Whole-Body Hyperthermia Provides Biphasic Cardioprotection Against Ischemia/Reperfusion Injury in the Rat

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Background—Hyperthermia increases cardiac tolerance to ischemia/reperfusion injury 24 hours after the heat stress. Free radicals and redox mechanisms have been implicated in such tolerance. However, the time course and its relation to the induction of antioxidative enzymes in the protection induced by whole-body hyperthermia against ischemia/reperfusion injury are unknown.

Methods and Results—Hyperthermia was induced in anesthetized rats by placement in a temperature-controlled water bath. After the defined recovery interval(s) at room temperature, ischemia was induced by occlusion of the left coronary artery for 20 minutes, followed by reperfusion for 48 hours. The exposure to hyperthermia led to a recovery interval–dependent, biphasic reduction in the incidence of ventricular fibrillation during ischemia and in the size of the myocardial infarct as determined after 48 hours of reperfusion. The time course of the late-phase (24- to 96-hour recovery interval) but not the early-phase (0.5 hour) cardioprotection depended on the degree of hyperthermia. The time course of the increase in myocardial manganese superoxide dismutase (Mn-SOD) activity corresponded to that of the cardioprotective effects, although an increase in the content of Mn-SOD and of heat shock protein 72 corresponded only to the late-phase effects. Administration of an antioxidant before hyperthermia abolished the early- and late-phase cardioprotection and the increase in Mn-SOD activity.

Conclusions—The activation of Mn-SOD mediated by free radical production during hyperthermia is important in the acquisition of early-phase and late-phase cardioprotection against ischemia/reperfusion injury in rats. (Circulation. 1998;98:1414-1421.)

Key Words: superoxide dismutase ■ proteins ■ free radicals ■ mercaptopropionyl glycine

Cardiac resistance to ischemia/reperfusion injury is increased by exposure to such sublethal stress as a brief period of ischemia or whole-body hyperthermia. Tolerance after exposure to a brief period of ischemia (ischemic preconditioning) is manifested in a biphasic manner, both soon after and 24 hours after preconditioning. Different mechanisms mediate cardioprotection in the early phase and in the late phase. The de novo synthesis of proteins is not involved in the early phase, but the induction of endogenous heat shock proteins (HSPs) or antioxidative enzymes such as manganese superoxide dismutase (Mn-SOD) is important in the late phase. However, the production of free radicals during ischemic preconditioning is involved in the mechanism of tolerance in both the early phase and the late phase.

Whole-body hyperthermia also induces tolerance of the heart to ischemia/reperfusion injury 24 hours after hyperthermia. Because free radicals are produced by heat stress and transduce the heat shock signal in mammalian cells, a redox mechanism may be involved in the acquisition of late-phase tolerance via the induction of rescue proteins, such as HSPs and endogenous scavengers of reactive oxygen species. However, heat stress induction of early-phase tolerance of the heart to ischemia/reperfusion injury and the role of free radicals in the acquisition of tolerance have not yet been determined.

The purpose of this study was to determine the role of redox mechanisms and the induction of rescue proteins in early-phase and late-phase cardioprotection against ischemia/reperfusion injury by whole-body hyperthermia. The relationship between the induction of Mn-SOD or HSP72 by heat stress and the protective effects of heat stress against incidence of ventricular fibrillation (VF) during ischemia and extent of myocardial necrosis after reperfusion were examined in a rat model of myocardial infarction.

Methods

Animals and Experimental Protocol

Male Wistar rats (300 to 350 g) were maintained on a 12-hour dark/light cycle, housed at 23 ± 1.5°C (45 ± 15% relative humidity), and allowed access to water and rat chow ad libitum. After the
induction of light anesthesia with sodium pentobarbital 5 to 10 mg/kg IP, whole-body hyperthermia was induced by placing the rats in a constant-temperature water bath as described previously. During whole-body hyperthermia, the animal was supported by a wire apparatus to prevent the aspiration of water and to facilitate the measurement of rectal temperature. Hyperthermia was maintained at 40.0±0.2°C for 5 minutes (40°C group), 41±0.2°C for 10 minutes (41°C group), or 42±0.2°C for 15 minutes (42°C group) (Figure 1). For hyperthermia at 42°C, ~5 minutes was required for the rat’s core temperature to reach 42°C. Rats in the sham-treated control group were placed in a water bath maintained at 36.5±0.2°C for 20 minutes. Rats were allowed to recover at room temperature for defined intervals (0.5, 3, 6, 12, 24, 36, 48, 60, 72, 84, 96, or 120 hours, n=10 to 12 each) before the induction of myocardial infarction. Some rats received neither the hyperthermic nor the normothermic water-bath treatments (untreated controls).

To determine the involvement of reactive oxygen species during hyperthermia in the acquisition of tolerance against ischemia/reperfusion injury, the low-molecular-weight synthetic antioxidant N-2-mercaptopropionyl glycine (MPG; 100 mg/kg IP) was infused 10 minutes before whole-body hyperthermia (42°C group) or normothermia. To facilitate the use of MPG without causing acidosis, the highly acidic aqueous solution of MPG was neutralized with NaOH to a pH of 7.3 to 7.4 before administration.

Infarction Protocol

At the end of the recovery interval, rats were anesthetized with sodium pentobarbital 25 mg/kg IP, intubated, and ventilated with a small-animal respirator (model SN-480-7-10, Shimano Seisakusyo). The right femoral artery was cannulated with polyethylene tubing for the continuous measurement of arterial blood pressure with a pressure transducer (TP-300T; Nihon Kohden). The heart rate, incidence of arrhythmias, and ST-segment changes were monitored. Hemodynamic variables were recorded continuously (model WT-645G recorder; Nihon Kohden). The chest was opened with a midline sternotomy. Silk thread (7-0) was passed around the left coronary artery (LCA) 3 to 4 mm distal to the LCA origin. After a 10-minute period of stabilization, measurement of arterial pressure was initiated and the LCA was ligated. After 20 minutes of coronary occlusion, the snare was released. Reperfusion was indicated by a change in the color of the ventricular surface. The silk thread around the coronary artery was left in place. The surgical wounds were repaired 60 minutes after reperfusion, and the rats were returned to individual cages to recover. Aseptic surgical techniques were used throughout. Benzylpenicillin 30 000 U/kg IM was injected as prophylaxis against infection. Rats were reanesthetized with sodium pentobarbital 25 mg/kg IP 48 hours after surgery and were intubated and ventilated with a respirator. After the heart was exposed and the LCA was reoccluded, Evans blue dye (2%) was injected via the right femoral vein to estimate the area perfused by the occluded artery (ischemic region). Rats were killed by an overdose of sodium pentobarbital. The left ventricle was then cut into 6 pieces perpendicular to the apex–base axis. These specimens were incubated with 1% triphenyltetrazolium chloride (TTC) at 37°C to stain the nonischemic region. The ischemic, infarcted, and nonischemic areas of tissue were separated with scissors and weighed. The area at risk and the size of the infarct were defined as the ratio of the mass of the ischemic region to the left ventricular mass and the ratio of the mass of the infarct region to the mass of the ischemic region, respectively, expressed as a percentage.

Arrhythmias were monitored by ECG. VF was defined according to the criteria of the Lambeth Conventions. If VF occurred during ischemia and did not resolve spontaneously within 3 seconds, manual cardioversion was attempted by gentle palpation of the nonischemic region of the heart. We excluded from infarct-size analysis rats in which VF persisted for >6 seconds or in which cardioversion had to be performed >3 times. Incidence of VF was evaluated as it occurred (yes/no).

Myocardial Tissue Sampling

To obtain tissue samples for measurement of enzyme content and activity, rats were killed by an overdose of sodium pentobarbital. The myocardial tissue was rinsed in PBS, and both atria and the right ventricle were removed. Left ventricular myocardial samples were rapidly frozen by immersion in liquid nitrogen and stored at −80°C. To measure myocardial SOD activity and content, blood remaining in the left and right coronary arteries was washed out by retrograde infusion of PBS through the ascending aorta before myocardial tissue was sampled.

Measurement of Activity and Content of Mn-SOD

Myocardial levels of Mn-SOD were determined in rats killed after recovery intervals of 0.5, 3, 12, 24, 48, 72, 96, or 120 hours and in control rats that did not receive water-bath treatment. SOD activity of the myocardial samples was determined by the nitro blue tetrazolium (NBT) method. Myocardium was homogenized with 20 mmol/L PBS containing 1 mmol/L EDTA and centrifuged at 900 g for 15 minutes. The supernatant was sonicated and added to the reaction mixture of NBT with xanthine–xanthine oxidase. SOD activity in the supernatant was measured colorimetrically as inhibitory activity against the formation of blue formazan by SOD in the reaction mixture. To evaluate Mn-SOD, the activity was assayed in the presence of potassium cyanide (1 mmol/L) to inhibit copper–zinc–superoxide dismutase (Cu,Zn-SOD) activity. Cu,Zn-SOD activity was determined by subtracting the activity of Mn-SOD from the total SOD activity. Pentobarbital sodium had no effect on the SOD activity of normal rat hearts in our preliminary study. The activity and content of Mn-SOD were expressed relative to the protein concentration in the supernatant.
Measurement of HSP72 Content
HSP72 content in heart tissue from sham-treated controls and from rats exposed to whole-body hyperthermia at 42°C was evaluated for recovery intervals of 0.5 and 72 hours. HSP72 content in the supernatant of unsonicated heart tissue homogenate was determined by means of Western blot analysis. Protein samples were diluted into a 1X Laemmli sample buffer solution. Equal total protein loads (40 μg) were separated by SDS-PAGE on 1-mm-thick, 7.5% polyacrylamide gels. The gels were run in duplicate, and 1 gel was stained with Coomassie blue to determine equivalence of loading and adequacy of sample preparation. The proteins were transferred onto a nitrocellulose membrane by Western blotting. Membranes were washed in PBS with 0.2% BSA to block nonspecific binding sites. The blocked membranes were incubated at 4°C overnight in PBS containing mouse monoclonal IgG cross-reactive to the inducible 72-kD heat-shock protein (Stressgen) at 1:500 dilution and then incubated in PBS containing horseradish peroxidase–conjugated rabbit anti-mouse IgG (Kirkegaard & Perry Laboratories) at 1:5000 dilution at room temperature for 2 hours.

Materials
Chemicals were purchased from Sigma Immunochemicals and Wako Fine Chemicals.

Statistics
Data are expressed as mean±SEM. The significance of the differences between shams and each hyperthermic group was assessed by 1-way ANOVA and the Fisher protected least significant difference post hoc test. The significance of differences in the incidence of VF was determined by χ² testing with Yates’ correction. A value of P<0.05 was considered statistically significant.

Results
Exclusion Due to VF and Death
A total of 24 rats that developed serious VF during occlusion (5 in the sham-treated control group, 4 in the 40°C group, 4 in the 41°C group, 8 in the 42°C group, 2 in the 42°C group pretreated with MPG, and 1 in the sham-treated control group pretreated with MPG) were excluded from the evaluation of myocardial infarct size. Eleven rats died prematurely during the 48-hour reperfusion period (2 in the sham-treated control group, 2 in the 40°C group, 3 in the 41°C group, 2 in the 42°C group, 1 in the 42°C group pretreated with MPG, and 1 in the sham-treated control group pretreated with MPG).

Hemodynamic Data, Area at Risk, and Rectal Temperature
No significant differences were observed in the rate-pressure product or in the rectal temperature during the infarct protocol among the groups before ischemia, at the end of the ischemic period, or 0.5 hour after reperfusion (data not shown).

The size of the area at risk expressed as a percentage of left ventricular area did not differ significantly among the groups (data not shown).

Incidence of VF
The sham-treated control animals that were placed in a water bath at 36.5°C exhibited no significant differences in the incidence of VF (yes/no) for recovery intervals of 0.5 to 72 hours. Prior exposure to whole-body hyperthermia resulted in a time-dependent biphasic tolerance against VF during myocardial ischemia (Figure 2). Hyperthermia at 40°C for 5
Size of Myocardial Infarct

There were no significant differences in the size of the myocardial infarct for recovery intervals of 0.5 to 72 hours among the sham-treated control groups. The induction of whole-body hyperthermia reduced the size of the ischemia/reperfusion–induced myocardial infarct in a time-dependent, biphasic manner (Figure 3). Protection against ischemic damage was evident in rats allowed a 0.5-hour recovery interval but was no longer evident by 6 hours in all 3 groups. As the recovery interval increased beyond 6 hours, protection from damage was again observed and followed a time course similar to that for tolerance to VF (Figure 3). Although not shown, the size of the infarct assessed by TTC staining did not differ significantly from that assessed by histology in additional rats with 48-hour reperfusion.

Induction of Mn-SOD After Hyperthermia

In the myocardia from sham-treated controls, both Mn-SOD activity (data not shown) and Mn-SOD content remained constant during the time course of the experiment. The biphasic time course of changes in Mn-SOD activity in hyperthermia-treated myocardium (Figure 4) was complementary to the time course for protection against VF (Figure 2) and protection against ischemic damage (Figure 3) for each group. Mn-SOD activity increased at the 0.5-hour recovery interval but was not different from control for recovery intervals of 3, 12, or 120 hours. Mn-SOD content in rats of the 42°C hyperthermia group (Figure 5) did not change relative to control for recovery intervals, thereafter, Mn-SOD content increased significantly and reached 145% of sham-treated control values 72 hours after hyperthermia at 42°C. The activity of the cytosolic isoform of SOD (Cu,Zn-SOD) did not change after hyperthermia (data not shown).

Effect of MPG Treatment

Because the half-time for elimination of MPG is ≈7 minutes in vivo,26 MPG was considered to be effective as an antiox-
idant only during hyperthermia. The infusion of MPG did not alter the incidence of VF or the size of the myocardial infarct in sham-treated control rats (Figure 6). However, MPG completely abolished the protection against ischemic damage and the occurrence of VF at both 0.5 and 72 hours after hyperthermia at 42°C.

MPG did not alter Mn-SOD activity at recovery intervals of 0.5 or 72 hours in sham-treated control rats (Figure 7). However, MPG completely abolished both the early peak of Mn-SOD activity (0.5 hour after hyperthermia) and the late-phase peak of Mn-SOD activity (72 hours after hyperthermia) in the 42°C group (Figure 7). Moreover, MPG completely abolished the increase of Mn-SOD content 72 hours after hyperthermia in the 42°C group (data not shown).

**Induction of HSP72**

In sham-treated control rats, HSP72 levels did not increase after recovery intervals of either 0.5 or 72 hours after normothermic treatment (data not shown). Representative results regarding HSP72 induction are shown in Figure 8. HSP72 content was not different from control 0.5 hour after 42°C hyperthermia. However, HSP72 content was markedly increased at 72 hours after hyperthermia. MPG did not alter the HSP72 levels after normothermic treatment. However, MPG abolished the increased levels of HSP72 observed in myocardium at 72 hours after hyperthermia.

**Discussion**

In ischemic preconditioning, cardioprotection at both the early phase and the late phase is mediated by the oxygen free radicals that are produced during preconditioning. In particular, Baines et al. clearly demonstrated the role of free radicals in classic preconditioning, albeit generated by ischemia or the addition of hypoxanthine and xanthine oxidase to the perfusate. The generation of superoxide by the mitochondria of rat muscle increases in a temperature-dependent manner from 37°C (muscle temperature at rest) to 45°C (muscle temperature after exercise). Pretreatment with a radical scavenger, MPG, abolished both the early-phase and late-phase protection against ischemic damage.

**Figure 5.** Effects of hyperthermia and recovery interval on Mn-SOD content in rat myocardium. Mn-SOD content was determined by ELISA in cardiac tissue homogenates from rats exposed to treatments as described in Figure 1. Untreated control indicates value from rats that received neither hyperthermic nor normothermic water-bath treatment. ○, Sham-treated control group; ●, hyperthermia (42°C)–treated group. Each data point represents mean ± SEM of values from ≥4 rats. *P < 0.05 vs corresponding sham-treated control by ANOVA and Fisher protected least significant difference test.

**Figure 6.** Effects of MPG on incidence of VF during ischemia and on infarct size in rats. Arrhythmias were monitored by ECG during ischemia, and infarct size was calculated as ratio of mass of infarct region to mass of ischemic region after reperfusion in rats exposed to treatments as described in Figure 1. Sham indicates sham-treated controls; MPG, sham-treated controls receiving MPG 100 mg/kg IP; 42°C, rats from 42°C group; and MPG+42, rats receiving MPG before hyperthermic treatment at 42°C. Number of rats used is indicated in each column. *P < 0.05 vs sham-treated controls by χ² analysis with Yates’ correction (incidence of VF) and by ANOVA (infarct size).

**Figure 7.** Effects of MPG and hyperthermic treatment on Mn-SOD activity in rat myocardium. Activity of Mn-SOD was determined by NBT method in cardiac tissue homogenates from rats exposed to treatments as described in Figure 1. Data are mean ± SEM values from at least 6 rats. *P < 0.05 vs sham-treated controls by ANOVA and Fisher protected least significant difference test.
late-phase beneficial effects of hyperthermia on ischemia/ reperfusion injury, indicating that the oxygen free radicals produced during hyperthermia were cardioprotective, not cardiotoxic. Numerous studies have shown that small changes in thiol redox potential can exert signaling functions that can be blocked by high levels of thiol antioxidants. The transcription factors activating protein-1 and nuclear factor (NF)-κB and the activity of several kinases and phosphatases are subject to redox regulation.21,22 The alteration of a redox state in mammalian cells is also important in the heat shock signal transduction.12 Previous studies have shown that transient thiol oxidation results in activation of NF-κB by tumor necrosis factor-α and that N-acetylcysteine, a glutathione precursor and antioxidant, blocks both the decrease in glutathione and the activation of NF-κB.23 In the present study, MPG abolished the increase in levels of HSP72 observed in myocardium at 72 hours after hyperthermia. MPG also abolished the early-phase and late-phase peaks of Mn-SOD activity and the late-phase induction of Mn-SOD. These data indicate that the thiol redox potential and the generation of oxygen free radicals are important in signal transduction after heat stress.

The mechanism that underlies the cardioprotection observed at both the early phase and the late phase after hyperthermia appeared to be related to an increase in Mn-SOD activity. The time course of cardioprotection coincided with that for the increase of Mn-SOD activity after hyperthermia for various magnitudes of heat stress or after treatment with an antioxidant. Brief, sublethal ischemic or anoxic insults have been shown to increase Mn-SOD activity and to induce cardioprotection or myocyte protection in a biphasic manner.5,7 Other studies have also attempted to measure Mn-SOD activity after sublethal ischemia and reperfusion but have been less successful at establishing the correlation of the level of Mn-SOD activity with the degree of cardioprotection.24 Das et al25 reported that mammalian hearts subjected to heat shock increase expression of Mn-SOD mRNA. Heat shock also enhances SOD activity in the pig heart.26 Mn-SOD is directly associated with the protection of the myocyte against hypoxia-reoxygenation injury.6,19 However, the causal relation between the hyperthermia-induced cardioprotection and the elevation of Mn-SOD activity remains to be elucidated.

Whole-body hyperthermia significantly increased the late-phase levels of HSP72. Treatment with a radical scavenger during hyperthermia reduced the induction of HSP72. These data suggest that myocardial HSP72 and Mn-SOD may be induced via a common pathway that involves the production of free radicals during hyperthermia. However, HSP72 induction and hyperthermia-induced cardioprotection are not correlated,14,15 although some reports have demonstrated a positive correlation between heat-induced HSP72 expression and infarct size reduction.2,10,27,28 Data from in vitro experiments19 indicate that Mn-SOD and HSP72 are induced in cardiac myocytes after hyperthermia, but only the inhibition of Mn-SOD (by antisense oligodeoxyribonucleotides) abolishes the late-phase cardioprotective effect. Beckmann et al29 showed that members of the HSP70 family bind transiently to nascent proteins and act as intracellular chaperones, helping to stabilize these proteins until they achieve their final conformation. Voos et al30 suggested that the constitutively expressed HSP70 in yeast cells may be an unfoldase that facilitates protein transport through the membranes of the endoplasmic reticulum and mitochondria. The induced HSP72 may have chaperon functions and help refold partially denatured proteins after stress. Thus, induction of HSP72 may promote the maturation of Mn-SOD. Overexpression of HSP70 in rat hearts is reported to induce cardioprotection against ischemia/reperfusion injury associated with increased levels of myocardial Mn-SOD content.31 However, it remains to be determined whether HSP72 and Mn-SOD are cooperative or interacting factors in acquired ischemic tolerance.

Kingma et al14 reported that endogenous catalase activity in myocardium is increased after heat stress and could protect the heart against ischemia-reperfusion injury. Although we did not examine myocardial catalase activity in the present study, it is conceivable that a heat shock–induced increase in endogenous catalase activity in myocardial tissue could facilitate or enhance the detoxification of the superoxide in mitochondria and thus result in reduced tissue injury during the process of ischemia-reperfusion.

Induction of Mn-SOD has been demonstrated in eukaryotes in conditions that favor the production of free radicals such as superoxide.32 The induction of Mn-SOD by tumor necrosis factor-α was shown to be mediated by oxygen free radicals.33 Oberley et al33 reported that the local x-irradiation of mouse heart, which is known to produce oxygen free radicals, causes a large biphasic increase in Mn-SOD activity; this increase was dose- and time-dependent. In the present study, the late-phase increase in Mn-SOD activity shifted to a later period as the magnitude of the prior heat stress increased, although the reason for this shift is unclear. Also unclear is the mechanism responsible for early-phase increased Mn-SOD activity in the absence of an increase in Mn-SOD content. Modulation of early-phase Mn-SOD activity by antioxidants may indicate direct action of reactive oxygen species on the enzyme. In eukaryotic cells, Mn-SOD is preformed and stored as a precursor.35 It is also
possible that precursors of Mn-SOD that have antigenicity but lack enzyme activity are modulated by heat stress.

We used the macroscopic TTC staining technique for determination of necrotic tissue in this study, being fully aware of its documented limitation. Previous studies in rabbit and pig models have demonstrated that exogenous treatment of hearts with Cu,Zn-SOD induced the artifactual SOD-mediated preservation of the TTC reaction within 24 hours of reperfusion, whereas that preservation disappeared 72 hours after reperfusion. In our additional experiments, however, there were no differences in the sizes of the infarcts between 48-hour-reperfusion and 72-hour-reperfusion models assessed by TTC staining in the rats at 0.5 and 72 hours after 42°C whole-body hyperthermia (data not shown). Moreover, the Mn-SOD activity in the risk area of the myocardium decreased after reperfusion and did not exceed the level of that in control rats without ischemia-reperfusion (unpublished observations). Tanaka et al reported that TTC macrochemistry provided a reliable measurement of infarct size after 4 days or 4 hours of reperfusion in a dog model, even if hearts were treated with SOD exogenously. There may be differences in effects on measurement of infarct size assessed by TTC staining between endogenous and exogenous SOD, or between Cu,Zn-SOD and Mn-SOD.

Previous studies in rat and rabbit models indicate that heat stress at 42°C for 15 minutes induces maximal cardioprotection 24 hours after whole-body hyperthermia. In the present study, maximal late-phase protection was achieved 72 hours after hyperthermia at 42°C for 15 minutes. The apparent discrepancy may be explained by differences in the method used to induce hyperthermia. Rats were placed in a temperature-controlled water bath, rather than on a heating pad or blanket, to induce whole-body hyperthermia in the present study.

Conclusions

Whole-body hyperthermia protected against VF during ischemia and limited the extent of myocardial infarction after reperfusion both immediately and 24 to 72 hours after heat stress. The time course of the protective effect is similar to that for increases in myocardial Mn-SOD activity, although Mn-SOD content was increased only in conjunction with late-phase protection. Activation of Mn-SOD appears to be mediated through a common pathway that involves free radical production during hyperthermia. It remains to be determined whether elevation of Mn-SOD activity is causally related to the cardioprotection induced by heat stress against ischemia-reperfusion injury. Further studies should also be undertaken to clarify other mechanisms of cytoprotection evoked by hyperthermic preconditioning.

References


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