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N-2-Mercaptopropionyl Glycine Blocks Ischemic Tolerance and Synthesis of Heat Shock Protein 70 in Rabbit Hearts

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Abstract

Since oxidative stress is reported to be one of the mediators of synthesis of heat shock protein 70 (HSP70) induced by hyperthermia, we investigated the effects of N-2-mercaptopropionyl glycine (MPG), a diffusible antioxidant, in *in vivo* rabbit model of heat stress (HS) preconditioning. Three groups of rabbits were studied: Group I, control rabbits treated with anesthetic alone; Group II, rabbits subjected to HS by raising core temperature to 42℃ for 15 min; Group III, given MPG (50mg/kg/hr *i.v.*), beginning 30 min prior to HS and continued up to 15 min post HS. Twenty-four hours later, all animals were treated with 30 min of the left anterior descending coronary artery occlusion followed by 180 min of reperfusion under ketamine/xylazine anesthesia. Risk area was delineated by Evans blue and infarct size determined by tetrazolium staining. The risk area ranged from 59.3 ± 5.8 % to 66.3 ± 3.4 % with no significant difference among all the groups. Infarct size/area risk was 53.6 ± 5.9 % in control rabbits, and decreased significantly to 26.7 ± 4.3 % in the HS hearts. Treatment with MPG of HS rabbits resulted in a significant increase in the infarct size (48.6 ± 11.8 %). The results show that prior HS significantly reduced infarct size ($P<0.05$) which was inhibited by MPG ($P<0.01$). Western blot analysis revealed significant synthesis of HSP70 in HS rabbit hearts and that synthesis was blocked with MPG. We conclude that oxidative stress is one of critical mediators of HS induced myocardial protection *in vivo* and trigger expression of HSP70.

Key Words: Heat stress, Heat shock proteins, Free radical, N-2-mercaptopropionyl glycine, Heart infarct size

INTRODUCTION

When living cells are exposed to a short increase in temperature or other stressing conditions, a group of proteins known as heat stress or heat shock proteins (HSPs) are synthesized in the heart and other tissues[1~3]. Among these proteins, the most abundant subset is the 70-kD protein family. It has been shown that oxidative stress is one of the mediators of synthesis of heat shock protein 70 (HSP70)[4]. Marber *et al.*[5] demonstrated that myocardial stress protein induced by either sublethal thermal or ischemic injury is associated with myocardial protection in rabbit model.

Cardioprotection by ischemic preconditioning is mediated, in part, by oxygen radicals which are scavenged by SOD or N-2-mercaptopropionyl glycine (MPG) in rabbits[6]. Thus, it appears that oxygen

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radical, especially hydroxyl radical (HO·), generation induced by preconditioning may trigger events which lead to myocardiac protection. Therefore, we hypothesized that scavenging oxidative stress with MPG during heat stress may also block ischemic tolerance and inhibit synthesis of HSP70. The purpose of this study was to examine whether MPG, a powerful HO· scavenger, can block the ability of heat stress to limit infarct size and reduce synthesis of HSP70 in *in vivo* rabbit model of heat stress preconditioning.

Materials and Methods

General Surgical Preparation

Male New Zealand white rabbits weighing 2.7 - 3.2 kg were allowed *ad labium* access to standard laboratory stock diet and water. Animals were initially anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) given intramuscularly. Five ml of 1% lidocaine was subcutaneously injected as an additional local anesthetic during the initial surgical procedures. Tracheotomy was performed and rabbits were intubated with an uncuffed endotracheal tube (ID 3.5 mm). The animals were ventilated with room air supplemented with additional oxygen using mechanical ventilator (Shinano, SN-480-5, Tokyo, Japan) and a semi-closed breathing circuit (Shinano, SN-487, Tokyo, Japan). Inspired and expired anesthetic concentration, inspiratory O_2 percentage and end-tidal CO_2 partial pressures were continuously monitored using a multigas anesthetic monitor (Datex, Capnomac, Helsinki, Finland). Ventilation rate was 30 - 35 breaths/min and tidal volume was between 30 - 35 ml. The ventilation rate was frequently adjusted to maintain PaO_2 greater than 100 mmHg, $PaCO_2$ at 35 - 45 mmHg, pH 7.35 - 7.45, and Base Excess between -3 and +3. After the left jugular vein was exposed and cannulated with a polyethylene catheter, 0.9 % sodium chloride was continually administered (0.15 ml/min) during the experiments. A fluid-filled polyethylene tube was placed in the carotid artery, which was connected to the pressure transducer (Nihon-kohden Co, TP-400T, Tokyo, Japan) for arterial pressure recording. An electrocardiogram was recorded throughout the experiment *via* lead II of the standard electrocardiogram (Nihon-kohden Co, Life scope 11, Tokyo, Japan). Left thoracotomy was performed and pericardium was opened to expose the heart. A silk thread (K-890H, Ethicon, Somerville, NJ) with taper C-1 needle was passed around the left anterior descending coronary artery (LAD) and the end of the tie were threaded through a small vinyl tube to form a snare. The LAD was occluded by pulling the snare, which was then fixed by clamping the tube with a mosquito hemostat. The rabbits were given 500 units of heparin in the coronary artery during reperfusion for preventing thrombus formation. Myocardial ischemia was confirmed by regional cyanosis, ECG-ST segment elevation and decreased blood pressure. Reperfusion was confirmed by reactive hyperemia over the surface after releasing the snare.

Study Groups and Experimental Protocol

Fig 1 presents study groups and experimental protocol. Anesthesia was maintained with ketamine and xylazine solution (ketamine 35 mg/kg/hr, xylazine 5 mg/kg/hr *i.m.*; KX) with room air supplemented with additional pure oxygen. Anesthetic and respiration were frequently adjusted to maintain steady hemodynamics throughout the experiments in all groups of animals. After all the surgical procedures had been performed, a 15 min period was allowed for stabilization. Following the baseline measurements, the animals were randomized into one of the following experimental protocols: Group I, control rabbits (Control : n=11) treated with anesthetic alone; Group II (Heat Stressed: n=10) subjected to HS by raising core temperature to 42℃ for 15 min and Group III (MPG + Heat Stressed: n=7) given MPG (50 mg/kg/hr *i.v.*), beginning 30 min prior to HS and continued up to 15 min post HS. Twenty-four hours later, all animals treated with LAD occlusion followed by 3 hr of reperfusion.

Hemodynamic measurements

Hemodynamic measurements included systolic, diastolic, mean arterial blood pressures and heart rate. Rate pressure product was calculated as the product of heart rate and peak arterial pressure. Baseline he

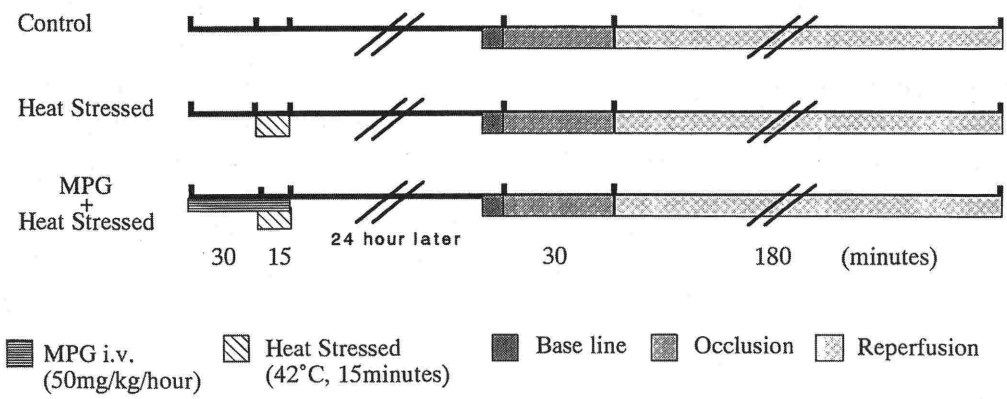


Fig 1. Design of experimental protocol. All animals were evaluated at a base line period. Evaluation of infarct size was carried out after 180 min of reperfusion.

modynamic measurements were taken prior to any experimental manipulations. Subsequently, the measurements were taken at 15 min of ischemia and 15, 60, 120 and 180 min of reperfusion.

Following completion of experimental protocol, the *in vivo* visualization of the myocardium at risk was accomplished with reocclusion of the coronary artery and injection of 10 % Evans blue into the venous cannula until the eyes turned blue. The Evans blue was allowed to circulate for about 30 sec to demarcate the risk and non-risk regions. The hearts were quickly excised under deep anesthesia and frozen. The frozen hearts were then cut into six transverse slices of equal thickness. The risk area was determined by negative staining with Evans blue. The slices were stained by incubation for 15 min in 1 % triphenyl tetrazolium chloride (TTC) in isotonic pH 7.4 phosphate buffer. After staining, the sections were placed in formalin for preservation, and measurements of risk area, infarct area and left ventricle were made with computer aided morphometry. From each section, the ischemic risk area (unstained by blue dye) and the infarcted area (unstained by TTC) were outlined and measured by planimetry. The area from each region was averaged from the slices. Infarct size was expressed both as a percentage of the ischemic risk area.

Gel electrophoresis and Western blotting

Six rabbits hearts (two controls, two Heat Stressed and two MPG + Heat Stressed) were used separately for stress protein determination. Three sets of animals went through the same protocol as that described above. Myocardial samples were placed in a lysis buffer of 5 % SDS and 1 % mercaptoethanol. The samples were homogenized with a polytron and boiled alternatively and passed through a 27-gauge needle and boiled again. Protein concentration was determined by Lowry's technique[7]. Equal amounts of proteins were loaded onto 12.5 % polyacrylamide gels. After electrophoresis, the proteins on the gel were transferred to nitrocellulose and probed by incubation with an antibody specific against HSP70 (Stressgen, Victoria, BC Canada). Alkaline phosphatase-conjugated goat anti-mouse IgGs were used as secondary antibodies (Vector Laboratories, Ins. Burlingame. CA).

Statistical analysis

Comparisons of myocardial tissue weights and necrosis data were made by one way analysis of variance (ANOVA). Statistical comparisons of individual hemodynamic parameters between groups were made using one-way ANOVA followed by Fisher's protected least significant difference. Bartlett's test for equality of variances was used to ensure the validity of statistical comparison using the one-way ANOVA. All data are reported as group mean ± SE, and were considered statistically significant at a probability value (P) less than 0.05.

RESULTS

Hemodynamic parameters

Heart rate, mean arterial blood pressure (MAP) and rate pressure product (RPP) are shown in Table 1 and Fig 2, respectively. No significant difference in the baseline levels of these parameters was observed between each group. The hemodynamics did not alter significantly throughout the reperfusion period at any data point in all the groups. Mean values were not significantly different between the groups at any time point for all the groups.

Infarct Size and Risk Area

The risk area ranged from 59.3 ± 5.8 % to 66.3 ± 3.4 % with no significant difference among all the groups (Fig 3), suggesting that changes in the size of infarct observed between the groups were not related to the percentage of area of left ventricle occluded by our technique. Fig 4 shows the infarct size expressed as percentage of risk area in three groups. Infarct size/risk area was 53.6 ± 5.9 % in control; the size was decreased significantly to 26.7 ± 4.3 % by pretreatment with heat stress. Treatment with MPG of heat stressed rabbits resulted in a significant increase in the infarct size (48.6 ± 11.8 %). The results show that prior HS significantly reduced infarct size ($P<0.05$) which was blocked by MPG ($P<0.01$).

Western blot analysis of HSP70

Western blot analysis revealed significant synthesis of HSP70 in heat stressed rabbit hearts and that synthesis was inhibited by MPG (Figs. 5, 6).

Discussion

Our results show that MPG, the cell diffusible free radical scavenger, administered prior to and during whole body hyperthermia blocked the protective effect of heat stress for ischemia with the expression of HSP70 *in vivo* rabbit hearts. Initially, we observed that heat stress by raised body temperature to 42℃ for

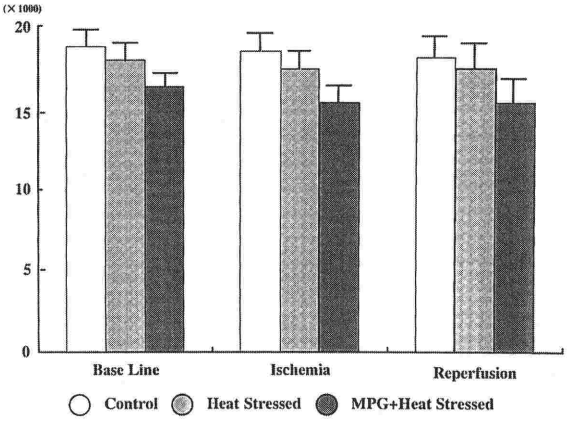


Fig 2. Effects of MPG on heat stress induced-rate pressure product..

Table 1. Hemodynamics during ischemia and reperfusion

Group	Base line	Ischemia	Reperfusion			
		15	15	60	120	180
Control(Vehicle)						
HR(beats/min)	202±9	207±12	208±13	212±14	210±9	210±16
MAP(mmHg)	81±6	79±6	72±5	75±6	67±4	63±5
Heat Stressed						
HR(beats/min)	217±9	221±8	220±11	235±10	217±10	238±13
MAP(mmHg)	76±5	72±5	73±4	70±5	68±5	65±5
MPG-Heat Stressed						
HR(beats/min)	203±9	204±8	205±12	211±23	204±16	209±22
MAP(mmHg)	67±5	63±6	68±5	67±7	65±5	57±5

HR-Heart rate; MAP-Mean arterial blood pressure. Results are expressed as mean±SEM from 7-11 animals in each group.

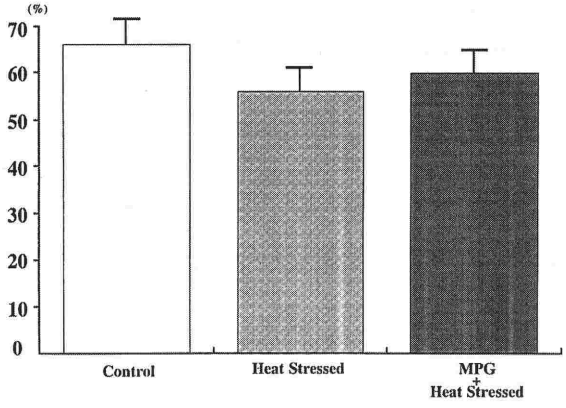
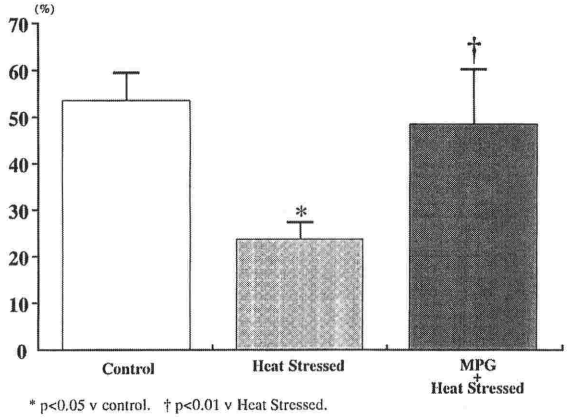


Fig 3. The effects of MPG on heat stress induced-risk area as percentage of anatomic left ventricle. Data are expressed as mean± SEM (n=7-11).



* p<0.05 v control. † p<0.01 v Heat Stressed.

Fig 4. The effects of MPG on heat stress induced-infarct size expressed as percentage of anatomic risk area. *P<0.05 versus control, † P<0.05 versus Heat Stressed.

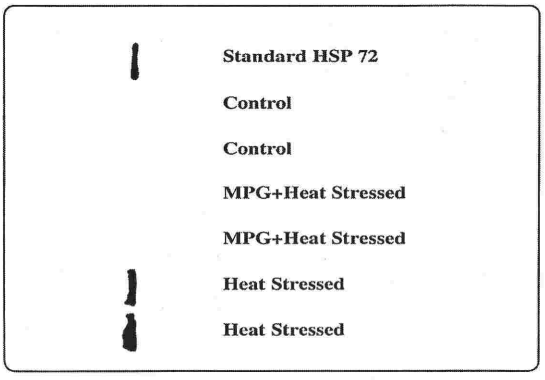


Fig 5. Densitometeric scan of Western bolt of HSP70.

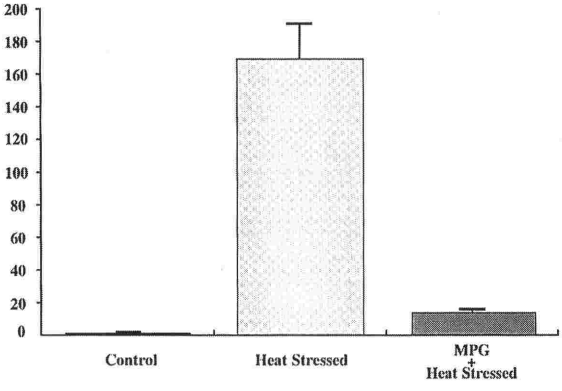


Fig 6. Effect of MPG on expression of heat stress induced-HSP70 in rabbit myocardium.

15 min resulted in significant reduction in myocardial infarct size, compared with that of control subjected to 30 minutes of coronary artery occlusion and 180 min of reperfusion. Thus, these results suggest an association of heat stress with ischemic tolerance observed during whole body hyperthermia. Whole-body hyperthermia significantly increased the expression HSP70. Our findings are consistent with the previous observations by Marber *et al.*[5,8] and Yamashita *et al.*[9] suggesting an important role of HSPs, particularly HSP 70 in myocardial protection.

In the present study, the infarct size was evaluated by staining with triphenyltetrazolium chloride (TTC) after 180 min of reperfusion. TTC reacts with NADH in the presence of lactate dehydrogenase enzymes causing the viable tissue (risk area) to stain a deep red color. The necrotic tissue dose not reacts with TTC, and remains a pale yellow color. This technique allows quantification of myocardial infarct after only a few hours of coronary occlusion and promises to be an accurate measure of ultimate infarct size at 2 to 48 hr of reperfusion when compared with subsequent histological analysis in animals not receiving further treatment[20,21]. TTC staining has been found to reveal equivalent infarct size values in comparison with histological determination in dogs[22], and rabbits[23] after 2 to 3 hr of reperfusion.

A brief period of ischemia or whole body hyperthermia increases cardiac resistance to ischemia reperfusion injury. Reactive oxygen species (ROS) have been proposed to participate in the induction of ischemic preconditioning[16]. Recently, Tritto *et al*[17] have shown that exposure to low concentrations of oxygen radicals can reproduce the beneficial effects of ischemic preconditioning on infarct size and post-ischemic recovery of left ventricular function. The data suggest that oxygen radicals might be potential contributors to ischemic preconditioning. It was shown that the limitation of infarct size by preconditioning is mediated by oxygen radicals which are scavenged by SOD or MPG in the rabbit[6,18], suggesting that oxygen radical generation appears to be involved in the triggering of ischemic preconditioning.

MPG acts as a scavenger of hydrogen peroxide but not of superoxide. Since the plasma half-time for elimination of MPG is less than 10 minutes *in vivo*, MPG is considered to be effective as an antioxidant only during hyperthermia[19]. MPG was not directly toxic to the hearts, as indicated by no significances in the hemodynamics. Pretreatment with MPG during hyperthermia reduced infarct size limiting-effect of hyperthermia on ischemia/reperfusion injury. These data indicate that the oxygen free radicals produced during hyperthermia induce cardioprotection independent of their hemodynamic effects, thus, suggesting that oxygen radicals contribute to myocardial protection in the rabbit heart.

Pretreatment with MPG during hyperthermia reduced the induction of HSP70 in the myocardium as well as the acquisition of thermotolerance. These data suggest that myocardial HSP70 may be induced during hyperthermia via a pathway that involves the production of free radicals which can be scavenged by MPG. It seems that HSP70 induced by heat stress is associated with hyperthermia-induced infarct limiting effects. Heat stress and the subsequent expression of HSPs has been shown to enhance post-ischemic functional recovery[10] and reduce infarct size[11,12]. Infarct size was highly inversively correlated with the amount of HSP70 induced as seen radiolabeled Western blots measured by optical densitometry. Recent studies[13~15] have shown that HSP70 transgene in the mouse heart protected against ischemia/reperfusion injury.

In conclusion, we have demonstrated that MPG, a diffusible antioxidant, given prior to heat stress blocked the protection normally provided by heat stress. It was suggested that oxygen free radicals trigger expression of HSP70 which eventually leads to myocardial protection following heat stress.

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