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Free-radical production triggered by hyperthermia contributes to heat stress-induced cardioprotection in isolated rat hearts

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Abbreviated running head: reactive oxygen species and heat stress response

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Abstract

1 Heat stress (HS) is known to protect the myocardium against ischaemic damage. It has been reported that reactive oxygen species (ROS) are abundantly produced during this stress. Since mechanisms triggering the HS-induced cardioprotection remain unknown, we investigated the role of ROS in the genesis of this protective phenomenon.

2 Rats were divided into 4 groups (n=8 in each group), subjected to either hyperthermia (42°C internal temperature for 15 min) or sham anaesthesia and treated or not with *N*-2-mercaptopropionyl glycine (MPG), a synthetic antioxidant, 10 min before HS. Twenty-four hours later, their hearts were isolated, retrogradely perfused, and subjected to a 30-min occlusion of the left coronary artery followed by 120 min of reperfusion. Myocardial Hsp 27 and 70 expression was assessed by western blot analysis (n=4). Cardiac activities of antioxidant enzymes (superoxide dismutase and glutathione peroxidase) were also examined (n=4).

3 Infarct-to-risk zone ratio was significantly reduced in HS ($17 \pm 1.3\%$) compared to Sham ($34.3 \pm 1.7\%$) hearts. This effect was abolished by MPG pretreatment ($40.6 \pm 1.9\%$ in HS+MPG *vs* $39.8 \pm 2.5\%$ in Sham+MPG hearts). This cardioprotection was associated with an enhanced Hsp 27 and 70 expression, which was not modified by MPG pretreatment. Antioxidant enzyme activities was not modified by heat stress or MPG pretreatment.

4 Free radical production following hyperthermia appears to play a role in the heat stress induced cardioprotection, independently of Hsp levels. Antioxidant enzyme activities do not seem to be implicated in this cardioprotective mechanism.

Keywords: cardiovascular system, animal models, heat stress, heat shock proteins, free radicals.

List of abbreviations

CF: coronary flow

GPx: glutathione peroxidase

HR: heart rate

HS: heat stress

Hsp: heat stress protein

I: infarct zone

IP: ischaemic preconditioning

LV: left ventricle

LVDP: left ventricular developed pressure

LVEDP: left ventricular end-diastolic pressure

MPG: *N*-2-mercaptopropionyl glycine

NO : nitric oxide

O₂⁻ : superoxide anion

·OH : hydroxyl radical

R: risk zone

ROS: reactive oxygen species

Cu,Zn SOD: copper, zinc superoxide dismutase

Mn SOD: manganese superoxide dismutase

VF: ventricular fibrillation

Introduction

Heat stress (HS) is known to increase myocardial tolerance to a subsequent sustained period of ischaemia by preserving myocardial function (Currie et al., 1988) and reducing myocardial necrosis (Donnelly et al., 1992; Yellon et al., 1992). Various mechanisms seem to initiate this cardioprotective effect (Joyeux et al., 1999). Among them, nitric oxide (NO), which constitutes the most abundant free radical in the body (Moncada et al., 1991), has been recently shown to trigger the resistance to myocardial infarction induced by heat stress in the isolated rat heart (Arnaud et al., 2001).

Reactive oxygen species (ROS), like superoxide anion ($O_2^{\cdot-}$), are also produced during hyperthermia (Salo et al., 1991; Flanagan et al., 1998). It has been observed that these ROS play an essential role in the genesis of the delayed protection conferred by ischaemic preconditioning (IP) (Sun et al., 1996; Zhou et al., 1996). Since the cardioprotection induced 24-48 h following heat stress resembles that observed during the late phase of IP, triggers under investigation for their role in IP may provide a potential mechanism for HS-induced protection (for review, see Bolli (2000)). Hence, the initial oxidative stress occurring following hyperthermia could potentially trigger the HS-response.

Many candidates have been proposed as end-effectors of the heat stress response (for review, see Joyeux et al. (1999)). Among them, the increase in endogenous cardiac antioxidant defences has been reported. Indeed, the activation of the manganese superoxide dismutase (Mn-SOD) seems to be associated with the HS-induced cardioprotection (Yamashita et al., 1998). The *de novo* synthesis of heat shock proteins (Hsp) seems also to mediate this protective effect. A direct correlation between the amount of Hsp 70 expression and the degree of HS-induced myocardial protection has been observed in the rat (Hutter et al., 1994) and in the rabbit (Marber et al., 1994). Furthermore, small Hsp (Hsp 27) overexpression seems to confer protection against ischaemic injury in cardiac myocytes (Martin et al., 1997).

The purpose of this study was thus to determine the role of the initial oxidative stress occurring following hyperthermia in the HS-induced cardioprotection and its potential interaction with end-effectors of this response. Therefore, we examined the effect of a synthetic antioxidant, *N*-2-mercaptopropionyl glycine (MPG), on the resistance to myocardial infarction conferred by heat stress in the isolated rat heart. The effect of MPG treatment on HS-induced increase in cardiac Hsp synthesis and antioxidant enzyme activities has also been investigated.

Methods

Experimental treatment groups

Male Wistar rats (280-340 g) were used for this study. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication n° 85-23, revised 1996).

First, rats were submitted to either heat stress (HS groups) or anaesthesia without hyperthermia (Sham groups). Prior to this procedure, the animals were treated with either MPG, a thiol compound that avidly scavenges oxidant species and especially hydroxyl radical ($\cdot\text{OH}$) (Bolli et al., 1989; Sun et al., 1993), or saline, as previously described (Yamashita et al., 1998). Subsequently, all animals were allowed to recover for 24 h. Then, ischaemia-reperfusion was performed in isolated hearts. The four following experimental groups (n=8 per group) were studied.

Group HS - rats were sham pretreated with saline 10 min before undergoing heat stress;

Group HS+MPG - rats were pretreated with MPG (100 mg kg^{-1} , ip) 10 min before undergoing heat stress;

In Groups Sham and Sham+MPG - rats were similarly pretreated before being sham anaesthetised.

The experimental protocols are summarised in Figure 1.

Heat stress protocol

Heat stress was achieved, as previously described (Joyeux et al., 1998), by placing anaesthetised (with 25 mg kg^{-1} , ip, sodium pentobarbitone) rats in an environmental chamber under an infrared light. Their body temperature, recorded with a rectal probe, was increased to

42 ± 0.2°C for 15 min. Sham control animals were anaesthetised only. All rats were allowed to recover for 24 h.

Ischaemia-reperfusion protocol

Twenty-four hours after heat stress, rats were re-anaesthetised (60 mg kg⁻¹, ip, sodium pentobarbitone) and treated with heparin (500 U kg⁻¹, iv). The heart was rapidly excised and immediately immersed in 4°C Krebs-Henseleit buffer solution (NaCl 118.0, KCl 4.7, CaCl₂ 1.8, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.2 and glucose 11.0 mM). The aortic stump was then cannulated and the heart perfused retrogradely using the Langendorff technique at a constant pressure (75 mmHg) with oxygenated Krebs-Henseleit buffer. A water-filled balloon (Hugo Sachs, n°4), coupled to a pressure transducer (Statham), was inserted into the left ventricular cavity *via* the left atrium for pressure recording. Left ventricular end-diastolic pressure (LVEDP) was adjusted between 5-10 mmHg. Myocardial temperature was measured by a thermoprobe inserted into the left ventricle and was maintained constant close to 37°C. For temporary occlusion of the left coronary artery (LCA), a 3/0 silk suture (Mersilk W546, Ethicon) was placed around the artery a few millimetres distal to the aortic root. After 20 min of stabilisation, regional ischaemia was induced by tightening the snare around the LCA for 30 min. Thereafter the heart was reperfused for 120 min. Coronary flow (CF) was measured throughout the ischaemia-reperfusion procedure, by collecting the effluent. Heart rate (HR) and left ventricular developed pressure (LVDP = difference between left ventricular systolic pressure and LVEDP) were continuously recorded on a polygraph (Windograph, Gould Instrument). At the end of the reperfusion period, the coronary artery ligature was retied and unisperse blue (Ciba-Geigy) dye was slowly infused through the aorta to delineate the myocardial risk zone. After removal of the right ventricle and connective tissues, the heart was frozen and then cut into 2 mm transverse sections from apex to base (6-7 slices/heart).

Following defrosting, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 10-20 min and fixed in 10% formaldehyde solution to distinguish stained viable tissue and unstained necrotic tissue. Left ventricular infarct zone (I) was determined using a computerised planimetric technique (Minichromax, Biolab) and expressed as a percentage of the risk zone (R) and of the left ventricle (LV).

Western Blot analysis of myocardial Hsp 27 and 70

To determine Hsp 27 and 70 expression, additional animals (n=4 in each group) were submitted to either HS or sham anaesthesia, pretreated or not with MPG. Twenty-four hours later, animals were re-anaesthetised and treated with heparin as described above, before their heart was quickly excised. Hearts were minced and homogenized in a lysis buffer pH 7.4 containing 50 mM Tris, 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, 100 µM Na₂MoO₄, 5 µl ml⁻¹ protease inhibitors, 1‰ microcystine and 1% Triton. Samples were then centrifuged for 5 min at 14 000 rpm, 4°C. The supernatant were diluted in sample buffer containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 62.5 mM Tris HCl (pH 6.8), 100 mM DTT, 0.1% bromophenol blue. The protein concentration in samples was determined using the BCA protein assay Kit (Pierce). Samples were loaded at 5 µg/lane and proteins were separated by electrophoresis on 12% acrylamide gels. Proteins were transferred from gel to nitrocellulose membrane, which was then incubated with antibodies to either Hsp 27 (Upstate Biotechnology) or Hsp 70 (Santa Cruz Biotechnology). The second antibody was peroxidase-conjugated anti-rabbit for Hsp 27 antisera, and peroxidase-conjugated anti-goat for Hsp 70 antisera. The membrane was developed using an enhanced chemiluminescence system (Pierce) to obtain an autoradiogram.

The relative levels of Hsp 27 and 70 were determined by densitometry of the autoradiogram (Sony video system coupled with a Minichromax digitization system and Biolab software).

Determination of myocardial antioxidant enzyme activities

To measure myocardial levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx), the frozen tissue samples were homogenized on ice in 10 mM Tris, 1 mM diethylenetriaminepentaacetic acid and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. SOD activity was determined using the pyrogallol assay following the procedure described by Marklund et al. (1974), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, photometrically read at $\lambda = 420$ nm. GPx activity was measured after the rate of NADPH oxidation by t-butyl hydroperoxide at 340 nm (Turrens et al., 1992). Activities were corrected for the concentration of protein.

Statistical analysis

The data are presented as mean \pm s.e.mean. Comparisons in CF, HR and LVDP were performed using two-way repeated measures ANOVA. Post-hoc comparisons were done using Tukey tests. Infarct size analysis, semi-quantitative Hsp analysis and comparison in antioxidant enzyme activities were performed by a one-way ANOVA. *P* values ≤ 0.05 were considered significant.

Materials

MPG were purchased from Sigma (France). Unisperse blue dye was obtained from Ciba-Geigy (France). Anti-Hsp 27 antibody was from Upstate biotechnology and anti-Hsp 70 from Santa Cruz Biotechnology Inc. The enhanced chemiluminescence reaction kit assay was from Pierce.

Results

Hemodynamic data

Table 1 summarises CF, HR and LVDP data recorded in the four experimental groups during the stabilisation and ischaemia-reperfusion periods. Twenty-four hours after heat stress or sham anaesthesia, there was no statistically significant difference in hemodynamic performance measured between the four groups.

Infarct data

Heat stress significantly reduced infarct size from $34.3 \pm 1.7\%$ in Sham to $17 \pm 1.3\%$ in HS hearts ($p < 0.001$, one-way ANOVA). This infarct size-reducing effect of heat stress was abolished by MPG pretreatment ($40.6 \pm 1.9\%$), whereas in non-heat stressed rats, MPG had no effect on infarct size ($39.8 \pm 2.5\%$) (Figure 2). Similar results were observed concerning I/LV ratio between the four groups (data not shown). Myocardial risk zone expressed as the percentage of the left ventricle (R/LV) was similar for all groups: $45.9 \pm 2.3\%$ in Sham, $44.7 \pm 2.6\%$ in HS, $44.1 \pm 1.5\%$ in Sham+MPG and $42.6 \pm 1.1\%$ in HS+MPG groups. Therefore, differences in infarct size did not result from variability in the risk zone.

Western blot analysis of Hsp 27 and 70

Western blot analysis of myocardial Hsp 27 and 70 (Figures 3 and 4) expression showed a marked increase of these proteins, 24 hours after heat stress. MPG-pretreatment did not modify the HS-induced increase in Hsp 27 and 70 expression (Figures 3 and 4).

Antioxidant enzyme activities data

No differences in SOD and GPx activities was seen between the different groups. Results are summarised in Table 2.

Discussion

In our *in vitro* rat model of myocardial infarction, prior heat stress significantly reduced infarct size performed 24 h after, in accordance with previous studies (for review, see Joyeux et al. (1999)). The mechanism for this delayed protection is currently unknown. The pertinent finding of this work is the implication of ROS as triggers of this cardioprotective mechanism, since MPG, administered before HS, abolished the HS-induced-infarct size reduction, in this isolated rat heart model.

HS-induced reduction on infarct size

In our study, we investigated the effect of MPG pretreatment on the HS-induced reduction of infarct size, in the isolated rat heart. The effect of heat stress is well documented in the literature. Actually, it is known that hyperthermia (42°C, during 15 to 20 min) reduces the infarct size assessed 24 h later, at the end of a 30 min left coronary artery occlusion-120 min reperfusion. This result has been observed *in vivo* in the rat (Donnelly et al., 1992; Hutter et al., 1994) and in the rabbit (Marber et al., 1993) as well as in the isolated rat heart (Joyeux et al., 1997 and 1998). Controversially, Yamashita et al. (1998) do not observe a significant decrease in infarct size 24 h but only 48 to 72 h after heat stress (42°C). However, the decrease in infarct size we observed cannot be compared with this study, since reperfusion durations are different (120 min *versus* 48 h). Thus, the discrepancy between our study and the one by Yamashita et al. (1998) may be explained by the difference in reperfusion duration.

Delayed preconditioning mediated by ROS

ROS have yet been shown to be involved in cardioprotection. Indeed, Yamashita et al. (1998) showed, in conscious rat, that oxygen free radicals production during hyperthermia is necessary to the reduction of infarct size, as well as of ventricular fibrillation, investigated

both 30 min or 48 h after HS. Moreover, it has been observed in cardiomyocytes that a burst of ROS, generated during the initial periods of brief repetitive anoxia, contributes to the late cytoprotection induced by this preconditioning (Zhou et al., 1996). In addition, antioxidant treatment completely blocks the development of a late protection induced by IP against stunning in conscious pig, indicating that the initial production of ROS is one of the mechanism of the late protective response (Sun et al., 1996). ROS seem also to trigger the delayed protective effect of nitric oxide (NO) donors in conscious rabbit, since MPG coadministration abolishes the protection against myocardial stunning and infarction conferred by NO donors alone (Takano et al., 1998).

HS-induced production of ROS

Our study provides indirect demonstration that a burst of free radicals during hyperthermia is necessary for the HS-induced cardioprotection. In previous studies, hyperthermia was shown to induce production of free radicals. A sharp increase in ROS generation has been observed after hyperthermia in a cellular model, using electron paramagnetic resonance spin trapping (Flanagan et al., 1998). Moreover, oxidative stress, assessed by the efflux of both glutathione and oxidised glutathione, has been shown to be significantly increased by hyperthermia, in the rat liver (Powers et al., 1992). Zuo et al. (2000) also suggested that HS stimulates superoxide production in the mouse skeletal muscle. Finally, Salo et al. (1991) showed in the rat muscle that generation of O_2^- by the mitochondria increases in a temperature-dependant way, from 37°C to 45°C.

***De novo* synthesis of proteins following heat stress**

The fact that HS-induced cardioprotection requires 24 h to occur suggests that this phenomenon is related to *de novo* synthesis of proteins which might mediate this response.

Hsp have been first proposed as effectors of this response, since a positive correlation between HS-induced Hsp 70 expression and infarct size reduction was described (Marber et al., 1993; Hutter et al., 1994; Marber et al., 1994). Our western blot analysis of myocardial Hsp 27 and 70 expression showed a marked increase of these proteins, 24 h after HS, which was not modified by ROS scavenger pretreatment. Thus, it seems here that HS-induced cardioprotection is dissociated from induction of Hsp since pretreatment with MPG abolished cardioprotection while having no effect on myocardial Hsp content. In the same manner, previous studies also showed that the cardioprotection conferred by HS could be abolished by blocking different mediators of this response independently of the myocardial Hsp expression (Arnaud et al., 2001; Joyeux et al., 1998; Joyeux et al., 1997). However, Yamashita et al. (1998) showed that treatment with a radical scavenger reduces the increase in Hsp 70 expression as well as inhibits the cardioprotection induced 72 h following HS. Thus, the exact functional role of Hsp in cardioprotection is still unknown and remain to be determined. Furthermore, we have only observed here the expression of Hsp, by western blot, and we cannot exclude possible post-translational modifications of these proteins in response to HS. Other proteins could be upregulated in response to hyperthermia. Thus, it seems that HS also induces an increase in myocardial antioxidant activity. Indeed, it has been shown in the rat that Mn-SOD was significantly enhanced 24 h after HS in cardiomyocytes (Yamashita et al., 1997) or 48 h after HS in the heart (Yamashita et al., 1998). Maulik et al. (1995) have observed an increase in Mn-SOD activity 40 h after drug-induced HS-preconditioning in pig myocardium. However, controversy still exists on HS-enhanced antioxidant activity which seems to be animal species and time dependant. Indeed, it has also been reported that myocardial SOD activity is not modified 24 h after HS in the rat (Currie et al., 1988) and in the mouse (Xi et al., 1998). Moreover, GPx activity seems not to be influenced by prior HS in the rat myocardium (Currie et al., 1988). Our results confirm this controversy. Indeed, we do

not observe any modification in myocardial SOD or GPx activities, 24 h after HS in our rat model. Thus, antioxidant enzymes seem not to be effectors of the HS-induced cardioprotection in our rat heart model.

Conclusions

This study demonstrates that an initial burst of oxygen free radicals is necessary to initiate the HS-induced late cardioprotection, since MPG, a free radical scavenger, abolished the HS-response. This protection seems not to be related to the HS-induced increase in myocardial Hsp synthesis or antioxidant enzyme activities. Further experiments are needed to clarify the exact mechanisms occurring following ROS production and mediating the HS response.

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Table 1. Hemodynamic data.

	<i>Group</i>	<i>Stabilisation</i>	<i>Ischaemia</i>		<i>Reperfusion</i>		
			<i>5 min</i>	<i>29 min</i>	<i>15 min</i>	<i>60 min</i>	<i>120 min</i>
CF (ml min ⁻¹)	Sham	13.2 ± 0.5	8.4 ± 0.3	8.7 ± 0.4	12.6 ± 0.7	11.6 ± 0.5	10.0 ± 0.5
	HS	13.5 ± 0.3	8.1 ± 0.5	8.8 ± 0.4	12.0 ± 0.6	10.8 ± 0.4	9.6 ± 0.4
	Sham+MPG	14.0 ± 0.3	8.8 ± 0.5	9.1 ± 0.8	11.6 ± 0.7	9.5 ± 0.5	8.1 ± 0.3
	HS+MPG	14.6 ± 0.5	8.1 ± 0.5	8.0 ± 0.5	11.1 ± 0.8	9.9 ± 0.8	8.6 ± 0.6
HR (beats min ⁻¹)	Sham	300 ± 6	297 ± 6	284 ± 6	289 ± 8	287 ± 8	285 ± 11
	HS	302 ± 7	293 ± 5	291 ± 11	290 ± 8	283 ± 10	284 ± 11
	Sham+MPG	295 ± 8	286 ± 10	277 ± 9	272 ± 9	277 ± 1	281 ± 12
	HS+MPG	293 ± 9	281 ± 7	275 ± 11	277 ± 9	282 ± 11	278 ± 13
LVDP (mmHg)	Sham	104 ± 2	51 ± 4	63 ± 5	83 ± 3	74 ± 2	63 ± 3
	HS	106 ± 7	44 ± 4	62 ± 5	81 ± 9	76 ± 5	66 ± 5
	Sham+MPG	115 ± 5	58 ± 7	78 ± 5	97 ± 6	85 ± 4	68 ± 5
	HS+MPG	117 ± 9	43 ± 4	72 ± 6	85 ± 9	75 ± 8	64 ± 7

CF - coronary flow, HR - heart rate, LVDP - left ventricular developed pressure. Sham - sham-anaesthetised, HS - heat-stressed, MPG - *N*-2-mercaptpropionyl glycine-treated. Data are mean ± s.e.mean.

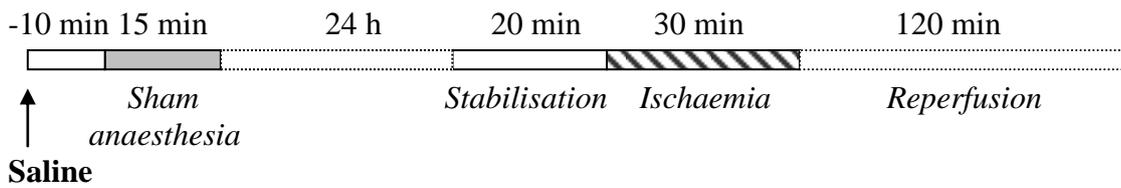
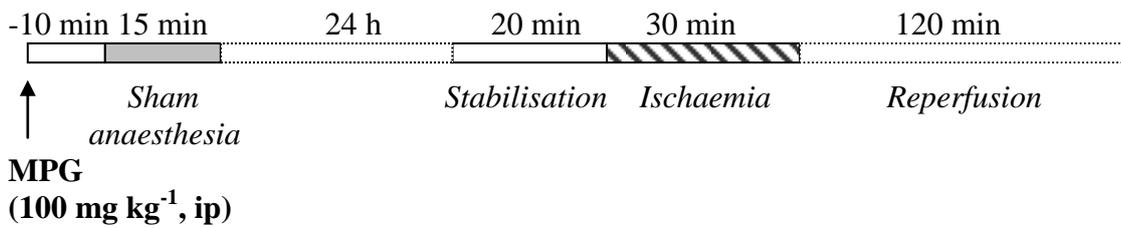
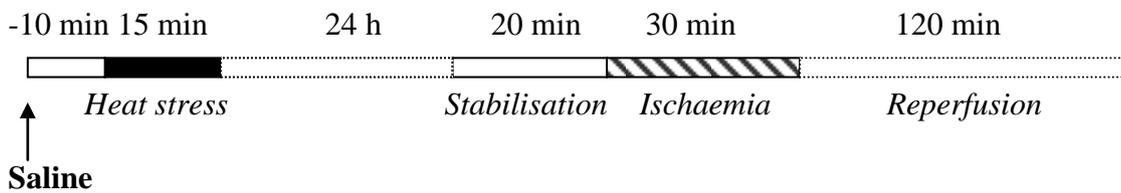
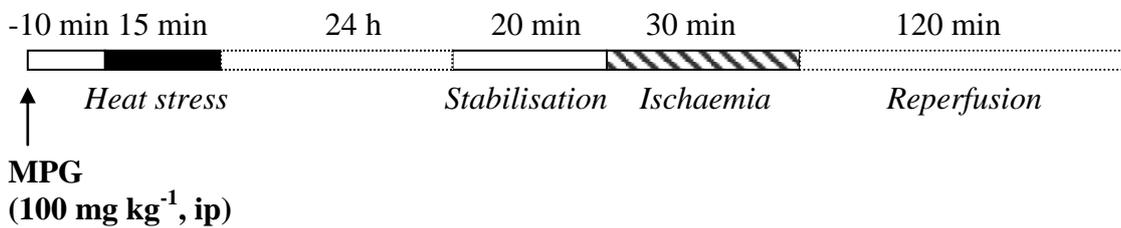
Table 2. Myocardial antioxidant enzyme activities.

	<i>Sham</i>	<i>HS</i>	<i>Sham+MPG</i>	<i>HS+MPG</i>
Total SOD	16.5 ± 0.4	16.3 ± 0.9	16.7 ± 1.6	15.4 ± 0.5
Mn SOD	6.6 ± 0.5	6.8 ± 0.5	6.6 ± 0.1	6.9 ± 0.2
GPx	1129 ± 49	1069 ± 82	1022 ± 26	1217 ± 74

SOD - superoxide dismutase, Mn SOD - mitochondrial SOD, GPx - glutathione peroxidase. Sham - sham-anaesthetised, HS - heat-stressed, MPG - *N*-2-mercapto-propionyl glycine-treated. n=4 in each group. Values are expressed as mean ± s.e.mean in U/mg protein.

Figure legends

- Figure 1.** Experimental protocol.
MPG - *N*-2-mercaptopropionyl glycine.
- Figure 2.** Effect of *N*-2-mercaptopropionyl glycine (MPG) pretreatment on myocardial infarct size assessed after 30-min coronary occlusion followed by 120-min reperfusion in isolated hearts from rats subjected to sham-anaesthesia (Sham) or heat-stress (HS). Infarct size (I) is expressed as a percentage of the risk zone (R). **P* < 0.001 vs the other groups (one-way ANOVA).
- Figure 3.** Western blot analysis of myocardial Hsp 27 and 70 in the 4 experimental groups. Sham - sham-anaesthetised; HS - heat-stressed; MPG - *N*-2-mercaptopropionyl glycine-treated.
- Figure 4.** Semi-quantitative analysis of myocardial Hsp 27 and 70 levels obtained by autoradiogram in the 4 experimental groups, n=4 in each group). Sham - sham-anaesthetised; HS - heat-stressed; MPG - *N*-2-mercaptopropionyl glycine-treated. **P* < 0.001 vs Sham and Sham+MPG (one-way ANOVA).

Group Sham**Group Sham+MPG****Group HS****Group HS+MPG****Figure 1.**

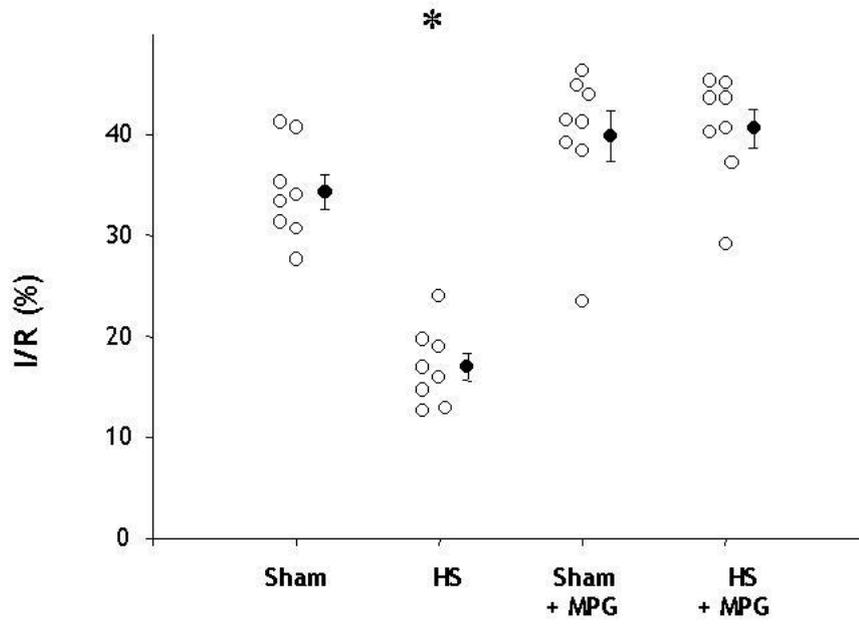


Figure 2.

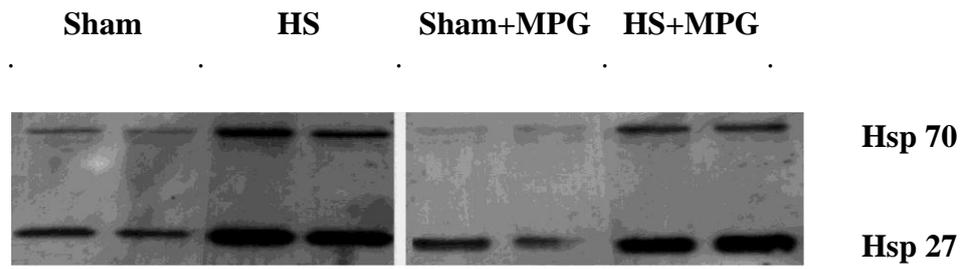


Figure 3.

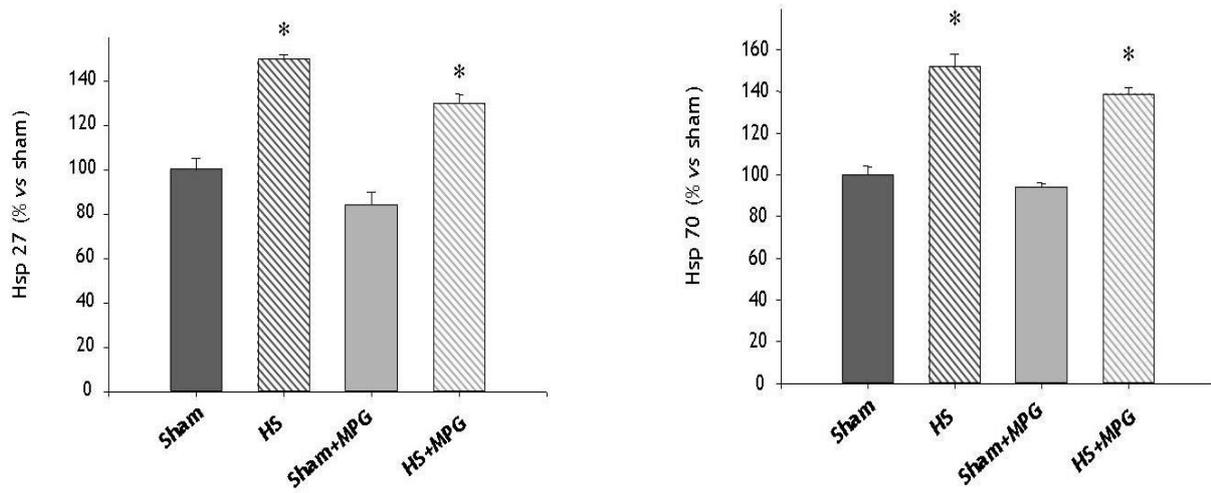


Figure 4.