Journal of the American College of Cardiology
© 2002 by the American College of Cardiology Foundation
Published by Elsevier Science Inc.

Vol. 40, No. 4, 2002
ISSN 0735-1097/02/$22.00
PII S0735-1097(02)02001-6

# Repeated Physiologic Stresses Provide Persistent Cardioprotection Against Ischemia-Reperfusion Injury in Rats

Shiro Hoshida, MD, PHD,*† Nobushige Yamashita, MD, PHD,† Kinya Otsu, MD, PHD,† Masatsugu Hori, MD, PHD†

*Osaka, Japan*

**OBJECTIVES** We investigated the time course of myocardial tolerance to ischemia-reperfusion injury after repeated physiologic or pharmacologic stresses.

**BACKGROUND** Sublethal stress provides cardiac tolerance to ischemia-reperfusion injury and increases the activity of manganese superoxide dismutase (Mn-SOD) in the myocardium in a biphasic manner. However, few studies have investigated the time course of the cardioprotective effects after repeated stresses.

**METHODS** One or two episodes of the same physiologic or pharmacologic stress (exercise, whole-body hyperthermia, or tumor necrosis factor-alpha treatment), or a combination of two different types of stress, were induced after a 48-h interval. The rats were then subjected to 20 min of left coronary artery occlusion, followed by 48 h of reperfusion. The interval between the last stimulus and the induced ischemia was between 0.5 h and 168 h. The incidence of ventricular fibrillation during ischemia and the size of the myocardial infarct after reperfusion were then examined.

**RESULTS** When two episodes of physiologic or pharmacologic stress were induced, the beneficial effects against ischemia-reperfusion injury were observed in a monophasic manner. These effects persisted for a period of 0.5 to 60 h. One episode of sublethal stress provoked the same beneficial effects, but in a biphasic manner. The increase in Mn-SOD activity in the cardiac tissue resembled the time course for cardioprotection against ischemia-reperfusion injury.

**CONCLUSIONS** Two episodes of physiologic or pharmacologic stress can provide persistent cardioprotective effects against ischemia-reperfusion injury. (J Am Coll Cardiol 2002;40:826–31) © 2002 by the American College of Cardiology Foundation

We and others have reported that physiologic stress, such as exercise (1), whole-body hyperthermia (2–4) and sublethal ischemia (2,5), or pharmacologic stress, such as treatment with monophosphoryl lipid A (6,7) or tumor necrosis factor (TNF)-alpha (1), can provide cardioprotection against ischemic injury in a biphasic manner. The obtainment of cardioprotection by these stresses may involve a common mechanism that functions through the induction and activation of manganese superoxide dismutase (Mn-SOD) through the production of reactive oxygen species and inflammatory cytokines (1,8,9). Acute (10,11) or endurance exercise training (12,13) has been shown to provide myocardial protection against ischemia-reperfusion injury, as assessed by left ventricular function. Prolongation of the delayed phase of cardioprotection was also observed by repetitive adenosine $A_1$ receptor activation (14). Our hypothesis is that various repeated stresses may provoke a persistent tolerance to ischemia-reperfusion injury, especially in terms of the size of the myocardial infarct and the incidence of ventricular fibrillation (VF). In this report, we investigated whether two episodes of the same physiologic or pharmacologic stress (exercise, whole-body hyperthermia or administration of TNF-alpha), or a combination of two different types of stress, could provide prolonged protection against myocardial ischemia-reperfusion.

From the *Division of Cardiology, Osaka Rosai Hospital, Sakai; and †Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Osaka, Japan.

Manuscript received June 12, 2001; revised manuscript received April 19, 2002, accepted May 20, 2002.

## METHODS

**Animals and experimental protocol.** Male Wistar rats (240 to 320 g) were maintained in a 12-h dark/light cycle. The rats were housed in a facility at 23 ± 1.5°C and 45 ± 15% relative humidity, with access to food and water ad libitum.

One or two episodes of the same physiologic or pharmacologic stress (exercise, whole-body hyperthermia, or an intravenous injection of 1.5 μg/kg body weight of TNF-alpha), or a combination of two different types of stress, were induced after an interval of 48 h (Fig. 1). The protocols for the exercise and whole-body hyperthermia sessions have been previously reported (1,4). Briefly, exercise was induced by placing the rat on a motor-driven treadmill operating at a speed of 27 to 30 m/min, at 0% grade, for 30 min. The exercise session began and ended with a 5-min "warm-up" and "cool-down" period at a speed of 15 m/min. The duration of the exercise included the warm-up and cool-down periods. Whole-body hyperthermia was induced by placing the rats in a constant-temperature water bath after the induction of a light anesthesia with sodium pentobarbital (5 to 10 mg/kg intraperitoneally). Hyperthermia was maintained at 41 ± 0.2°C (rectal temperature) for 10 min.

**Abbreviations and Acronyms**

MI = myocardial infarction
Mn-SOD = manganese superoxide dismutase
TNF = tumor necrosis factor
VF = ventricular fibrillation

The rats were allowed to recover at room temperature for 48 h before the second stress or for a defined interval before the induction of myocardial infarction (MI) or the measurement of Mn-SOD activity. Recombinant murine TNF-alpha was obtained from Genzyme (Framingham, Massachusetts). The recombinant cytokine was diluted in pathogen-free saline on the day of injection.

**Infarction protocol.** The infarction protocol was performed according to a previously reported method (1,4). Briefly, all rats underwent 20 min of left coronary artery ligation, followed by release of the ligature. Forty-eight hours after reperfusion, the heart was excised, and the size of the infarct was evaluated using a double-staining method with Evans' blue dye and triphenyltetrazolium chloride. The size of the myocardial infarct (area not containing triphenyltetrazolium chloride staining) was expressed as a percentage of the ischemic risk area (area not containing Evans' blue dye).

Blood pressure was monitored through a polyethylene cannula placed in the right femoral artery. When the VF occurring during left coronary artery ligation did not resolve spontaneously within 3 s, the nonischemic region of the heart was manually "flicked" to produce cardioversion. If the VF persisted for more than 6 s or if cardioversion was performed more than three times, the animal was excluded from the assessment of infarct size.

This study was performed under the supervision of the Animal Research Committee in accordance with the Guidelines for Animal Experiments of Osaka University.

**Measurement of Mn-SOD activity.** We measured myocardial Mn-SOD activity 0.5 to 168 h after the last stress stimulus. The blood remaining in the left and right coronary arteries was washed out by retrograde infusion of phosphate-buffered saline through the ascending aorta, before the myocardial tissue was sampled. The myocardial tissue was then rinsed in phosphate-buffered saline, and both the atria and right ventricle were removed. The SOD activity of the myocardial samples was determined according to a previously reported method (15). To evaluate Mn-SOD activity, the assay was performed in the presence of potassium cyanide (1 mmol/l) to inhibit copper- and zinc-SOD activity. The activity of Mn-SOD was expressed as a value relative to the protein concentration in the supernatant, as determined using Lowry's method.

**Statistical analysis.** Data are expressed as the mean value ± SEM. Intergroup comparisons were assessed for significance using one-way analysis of variance with repeated

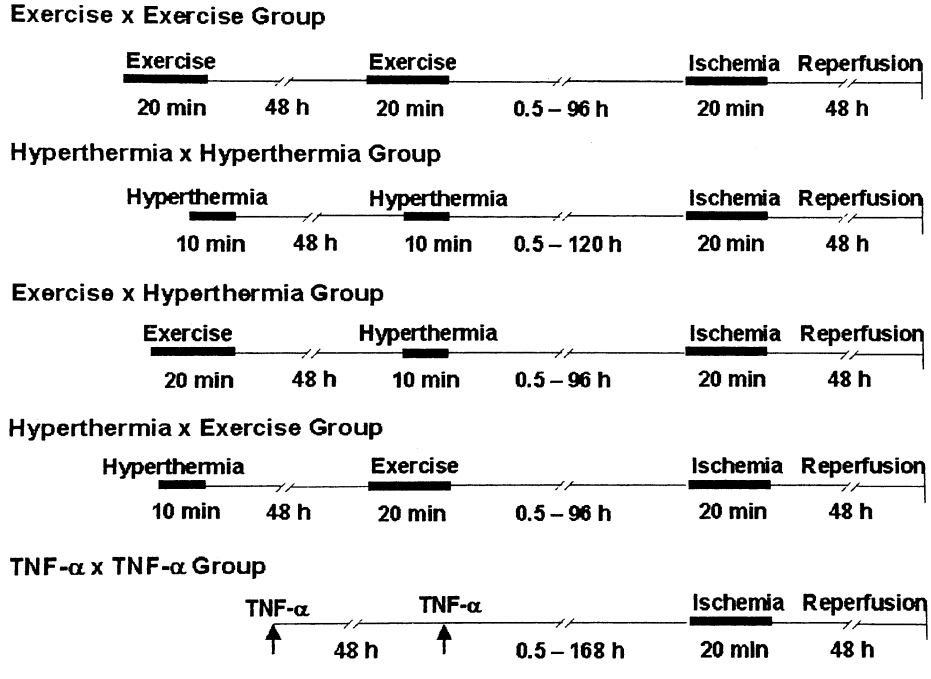


**Figure 1.** Experimental protocol for five repeated-stress groups. After two sessions of exercise, whole-body hyperthermia or tumor necrosis factor (TNF)-alpha injections, performed at an interval of 48 h, the rats were subjected to 20 min of left coronary artery occlusion, followed by 48 h of reperfusion. The interval between the second stimulus and ischemia was between 0.5 and 168 h. The incidence of ventricular fibrillation during ischemia and the size of myocardial infarction after reperfusion were then examined. Although not shown, in rats subjected to a single stress (three single-stress groups), the rats were subjected to 20 min of left coronary artery occlusion, followed by 48 h of reperfusion after one episode of exercise, whole-body hyperthermia or TNF-alpha injection. The interval between the single-stress stimulus and ischemia was between 0.5 and 120 h. Myocardial manganese superoxide dismutase activity was measured 0.5 to 120 h after the single-stress stimulus (single-stress group) or 0.5 to 168 h after the second-stress stimulus (repeated-stress group).

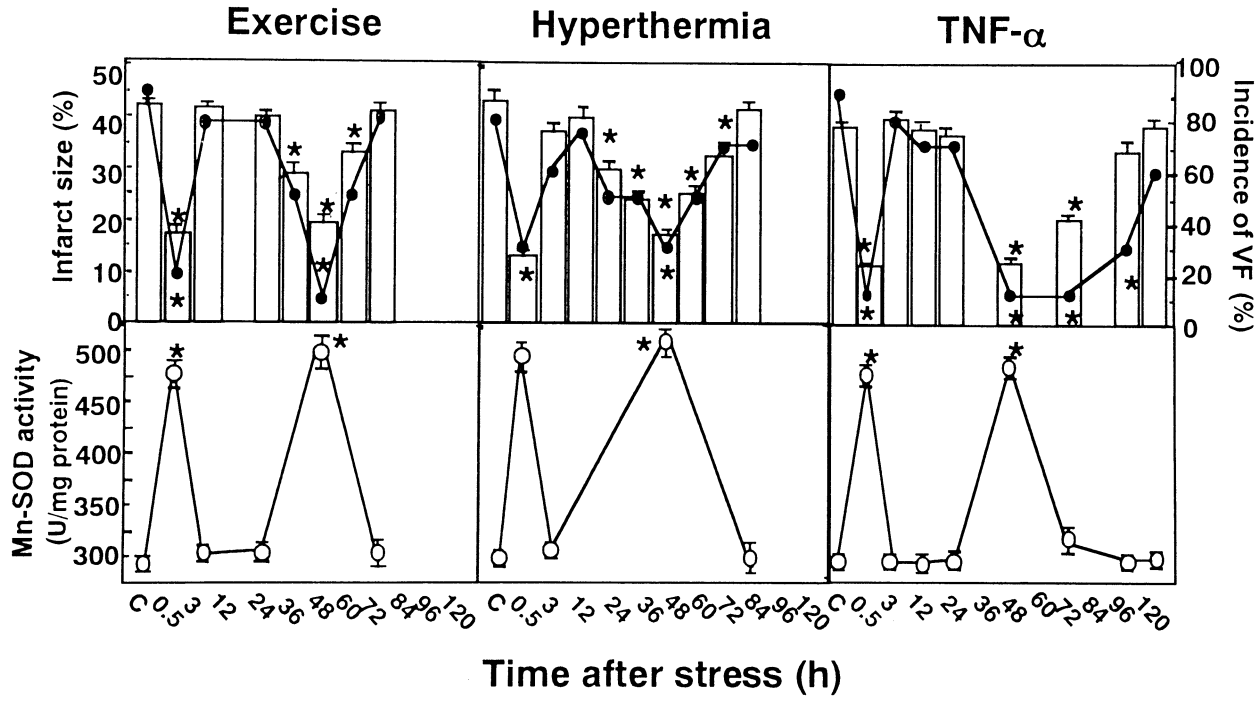


**Figure 2.** The effects of a single session of a sublethal stimulus and the recovery interval on the size of the myocardial infarct (**open bars**; mean ± SEM) and the incidence of ventricular fibrillation (VF) **(solid lines; upper panels)** and manganese superoxide dismutase (Mn-SOD) activity **(lower panels)** in rat myocardium. Between five and nine rats were included in each group. "**C**" indicates the values for control rats that did not receive a sublethal stimulus. *$p < 0.05$ vs. control rats.

measures (Dunnett's method). The incidence of VF between the groups was compared using the chi-square test with Yates' correction. A value of $p < 0.05$ was considered to be statistically significant.

## RESULTS

**Myocardial infarct size and VF incidence.** A single session of exercise, whole-body hyperthermia or TNF-alpha injection reduced both the incidence of VF and the size of the infarct in a biphasic manner, as we reported previously (1,4) (Fig. 2). Two sessions of exercise or whole-body hyperthermia markedly and persistently reduced both the incidence of VF and the extent of MI (Fig. 3). Ventricular fibrillation was almost completely suppressed when the interval between the second stimulus and the induced ischemia fell between 0.5 and 48 h. Although the incidence of VF began to increase thereafter, it remained lower than that of the sham-control rats, even after 72 h. The effect of the two sessions of exercise or whole-body hyperthermia on the extent of the myocardial infarct also exhibited a similar trend. In rats whose interval between the second stimulus and the induced ischemia ranged between 0.5 and 24 h, the size of the infarct was only ~10% of the ischemic risk area. Even in rats whose interval between the second stimulus and the induced ischemia was between 36 and 60 h, the size of the infarct was ~20% of the risk area; this infarct size is equal to that experienced by rats subjected to a single episode of exercise or whole-body hyperthermia, followed by the induction of ischemia after the most effective time interval (0.5 and 48 h) (1,4). Thus, two sessions of exercise or whole-body hyperthermia at an interval of 48 h produced a consistent and prolonged suppression of the incidence of VF and the extent of MI. Similar effects on the incidence of VF and the extent of MI were also observed in other groups receiving two stresses (Fig. 3). In particular, the TNF-alpha × TNF-alpha group exhibited a markedly consistent and prolonged suppression of these indexes, compared with the other four groups exposed to two sessions of sublethal stimuli.

The area at risk, expressed as a percentage of the left ventricular area after ischemia-reperfusion and the rate–pressure product during the infarct protocol, did not differ significantly among the groups (data not shown). No significant differences in the mortality rate were observed among the eight groups.

**Activity of Mn-SOD.** A single session of exercise, whole-body hyperthermia or TNF-alpha injection increased myocardial SOD activity in a biphasic manner, as previously reported (1,4). The persistent increase in Mn-SOD activity observed in the myocardium of rats exposed to two sessions of sublethal stimuli resembled the time courses for cardioprotection against ischemia-reperfusion injury in each group (Fig. 4).

## DISCUSSION

**Repeated stress-induced, persistent cardioprotection.** To the best of our knowledge, this is the first report to describe the time course of the beneficial effects on the

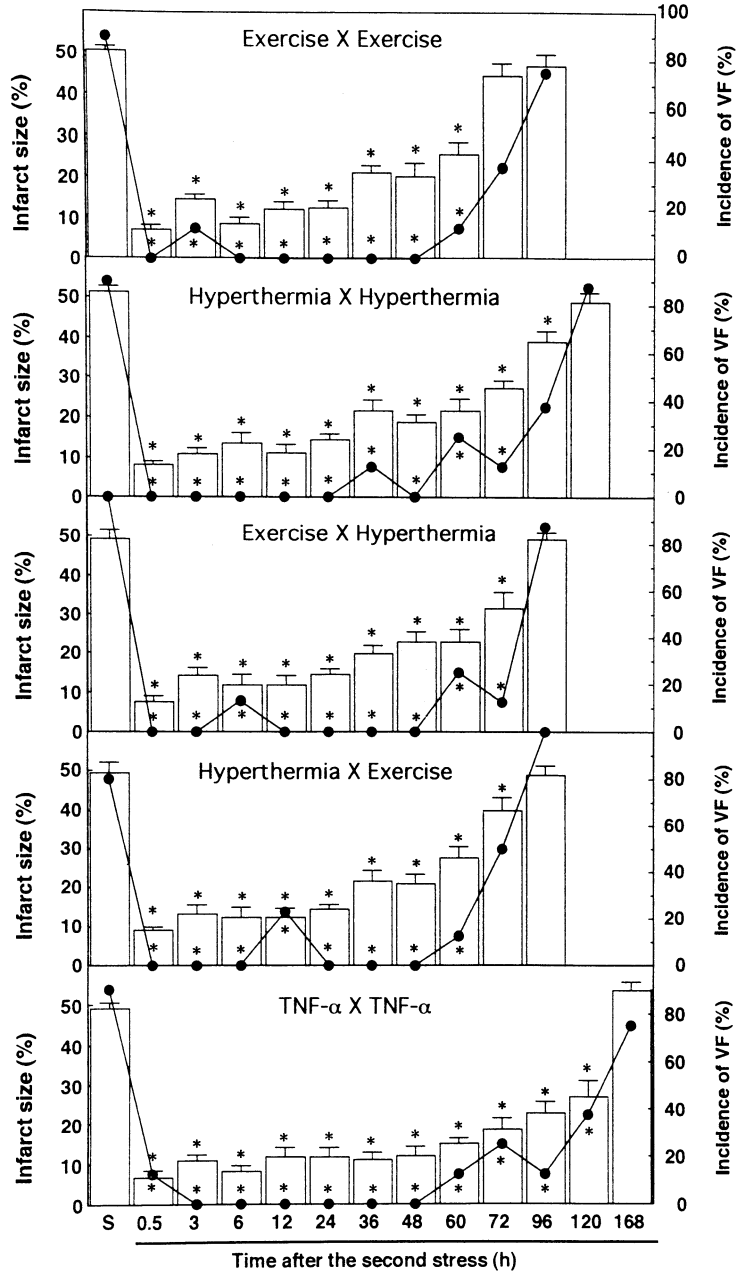


**Figure 3.** The effects of two episodes of sublethal stimuli and the recovery interval on the size of the myocardial infarct (**open bars**; mean ± SEM) and the incidence of ventricular fibrillation (VF) **(solid lines)**. Between 7 and 10 rats were included in each group. "**S**" indicates the values for sham-control rats that did not receive sublethal stimuli. *$p < 0.05$ vs. sham-control rats. TNF-$\alpha$ = tumor necrosis factor-alpha.

prevention of ischemia-reperfusion injury during acute MI after two sessions of various physiologic and pharmacologic stresses. Our results indicate that repeated physiologic stresses or repeated TNF-alpha injections produced a persistent cardioprotective effect against ischemic injury. Exercise, whole-body hyperthermia or TNF-alpha injections, repeated after an interval of 48 h, provided a prolonged protective effect that lasted for up to 60 h. Although some reports have studied the effects of repeated exercise sessions on ischemia-reperfusion injury (10–13), the optimal interval between stimuli producing the largest cardioprotective effect remains to be determined.

**Manganese-SOD and persistent cardioprotection.** We previously reported that the production of Mn-SOD, an intrinsic scavenger of superoxide anions, is induced at the same time as the acquisition of cardiac tolerance, 48 h after exercise (1). Furthermore, *N*-2 mercaptopropionyl glycine, a free radical scavenger, abolishes both the cardiac tolerance and the induction of Mn-SOD (1). The induction of Mn-SOD also plays a pivotal role in the second window of protection after ischemic preconditioning or whole-body hyperthermia (8,9,16). Exercise or whole-body hyperthermia induces the production of various proteins in heart tissue, including SOD (1,4,17), heat-shock proteins

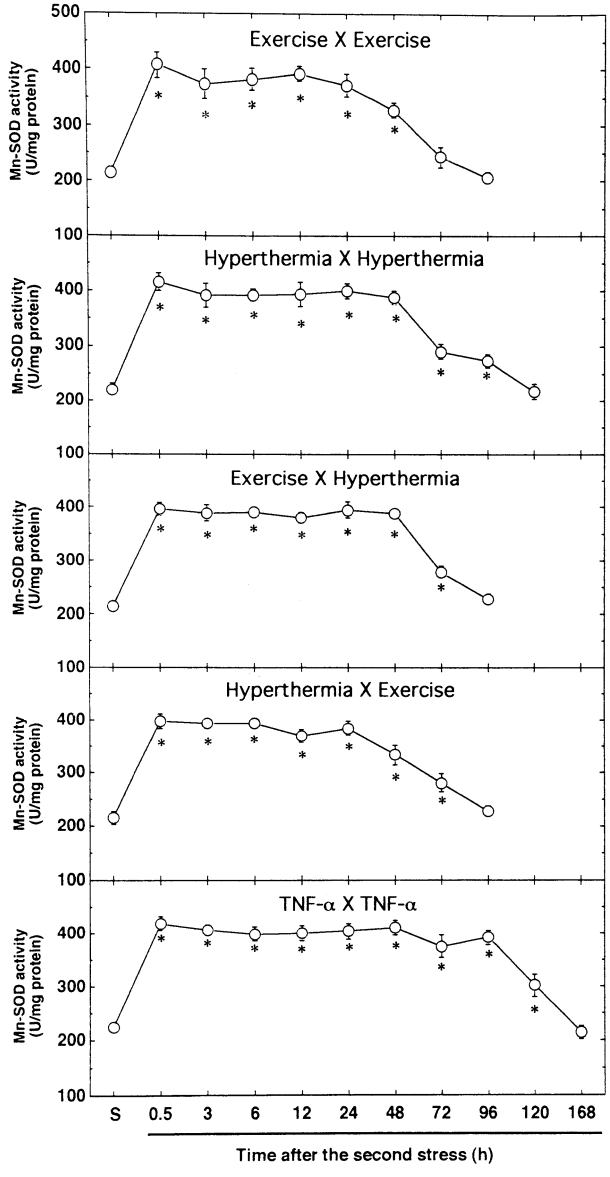


**Figure 4.** The effects of two episodes of sublethal stimuli and the recovery interval on manganese superoxide dismutase (Mn-SOD) activity in rat myocardium. Five or six rats were included in each group. "**S**" indicates the values for sham-control rats that did not receive sublethal stimuli. Data are expressed as the mean value ± SEM. *$p < 0.05$ vs. sham-control rats.

(2,3,10,18) and nitric oxide synthase (19); these proteins have been implicated in the mechanism of acquired tolerance to ischemia-reperfusion injury. Oxygen radicals are also known to induce the synthesis of proteins (20,21). Therefore, the oxygen radicals produced during these stresses may increase the tolerance of the heart to ischemia-reperfusion injury by inducing the production of intrinsic rescue proteins, such as Mn-SOD. Cytokines, such as TNF-alpha and interleukin-1 beta, have been shown to induce both Mn-SOD production (1,22,23) and cardioprotection against ischemia-reperfusion injury (24,25). The fact that the persistent increase in myocardial Mn-SOD activity after two sessions of stress stimuli resembled the time course for cardioprotection against ischemia-reperfusion injury supports the hypothesis that Mn-SOD is involved in the mechanism of cardioprotection. However, other factors, such as heat-shock proteins, nonprotein thiols and phosphofructokinase activity, may also be involved in this mechanism (26).

**Clinical implications.** The present findings showing that two sessions of sublethal stress stimuli extended the cardioprotective effect indicate that the signal transduction associated with cardioprotection and evoked by sublethal stress may be augmented by repeated stimuli separated by a period of 48 h. Understanding the precise mechanisms of signal transduction involved in this phenomenon will provide an important clue to maintaining the heart in a sustained or long-term preconditioned state and may lead to novel therapeutic clinical strategies.

**Conclusions.** The results of this study indicate that repeated physiologic stresses persistently protect the heart against ischemia-reperfusion injury in rats.

**Reprint requests and correspondence:** Dr. Shiro Hoshida, Vice Director, Yao Municipal Hospital, 2-1-55 Minami-Taishido, Yao, Osaka 581-0056, Japan. E-mail: hoshidas@city.yao.osaka.jp.

## REFERENCES

1. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation. J Exp Med 1999;189:1699–706.
2. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation 1993; 88:1264–72.
3. Hutter MM, Sievers RE, Barbosa V, Wolfe CL. Heat-shock protein induction in rat hearts: a direct correlation between the amount of heat shock protein induced and the degree of myocardial protection. Circulation 1994;89:355–60.
4. Yamashita N, Hoshida S, Taniguchi N, Kuzuya T, Hori M. Whole-body hyperthermia provides biphasic cardioprotection against ischemia/reperfusion injury in the rat. Circulation 1998;98:1414–21.
5. Kuzuya T, Hoshida S, Yamashita N, et al. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res 1993;72:1293–9.
6. Baxter GF, Goodwin RW, Wright MJ, Kerac M, Heads RJ, Yellon DM. Myocardial protection after monophosphoryl lipid A: studies of delayed anti-ischaemic properties in rabbit heart. Br J Pharmacol 1996;117:1685–92.
7. Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. Monophosphoryl lipid A provides biphasic cardioprotection against ischaemia-reperfusion injury in rat hearts. Br J Pharmacol 1999;128: 412–8.
8. Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. Involvement of cytokines in the mechanism of whole-body hyperthermia-induced cardioprotection. Circulation 2000;102:452–7.
9. Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. The involvement of cytokines in the second window of ischaemic preconditioning. Br J Pharmacol 2000;131:415–22.
10. Locke M, Tanguay RM, Klabunde RE, Ianuzzo CD. Enhanced postischemic myocardial recovery following exercise induction of HSP72. Am J Physiol 1995;269:H320–5.
11. Taylor RP, Harris MB, Starnes JW. Acute exercise can improve cardioprotection without heat shock protein content. Am J Physiol 1999;276:H1098–102.
12. Bowles DK, Farrar RP, Starnes JW. Exercise training improves cardiac function after ischemia in the isolated, working rat heart. Am J Physiol 1992;263:H804–9.

13. Powers SK, Demirel HA, Vincent HK, et al. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat. Am J Physiol 1998;275:R1468–77.
14. Dana A, Baxter GF, Walker JM, et al. Prolonging the delayed phase of myocardial protection: repetitive adenosine $A_1$ receptor activation maintains rabbit myocardium in a preconditioned state. J Am Coll Cardiol 1998;31:1142–9.
15. Hoshida S, Kuzuya T, Fuji H, et al. Sublethal ischemia alters myocardial antioxidant activity in canine heart. Am J Physiol 1993; 264:H33–9.
16. Yamashita N, Nishida M, Hoshida S, et al. Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 h after preconditioning. J Clin Invest 1994;94:2193–9.
17. Powers SK, Criswell D, Lawler J, et al. Rigorous exercise training increases superoxide dismutase activity in ventricular myocardium. Am J Physiol 1993;265:H2094–8.
18. Salo DC, Donovan CM, Davies KJ. HSP70 and other possible heat shock or oxidative stress proteins are induced in skeletal muscle, heart, and liver during exercise. Free Radic Biol Med 1991;11:239–46.
19. Bolli R. The late phase of preconditioning. Circ Res 2000;87:972–83.
20. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H202 for platelet-derived growth factor signal transduction. Science 1995;270:296–9.
21. Pinkus R, Wiener LM, Daniel V. Role of oxidants and antioxidants in the induction of AP-1, NF-kB, and glutathione S-transferase gene expression. J Biol Chem 1996;271:13422–9.
22. Wong GHW, Goeddel DV. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science 1988;242:941–3.
23. Tsan MF, White JE, Treanor C, Shaffer JB. Molecular basis for tumor necrosis factor-induced increase in pulmonary superoxide dismutase activities. Am J Physiol 1990;259:L506–12.
24. Maulik N, Watanabe M, Engelman D, et al. Myocardial adaptation to ischemia by oxidative stress induced by endotoxin. Am J Physiol 1995;269:C907–16.
25. Maulik N, Engelman RM, Wei Z, et al. Interleukin-1-alpha preconditioning reduces myocardial ischemia reperfusion injury. Circulation 1993;88 Suppl II:II387–94.
26. Powers SK, Locke M, Demirel HA. Exercise, heat shock proteins, and myocardial protection from ischemia-reperfusion injury. Med Sci Sports Exerc 2001;33:386–92.