophied myocardium is higher in rats with inherited hypertension [14, 16, 17], whereas the level of antioxidant enzymes is reduced [14, 18-20]. This also suggests higher susceptibility to the ROS effect.

In this study, we investigated susceptibility of the hypertrophied myocardium of SHRSP rats to acute oxidative stress induced by ischemia/reperfusion and analyzed the relationship between intensity of ROS generation and the zone of necrosis formed. Use of a combination of HPLC with a microdialysis technique allowed monitoring OH˙ output for a few hours in the myocardial tissue *in vivo* [5, 8, 21].

MATERIALS AND METHODS

Anesthetized 18-22-week-old male SHRSP (n = 7) and Wistar rats (n = 6) were used in experiments under conditions of open chest and artificial lung ventilation. During the whole experiment, arterial blood pressure (ABP) was registered through a catheter in the femoral artery connected to an electromanometer. Heart rate was monitored using an analog-number converter connected to an IMP PC. Myocardial ischemia was induced by ligation of the descending branch of the left main coronary artery at the level of the lower margin of the left atrial auricle. A microdialysis fiber (outer diameter of 0.3 mm; molecular mass cut-off 5000) was sewed into the wall of the left ventricle myocardium in the region of subsequent ischemia, which was detected by short-term occlusion of the descending branch of the left coronary artery. After implantation, the dialysis probe was perfused with Ringer solution (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$, pH 7.4) for at least 1 h at a flow rate 3 μl/min until steady state level of 2,3-DHBA and then several sequential dialyzate samples were collected. Then regional ischemia for 30 min followed subsequent reperfusion for 60 min was induced. During the whole ischemia/reperfusion period, dialyzate samples were continuously collected (fractions were collected for 5 or 10 min) (Fig. 1).

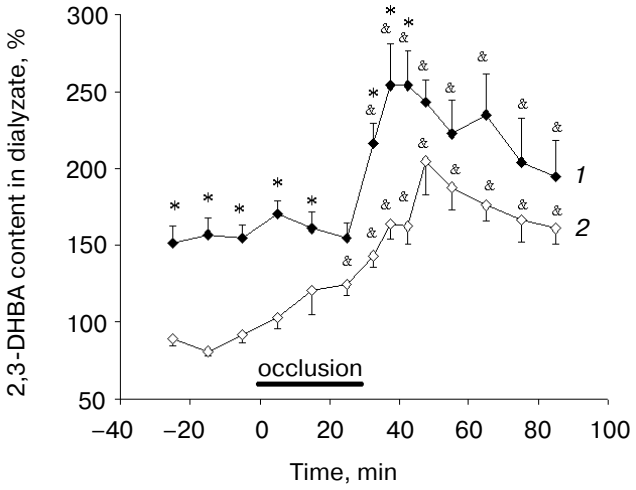


Fig. 1. Levels of 2,3-dihydroxybenzoic acid (2,3-DHBA) in myocardial dialyzate from Wistar rats (*1*) and SHRSP (*2*) (% of concentration in perfusate); &, statistically significant difference from the basal level; *, statistically significant difference between SHRSP and Wistar rats.

2,3-DHBA formed together with 2,5-DHBA in the reaction of hydroxyl radical with salicylic acid was used as the marker of OH˙ formation [7, 22]. Dialyzate aliquots (15 μl) were immediately used for analysis of 2,3-DHBA content by HPLC with electrochemical detection [5, 8, 21]. The HPLC equipment was from ESA (USA); it included a model 420 pump and Coulochem II electrochemical detector with Model 5010 Analytical Cell and HR-80 column (80 × 4.6 mm) (ESA). The sequential voltage at the first and the second potentiostats was −40 mV and +350 mV, respectively. The analysis was carried using the mobile phase consisting of 35 mM KH_2PO_4, 30 mM citric acid, 2 mM Na_2EDTA, 100 mM sodium octyl sulfate, 11% methanol, pH 2.5, in isocratic mode at a flow rate 1.1 ml/min. Retention time for 2,5-DHBA and 2,3-DHBA was 5 and 8.5 min, respectively, under complete separation of 2,3-DHBA from endogenous compounds in dialyzate. The detection limit of 2,3-DHBA was 2 pg at signal to noise ratio of 3 : 1.

In solution, sodium salicylate is readily oxidized with formation of hydroxybenzoic acids. To avoid oxidation of salicylate *ex vivo* (in the presence of metal ions) we used plastic microsyringes connected to plastic capillary. The whole pathway of perfusate flow was protected from light. For sample injection into the HPLC system, we also used a microsyringe with Teflon-tippet plunger. The sample was injected into the HPLC system quickly (with minimal retention time in the dosing loop of the injector). The content of 2,3-DHBA in the perfusate flowing into the myocardium was measured in the beginning and in the end of each experiment to monitor stability of the salicylate solution.

For each animal, the content of 2,3-DHBA in myocardial dialyzate was calculated as pg per sample and as percent of 2,3-DHBA concentration in the perfusate.

After termination of reperfusion, 2% methylene blue solution was injected into the jugular vein (under conditions of coronary artery occlusion). This allowed differentiating ischemic zones and intact myocardium. The heart was stopped by intravenous injection of 2% sodium chloride, and the heart was excised. After weighing, the left ventricle was frozen at –20°C. Each left ventricle was used for 5-6 cross sections (2-2.5 mm thick), which were incubated in 1% triphenyltetrazolium chloride solution in phosphate buffer (pH 7.4) for 5 min at 37°C. This allowed detection of zones of necrosis. The stained sections were scanned and zones of intact and ischemic myocardium and also zones of necrosis and their ratios were measured. The hypertrophy of left ventricle was evaluated by calculating the ratio of its weight (in grams) referred to the body weight of the rat (in kilograms).

Statistical treatment was carried out using nonpaired Student's *t*-test (for evaluation of differences between groups of animals) and paired Student's *t*-test (for evaluation of differences within each group). Results were expressed as mean ± SEM.

RESULTS

SHRSP were characterized by significantly higher ABP values (156 ± 7 versus 101 ± 10 mm Hg, $p < 0.01$) and pronounced left ventricle hypertrophy (2.6 ± 0.11 versus 2.1 ± 0.11 g/kg, $p < 0.01$) than control (normotensive) Wistar rats. There were no differences between heart rate values in these rats (265 ± 11 versus 286 ± 24 beats per min, $p > 0.05$).

The basal level of 2,3-DHBA in myocardial dialyzate was higher in SHRSP than in Wistar rats (Fig. 1). Myocardial ischemia for 30 min was accompanied by gradual increase in 2,3-DHBA level in Wistar rats. Under these conditions, the same parameter remained unchanged in SHRSP. Reperfusion caused significant increase of 2,3-DHBA level in myocardial dialyzates in both groups of animals, and total 2,3-DHBA output per 60 min of reperfusion was 1.8-fold higher in SHRSP than in Wistar rats (Fig. 2). However, during the whole reperfusion period the augmentation of 2,3-DHBA content over the basal level was the same in SHRSP and Wistar rats (181 ± 25 and 172 ± 36 pg, respectively).

Figure 3 shows that values of necrotic zones were significantly higher in hearts of spontaneously hypertensive rats (45.4 ± 4.3%) than in normotensive rats (32.8 ± 3.3%) ($p < 0.005$).

Correlation analysis revealed the existence of significant positive correlation between the basal level of 2,3-DHBA in myocardial dialyzates and the value of reperfusion output of 2,3-DHBA in SHRSP ($r = 0.752$, $p < 0.05$) (Fig. 4).

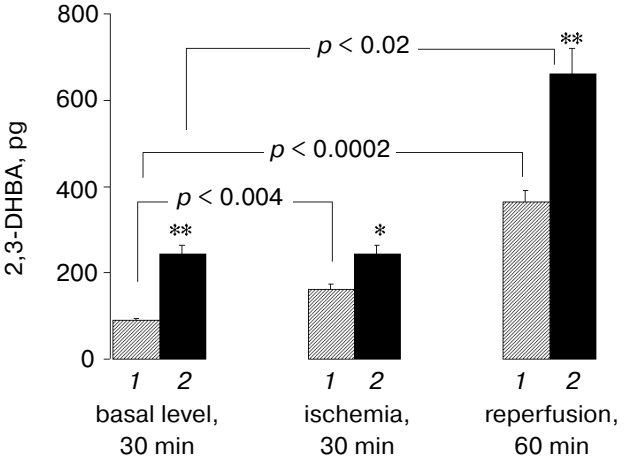


Fig. 2. Effect of ischemia–reperfusion on 2,3-dihydroxybenzoic acid (2,3-DHBA) (pg) in myocardial dialyzate in Wistar rats (*1*) and SHRSP (*2*). Asterisks show statistical significance between SHRSP and Wistar rats: * $p = 0.02$; ** $p \le 0.0002$.

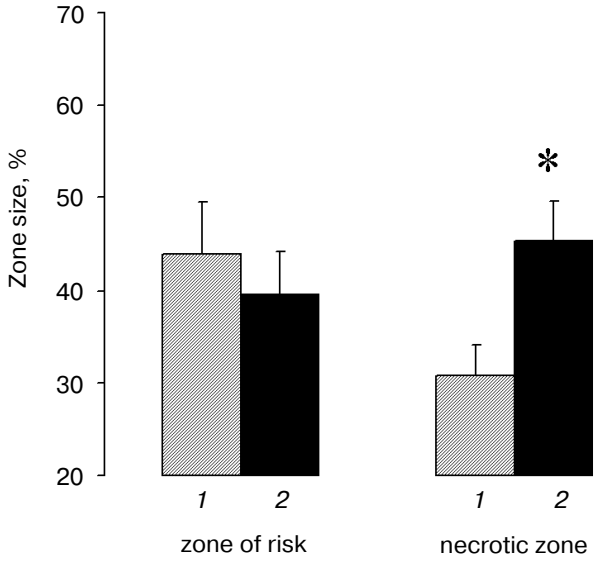


Fig. 3. Size of risk zone (ischemic zone to whole myocardium ratio, %) and necrotic zone (necrotic to ischemic zone ratio, %) in myocardium from Wistar rats (*1*) and SHRSP (*2*). Statistically significant difference between SHRSP and Wistar rats: * $p < 0.005$.

DISCUSSION

In myocardium, ROS can be generated by cardiomyocytes, endothelial cells, and neutrophils; they may also be produced during catecholamine autooxidation [23].

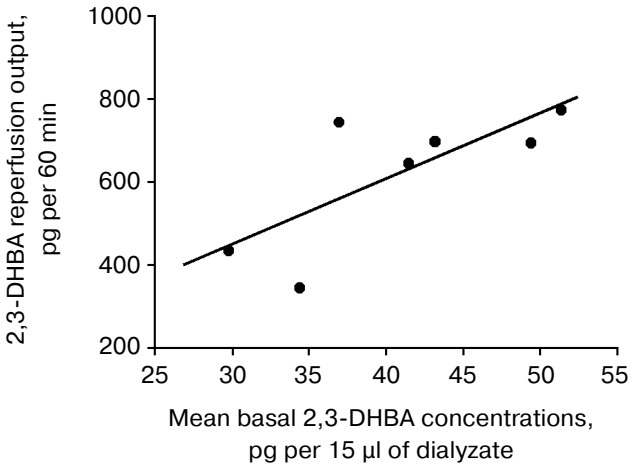


Fig. 4. The relationship between basal concentrations and total reperfusion output of 2,3-dihydroxybenzoic acid (2,3-DHBA) in myocardial dialyzate of SHRSP ($r = 0.752$, $p < 0.05$).

Superoxide radical ($O_2^{\cdot-}$) is produced in cells due to electron leak from the mitochondrial electron transport chain, due to activation of some oxidases, such as NAD(P)H-oxidase, xanthine oxidase, and also by cytochrome P450 and nitric oxide synthase [2]. Subsequent $OH^{\cdot}$ formation from products of $O_2^{\cdot-}$ conversion, hydrogen peroxide (in Fenton and Haber–Weiss reactions) and peroxynitrite may occur both inside cells and in the extracellular space. In this study, we used the method of quantitative evaluation of $OH^{\cdot}$ formation by the product of hydroxylation of salicylate, which enters myocardium through pores in the dialysis fiber. The permeability of biological membranes for salicylic acid and related compounds [24, 25] and stability of their hydroxylation products suggest that tissue levels of 2,3-DHBA and their equilibrium concentrations in the dialyzate reflect total (extra- and intracellular) $OH^{\cdot}$ generation. The correctness of interpretation of 2,3-DHBA concentration in the dialyzate as an indicator of $OH^{\cdot}$ production is strengthen by the fact that no specific antioxidant has been found for $OH^{\cdot}$, the most reactive radical among ROS [1, 2].

We found increased basal concentrations of 2,3-DHBA in myocardial dialysis samples from SHRSP versus Wistar rats; this corresponds to more intensive $OH^{\cdot}$ production. In agreement with previous observation [8], there was a direct relationship between tissue $OH^{\cdot}$ level and hypertrophy of the left ventricle in SHRSP ($r = 0.806$; $p < 0.05$).

Increased levels of ROS have been shown to accompany the development of left ventricle hypertrophy and heart failure; they are considered as important pathogenic factors underlying the development of hypertrophy [2, 23].

The hypertrophied myocardium is characterized by increased activity of some prooxidant enzymes and altered status of antioxidant defense system. For example, the activity of xanthine oxidase in the myocardium of SHRSP was 6.2-fold higher than that in normotensive WKY rats, whereas levels of glutathione and glutathione synthesizing enzyme, γ-glutamyl cysteine synthase were 1.3- and 2.4-fold higher, respectively [16]. Now certain evidence for increased NAD(P)H-dependent production of $O_2^{\cdot-}$ in cardiomyocytes and endothelial cells of hypertrophied myocardium exists: the development of pressure-induced hypertrophy of guinea pig left ventricle was accompanied by progressive increase NAD(P)H-dependent production of $O_2^{\cdot-}$ [26, 27].

Production of $O_2^{\cdot-}$ may also result from excess of catecholamines in myocardial interstitia [28]. Increase in noradrenaline level in myocardial tissues *in vivo* was shown to be accompanied by dose-dependent increase in $OH^{\cdot}$ level [29, 30]. Myocardium of SHRSP is characterized by higher density of noradrenergic nerve fibers in ventricle subepicardium and myocardium [31] and increased noradrenaline output during ischemia/reperfusion [15]. This suggests the existence of sympathetic hyperreactivity [32], which is the other reason underlying increased ROS production.

The state of the antioxidant system is age-related; it also changes during the development of hypertrophy. In 15-week-old SHRSP the activity of Mn- and Cu/Zn-dependent superoxide dismutases (SOD) is higher than in WKY rats, whereas in 31-week-old SHRSP the activity of Mn-SOD is lower than in WKY rats [20]. It is important that in SHRSP there is an age related decrease in mitochondrial SOD playing the key role in the control of $O_2^{\cdot-}$ production during oxidative phosphorylation; this SOD represents 70% of myocardial SOD activity and 90% of cardiomyocyte SOD activity [33]. Similar changes in myocardial antioxidant resources also take place after ligation of rat abdominal artery [9, 34]. The content of lipid peroxidation product, malondialdehyde, measured in the hypertrophied myocardium six weeks after the occlusion, was lower, whereas SOD activity was higher than in corresponding controls [9]. This probably reflects compensatory response of the antioxidant system to increased ROS production. But in a similar study three months after the occlusion the decrease in SOD activity and increased content of lipid peroxidation products were observed [34]. According to the hypothesis of Singal and Kirshenbaum [35], compensatory increase in activity of antioxidant system components does not abolish antioxidant deficit and subsequent depletion of myocardial antioxidant resources results in decompensation, which promotes the development of heart failure.

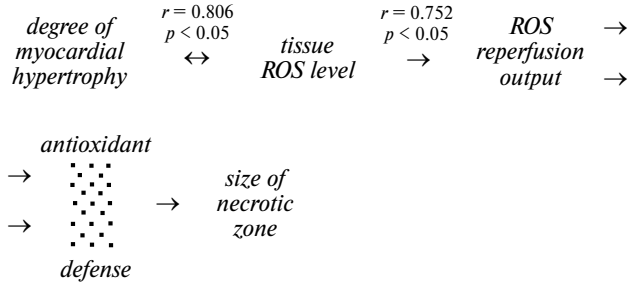
Thus, increased basal level of $OH^{\cdot}$ detected *in vivo* in left ventricle of SHRSP rats is consistent with known biochemical and morphological characteristics of their myocardium. It suggests chronic hyperproduction of

ROS. A direct relationship between increased basal level of $OH^{\cdot}$ and hypertrophy confirms a causal role of increased ROS production in pathogenesis of hypertrophy.

Our data clearly demonstrate that hypertrophied myocardium of SHRSP differs from myocardium of Wistar rats not only by increased basal level of $OH^{\cdot}$ generation but also by response to ischemia/reperfusion (Figs. 1 and 2). In Wistar rats, myocardial ischemia caused significant increase in $OH^{\cdot}$ level, whereas in SHRSP the same treatment did not influence the rate of $OH^{\cdot}$ generation in hypertrophied myocardium. Nevertheless, the total amount of $OH^{\cdot}$ formed during 30 min of ischemia was 1.5-fold higher in SHRSP than in Wistar rats. Reperfusion was accompanied by significant increase in $OH^{\cdot}$ level in myocardium of both strains of rats, but only SHRSP were characterized by both increased basal $OH^{\cdot}$ level and higher reperfusion output. This revealed a statistically significant positive correlation between these parameters (Fig. 4). In SHRSP total output of $OH^{\cdot}$ during 60-min reperfusion was 1.8-fold higher than in Wistar rats (Fig. 2). This was consistent with larger zone of necrosis (Fig. 3).

Myocardial ischemia is known to promote an increase in ROS [1, 2] due to: a) increased one-electron oxygen reduction in mitochondria, which stems from increased reduction of tricarboxylic cycle substrates; b) proteolytic conversion of xanthine dehydrogenase into xanthine oxidase accompanied by accumulation of the xanthine oxidase substrate, hypoxanthine, formed during ATP catabolism; c) autooxidation of catecholamines; d) activation of neutrophils; e) inhibition of antioxidant enzymes during acidification of their environment. The same processes are probably responsible for ROS output in reperfusion [23]. The larger amount of $OH^{\cdot}$ formed in SHRSP during ischemia/reperfusion (Fig. 2) may be attributed to increased activity of the prooxidant system of the hypertrophied myocardium [16, 20, 26, 27] and inadequate level of antioxidants representing the first line of antioxidant defense [14, 20]. This may result in augmentation of tissue levels of $OH^{\cdot}$ precursors—$O_2^{\cdot-}$ and H_2O_2. Reperfusion addition of $OH^{\cdot}$ to its pre-ischemic level was roughly the same in Wistar and SHRSP rats. The degree of tissue damage cause by oxidative stress is determined by the level of antioxidants representing the second and the third lines of antioxidant defense limiting lipid peroxidation. Taking into consideration larger necrotic damage of SHRSP rat hearts, we suggest the existence of deficit in the resources of the antioxidant system of hypertrophied myocardium.

Mean values for total $OH^{\cdot}$ output during reperfusion and the size of necrotic zones in left ventricle were 1.8- and 1.4-fold higher in SHRSP than in Wistar rats. Taking into consideration positive correlation between the intensity of $OH^{\cdot}$ generation in myocardium of SHRSP and the degree of its hypertrophy, we propose the following chain of relationships:



Although higher mean value of reperfusion $OH^{\cdot}$ output in SHRSP corresponded to mean value of larger damage of myocardium, there was no statistically significant correlation between the value of reperfusion $OH^{\cdot}$ output and the size of the necrotic zone. This is quite expectable because individual level of myocardial antioxidants could be subjected to significant individual variations, which may contribute to different effectiveness of neutralization of the reperfusion "wave" of ROS.

Thus, myocardial damage induced by ischemia/reperfusion and evaluated by the size of necrosis was higher in SHRSP than in Wistar rats. Based on these observations, we concluded that the hypertrophied myocardium is less resistant to oxidative stress *in vivo*. We believe that increased basal $OH^{\cdot}$ production by the hypertrophied myocardium and increased total $OH^{\cdot}$ reperfusion output are two of the main reasons for lower resistance to oxidative stress. More pronounced myocardial damage in SHRSP than in Wistar rats seen in response to equal reperfusion increase in ROS production over the basal level suggests the existence of deficit(s) in the antioxidant defense system in the hypertrophied myocardium of SHRSP.

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