Chinese Journal of Physiology 52(6): 419-425, 2009
DOI: 10.4077/CJP.2009.AMH048

Vascular Protection with Less Activation Evoked by Progressive Thermal Preconditioning in Adrenergic Receptor-Mediated Hypertension and Tachycardia

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Abstract

Thermal preconditioning may afford cardiovascular protection against oxidative injuries. However, hypertension and taychardia by sympathetic stimulation frequently occur during 42°C whole body thermal preconditioning (TP). We aimed to develop a modified TP to achieve cardiovascular protection with to reduced cardiovascular stimulation in the rat. We used a progressive thermal preconditioning (PTP) with three-step 5-min immersion of male Wistar rats in 42°C bath water. Treatment with phentolamine (α-adrenergic blocker), propranolol (β-adrenergic blocker) and atropine (muscarinic cholinergic blocker) was used to evaluate the effect and mechanism of PTP on systemic hemodynamics. Protective function was evaluated by $FeCl_3$-induced acute femoral arterial occlusion (TTO) and heat shock protein 70 expression. Our results show that TP enhanced body temperature, hypertension and tachycardia. However, PTP produced a similar increase in body temperature with significantly less enhancement of hypertension and tachycardia when compared with the TP group. TP- or PTP-induced increase of blood pressure and heart rate was inhibited by phentolamine and propranolol, respectively. PTP-induced attenuation of changes in hemodynamic parameters was *via* α- and β-adrenergic inhibition. $FeCl_3$ induced femoral arterial injury indicated by TTO at 416 ± 51 sec in the control rats. After 24 h of TP or PTP treatment with or without adrenergic blocker treatment, TP or PTP upregulated similar femoral arterial heat shock protein 70 expression and significantly ($P < 0.05$) delayed $FeCl_3$-induced femoral TTO to a similar degree. PTP may provide vascular protection against oxidative injuries with less activation in α-adrenergic receptor-mediated hypertension and β-adrenergic receptor-mediated tachycardia.

Key Words: progressive thermal preconditioning, $FeCl_3$, heat shock protein, adrenergic receptor, thrombosis

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Received: August 11, 2008; Revised (Final Version): February 27, 2009; Accepted: March 5, 2009.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

Increased hypoxic/ischemic tolerance in organs can be achieved by preconditioning with sub-lethal stresses such as hyperthermia (16). Whole body hyperthermia can elevate heat shock proteins and afford cardioprotection against reperfusion arrhythmias (18, 19) and ischemia/reperfusion injury (4) within a specific time window which places protection at 24 h after heat stress with disappearance of protection at 30-40 h (4). However, hyperthermia preconditioning within 3 h after hot-spring bathing sometimes induced acute myocardial infarction and cerebral infarction possibly due to transient change in blood pressure, heart rate, blood viscosity, fibrinolytic activity and platelet function (10). For example, an elevation in core temperature to 40-42°C for 15-20 min could lead to hypertension, tachycardia and polymorphonuclear cell migration into myocardium (9, 14). Sympathetic nerve activation resulting in a release of catecholamines from sympathetic nerve terminals and the adrenal medulla may contribute to the hyperthermia-induced cardiovascular system stimulation (17).

We hypothesize that achievement of protection with reduction in cardiovascular stimulation is important in the hyperthermia treatment. Previous study has shown that phentolamine (α-blocker) decreases increase in blood pressure and propranolol (β-blocker) decreases increased heart rates during heat stress, (17). Balneotherapy with high CO_2 content water significantly reduced thermal stimulation of enhanced heart rates in rats *via* the inhibition of cardiac sympathetic β1 adrenergic receptor activation (7). The coexistence of CO_2 and sodium ions in bathwater contributes to the sedative effect on human cardiovascular functions when bathing in CO_2-hot springs (22). Furthermore, the afferent signaling pathway to the brain, which is also involved in inducing reduced heart rates during immersion in CO_2-water, is located in the neuronal route and not in the bloodstream (22). Kataoka *et al*. (9) proposed that the optimum period of time for whole body bathing in 42°C water is 5 min. Our previous studies showed that local heating on the skin of the back overlying the kidneys with 42°C stimulation for 15 min was efficient to affect renal hemodynamics (2, 20). In the present study, we developed a progressive 42°C thermal preconditioning (PTP) model with three steps of 5-min whole-body immersion to minimize cardiovascular activation in the rat. We also compared potential protection of PTP and whole body 42°C thermal preconditioning (TP) on $FeCl_3$-induced acute arterial injury.

Materials and Methods

Animals

Male Wistar rats (200-250 g) were housed at the Experimental Animal Center, National Taiwan University, at a constant temperature of 23 ± 2°C and 55 ± 5% humidity and with a consistent light cycle (light from 0700 to 1800 h). All surgical and experimental procedures were approved by Institutional Animal Care and Use Committee of National Taiwan University College of Medicine and College of Public Health in accordance with the guidelines of the National Science Council, Republic of China (NSC, 1997). All efforts were made to minimize animal suffering and the number of animals used throughout the experiment.

Setup for PTP in the Rat

Under avertin anesthesia, a PE-50 catheter was catheterized in the carotid artery for monitoring arterial blood pressure and heart rate continuously by an ADI system (PowerLab/16S, ADI Instruments, Pty Ltd, Castle Hill, Australia). A temperature probe (TC-1000 Temperature controller, CWE Inc. Ardmore, PA, USA) was inserted into the colon for measurement of body temperature. The thermal preconditioning (TP) was based on the methods as previously described (7) with modifications (Fig. 1A). Animals were immersed in tap water and loosely fixed to plastic lattice plates using adhesive tape and the plates were set in a head-up position of 30° to horizontal in a Plexiglas animal cage that was used as a bathtub. Temperature of the bathtub water was maintained at 42°C throughout the experiment by immersing the bathtub into a water bath incubator. We divided the animals into three groups: control group at the room temperature, progressive thermal preconditioning (PTP) group with three stages (5 min each) of progressive immersion to 42°C water and whole body thermal preconditioning (TP) group for 15 min of 42°C water immersion (Fig. 1B). Animals in the TP group were directly immersed into water at Line 3 for 15 min. Animals in PTP group were progressively immersed into 42°C water bath at Line 1, Line 2 and Line 3 for respective 5 min.

Measurement of Hemodynamics during PTP

In *experiment* 1, we measured 5 min of baseline value and then 15 min of heart rate, blood pressure and body temperature during 42°C water immersion. All the singals were continuously record on a recording system (PowerLab/16S, ADI Instruments, Pty Ltd, Castle Hill, Australia).

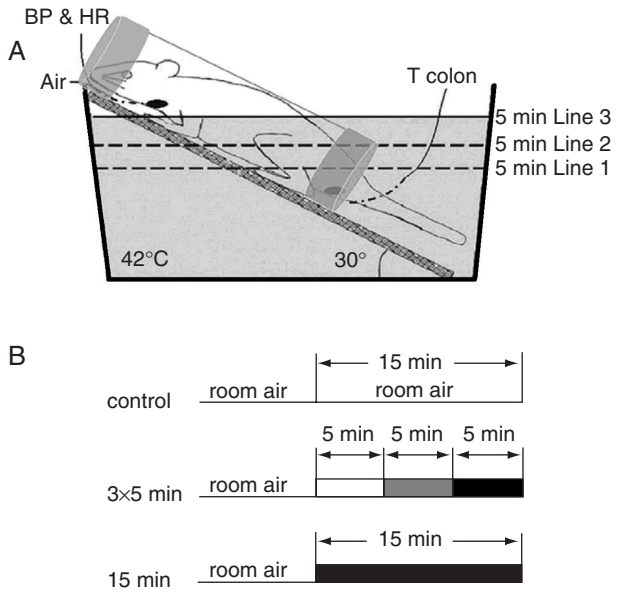

Fig. 1. The setup for TP (one stage of 15 min immersion, 15 min) and PTP (three stages of 5 min immersion, 3 × 5 min) in the rat is demonstrated in **A**. One thermosensitive probe was introduced in the colon for measuring colon temperature (T colon); a carotid artery catheter was used for measurement of blood pressure (BP) and heart rate (HR). The bath water temperature was maintained at 42°C by controlling the water bath incubator in which the bathtub was immersed. TP was performed at Line 3 for 15 min. PTP was performed progressively at Line 1 for 5 min, Line 2 for 5 min and Line 3 for 5 min. **B:** The schedule for water immersion experiments in the control with room air treatment, 3 × 5 min of 42°C water immersion of the PTP group and 15 min of 42°C water immersion of the TP group.

Effect of α-Adrenergic and β-Adrenergic Neurotransmission during PTP

To investigate which innervation is dominant for changes in heart rate and blood pressure in water bathing, sympathetic or parasympathetic autonomic nervous system blockade was performed. In *experiment* 2, the effect of intraperitoneal injection of phentolamine (10 mg/kg, α-adrenergic receptor blocker, Sigma, St. Louis, MO, USA), propranolol hydrochloride (1 mg/kg, β-adrenoceptor antagonist, Sigma) and atropine sulfate (1 mg/ml, muscarinic cholinergic antagonist; TBC, Taiwan Biotech. CO., LTD, Tao Yuan, Taiwan) on heart rate and blood pressure just before exposure to 15-min water immersion was evaluated in the control (C), TP and PTP groups (n = 6 in each group).

Evaluation of the Effect of PTP on $FeCl_3$-Induced Femoral Arterial Injury

In *experiment* 3, after 24 h of TP or PTP treatment, we evaluated $FeCl_3$-induced acute arterial thrombosis and heat shock protein 70 expression. The animals were anesthetized by subcutaneous injection of 1.2 g/kg urethane (Sigma-Aldrich Inc., St. Louis, MO, USA). After arterial isolation, a transonic flow probe (Probe# 0.5VBB517, Transonic Systems Inc., Ithaca, NY, USA) for femoral arterial blood flow measurement was applied and displayed on a small animal blood flow meter (Model 206, Transonic Systems Inc., Ithaca, NY, USA). The femoral arterial blood flow signals were continuously recorded by an ADI system (PowerLab/16S, ADI Instruments, Pty Ltd, Castle Hill, Australia). The femoral artery was then injured as previously described (11) with slight modifications. A filter paper (1 mm × 2 mm) soaked with 30% $FeCl_3$ solution (Ferric chloride, Sigma) was applied to the left femoral artery and the cavity was filled with saline immediately (11). The flow rate was monitored and recorded and the time to occlusion (TTO, arterial blood flow decreases to zero) was determined.

Immunoblotting of Heat Shock Protein 70 in the Femoral Artery

Right femoral arterial samples were homogenized in a buffer (1.5 M NaCl, 100 mM Tris-base [pH 8.0], 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.05% aprotinin and proteinase inhibitor cocktails). Each sample was mixed with 4X sample buffer (37.5% Tris-HCl, 9% SDS, 0.15% bromophenol blue and 30% glycerol) and boiled for 10 min. The samples were then separated by 12% sodium dodecyl sulfate-PAGE (1.5 M Tris [pH 8.8], 30% acryamide mix, and 10% SDS, 10% ammonium persulfate, TEMED) and transferred to nitrocellulose membrane (Amersham Biosciences, Euckinghamshire, UK). After blocking with 5% nonfat milk for 1 h, membranes were washed three times with TTBS (tween twenty buffer saline) for 10 min and incubated overnight at 4°C with heat shock protein 70 (1:2000 dilution in TTBS, H53220, Transduction Laboratories). β-actin was used as the control. After washing three times in TTBS, the membrane was incubated with a secondary antibody conjugated to heat shock protein 70 diluted 1:5000 in TTBS and incubated for 1 h at room temperature. The membranes were again washed with TTBS three times. Immune complexes were visualized with an enhanced chemiluminescence reagent (Amersham). Results were quantified by capturing the exposed X-ray film image and using area measurements from image analysis software.

Statistical Analysis

All values were expressed as mean ± mean of

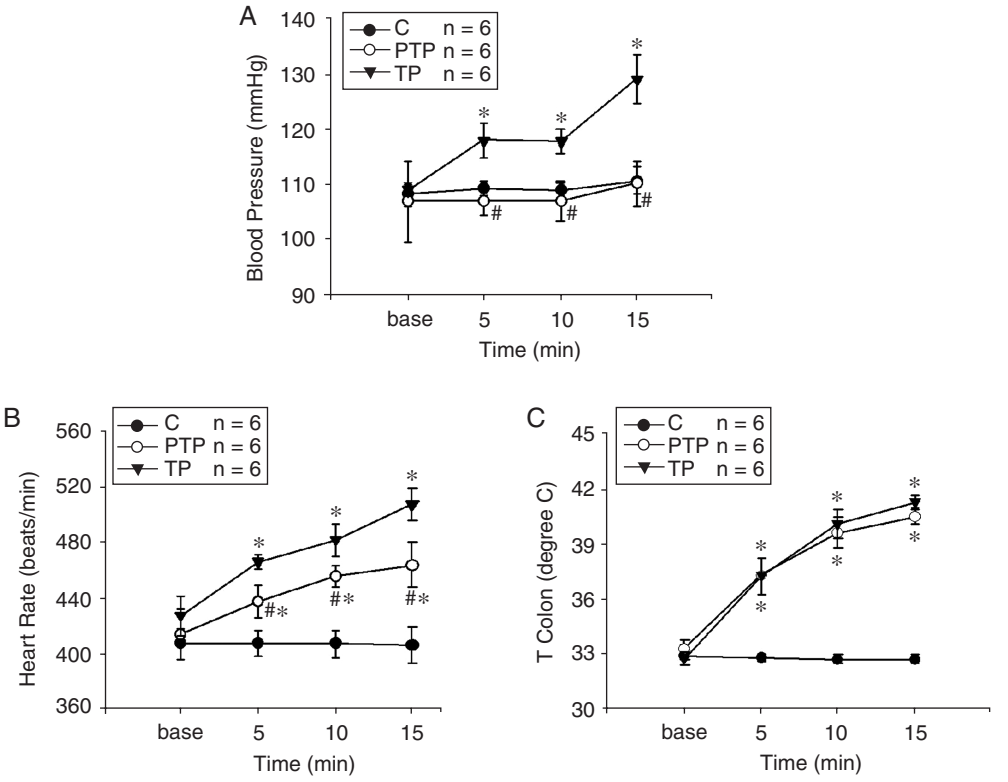

Fig. 2. Hemodynamic responses of 42°C TP and PTP on blood pressure (A), heart rate (B) and colon temperature (T colon) (C). 15 min of 42°C water immersion (TP) significantly increased blood pressure, heart rate and T colon when compared to the control rats. 3 steps of 5 min of 42°C water immersion (PTP) significantly attenuated 15 min 42°C water immersion (TP) enhanced blood pressure and heart rate in spite of a similar increase of T colon. $^{*}P < 0.05$ when compared to the baseline data (base). $^{\#}P < 0.05$ PTP *vs.* TP.

standard error (SEM). Differences within groups were evaluated by paired Student *t*-test. One-way analysis of variance was used for establishing differences among groups. Intergroup comparisons were made by Duncan's multiple-range test. Differences were regarded as significant if $P < 0.05$ was attained.

Results

PTP Displayed Less Activation in Heart Rate and Hypertension

Both TP and PTP increased colon temperature to 42°C in a similar profile (Fig. 2). However, a different response in blood pressure and heart rate to TP and PTP treatment was found (Fig. 2). With immersion in 42°C water, TP produced significant hypertension and tachycardia while PTP evoked less increases in heart rate without changes in blood pressure when compared with the control.

PTP Attenuated Enhanced Hemodynamic Parameters via α- and β-Adrenergic Inhibition

Figure 3 shows the effects of intraperitoneal injection of phentolamine, propranolol or atropine on the changes in heart rate (Fig. 3A) and blood pressure (Fig. 3B) during TP or PTP treatment. Blocker or saline was injected just before the start of water immersion. Phentolamine injection significantly ($P < 0.05$) suppressed the increase in blood pressure but not the increase in heart rate in the TP group as compared with the control group. In contrast, propranolol injection significantly ($P < 0.05$) suppressed the increase in heart rate without effect on the increase in blood pressure induced by TP treatment. Atropine had no effect on the increase in heart rate and blood pressure. The response of reduction in the increase in blood pressure by phentolamine and in the heart rate by propranolol was similar during PTP treatment.

PTP and TP Confers a Similar Vascular Protection in the Rat

Figure 4 shows the results after 24 h of the protective effect of TP or PTP with or without adrenergic blocker on femoral arterial heat shock protein

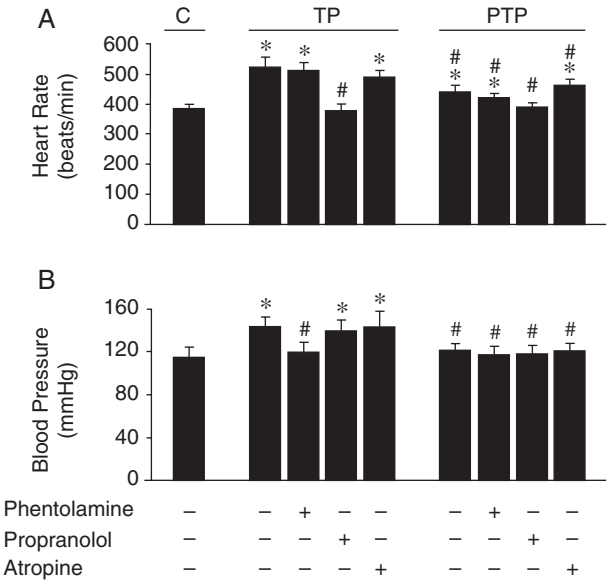

Fig. 3. Effects of intraperitoneal injection of phentolamine (α-adrenergic receptor blocker, 10 mg/kg), propranolol (β-adrenergic receptor blocker, 1 mg/kg) and atropine (1 mg/kg) on heart rate (A) and blood pressure (B) just before exposure to 15 min water immersion in the control (C) and the TP and TTP groups (n = 6 in each group). After 24 h of TP or PTP treatment, femoral TTO was compared in the rats. $^{*}P < 0.05$ when compared to the control data (C). $^{\#}P < 0.05$ *vs.* the TP group without any drug treatment. + represents drug treatment.

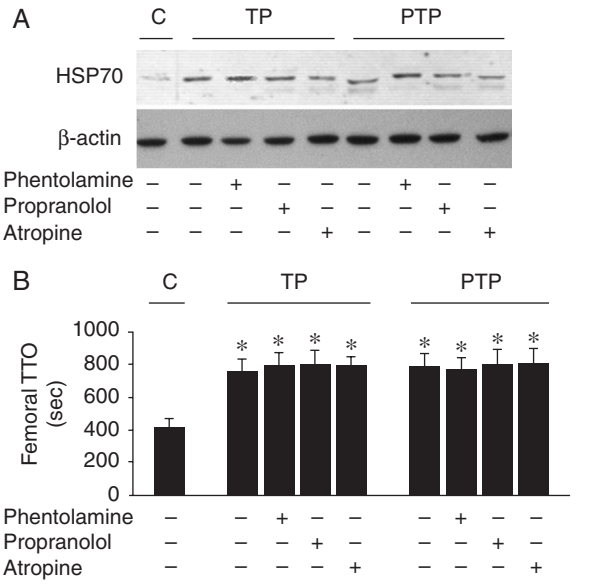

Fig. 4. Effects of intraperitoneal injection of phentolamine (α-adrenergic receptor blocker, 10 mg/kg), propranolol (β-adrenergic receptor blocker, 1 mg/kg) and atropine (1 mg/kg) on right femoral arterial heat shock protein 70 (HSP70) expression (A) and left femoral arterial TTO (B) in the control (C) and the TP and TTP groups (n = 6 in each group) after 24 h of TP or PTP treatment. $^{*}P < 0.05$, when compared to the control data (C). $^{\#}P < 0.05$, PTP *vs.* TP group without drug treatment.

70 protein expression (Fig. 4A) and TTO response (Fig. 4B). We found that the increased heat shock protein 70 protein expression in the femoral artery was similar in TP or PTP animals with or without drug treatment as compared with the control group. TP or PTP with or without drug treatment significantly ($P < 0.05$) delayed femoral arterial TTO as compared with the control animals.

Discussion

The present study provided evidence that PTP-induced attenuation of the degree of tachycardia and hypertension caused by 42°C heat stimulation was mediated *via* the decrease of α-adrenergic and β-adrenergic sympathetic transmission, respectively. PTP may provide mechanisms to upregulate the level of heat shock protein 70 expression in the femoral artery and to delay the femoral arterial TTO which was similar to that induced by TP treatment. We demonstrated that the PTP method achieves efficient cardiovascular protection against oxidative injury but reduces exaggerated cardiovascular stimulation.

TP with a whole-body hyperthermia at a level of 42-43°C for 15-20 min has been demonstrated to elicit the formation of heat shock proteins and improves cardiac recovery from subsequent ischemia/reperfusion (4, 16, 19, 20). However, these beneficial effects induced by hyperthermia are compromised by initial hemodynamic fluctuations and tissue injuries, which may, therefore, limit its clinical applicability (9, 10, 14, 20). During tap-water bathing, water temperature correlated positively with heart rate and negatively with mean arterial blood pressure (7). Changes in heart rate during recovery from heat stress are primarily mediated by the sympathetic but not vagal activity (7). Autonomic imbalance, a term used to indicate relative or absolute decrease in vagal activity or an increase in sympathetic activity, has been associated with an increased risk of death from cardiac (12) and from arrhythmic causes (13). A previous study has indicated that heart-rate changes during exercise and recovery is a strong predictor of sudden death (8). Therefore, prevention of cardiovascular system activation by enhanced sympathetic activity during heat stress is critically important. TP with 42-43°C whole-body hyperthemia or bathing at 40°C induced remarkable changes in the heart rate and blood pressure by the increasing core temperature for more than 10 min might result from an abruptly high surface area of peripheral thermal receptor stimulation (15, 16, 19). In the present study, we

developed a three-step 42°C PTP model, which was efficient in preventing or attenuating the increase of blood pressure and heart rate than those found in the rats with TP treatment. Our previous studies have implicated that local heating on the skin of the back overlying the kidneys with 42°C stimulation for 15 min did not significantly increase systemic blood pressure (2, 20). We suggested that PTP attenuated heat-enhanced hemodynamic parameters possibly *via* the following mechanisms: [1] a mild and progressive, not abrupt, stimulation of 42°C peripheral thermal receptor stimulation; [2] a total lower amount of thermal receptor stimulation because of a lower surface area of thermal stimulation during the course of PTP treatment and [3] less activation in sympathetic nervous activity in PTP treatment. However, these mechanisms require further experiments to confirm.

Mild hyperthermia (39°C preconditioning) has been demonstrated to be as effective as acute heat shock in inducing the heat shock response and *via* heat shock protein upregulation induces a beneficial adaptive response including thermotolerance and oxidative resistance, but caused no detectable adverse effects (20). CO_2 balneotherapy using hot springs containing high oncentrations [$\geqq$1,000 parts/million (ppm)] of free carbon dioxide (CO_2-hot spring) has been applied clinically to improve cardiovascular symptoms in European countries. Previous studies have shown that increased CO_2 concentration in the 30°C warm water can reduce the degree of tachycardia induced by the enhanced sympathetic tone (7, 21, 22). Decreased heart rate in CO_2-rich water was inhibited by infusion of atenolol (β_1-adrenoceptor blocker) but was unaffected by atropine (muscarinic cholinoceptor blocker) suggesting that bradycardia in CO_2 hot-spring bathing is probably caused by an inhibition of cardiac sympathetic innervation (7). This notion is compatible with the concept that phentolamine (α-blocker) can decrease elevation in blood pressure and propranolol (β-blocker) can decrease increased heart rate during heat stress (17). Our present data showing that PTP could attenuate these hemodynamic parameters *via* the inhibition of α- and β-adrenergic transmission are compatible with those of previous reports and may also indicate that there is a safe strategy of PTP implication with a three-stepwise increase in hyperthermia.

Cardiovascular protection can be achieved by thermal preconditioning (4, 16, 19, 20). Increased heat shock proteins have been associated with reduction in infarction size and improved postischemic recovery (5). Heat shock protein 70-enriched cells exhibit marked resistance to oxidative challenges including exposure to hydrogen peroxide (H_2O_2), hydroxyl radical, menadione and hypoxia/reoxygenation suggesting that heat shock protein 70 may actively participate in the heat shock-induced oxidative protection (4). In this regard, Amrani *et al*. (1) revealed that some essential functions in rat coronary artery endothelial cells were protected against ischemia and reperfusion preceding a whole body heat treatment and that this protection is probably associated with enhancement of heat shock protein 70 levels. Su *et al*. (19) used H9c2 cells to investigate the resistance against H_2O_2 after heat pretreatment and found that full protection against moderate H_2O_2 concentrations occurred only after 20-24 h. In the present study, upregulation of heat shock protein 70 in the femoral artery after 24-h PTP treatment was presented and was similar to that of the TP group suggesting that both PTP and TP may share a common mechanism in terms of cardiac protection induced by hyperthermia. The application of cholinergic blocker, α- and β-adrenergic blockers did not influence the increase in heat shock protein 70 expression in the PTP or TP animals suggesting that heat shock protein 70-induced cardiovascular protection may have been dependent on temperature increase but not on neural factors per se.

We used a rat model treated with $FeCl_3$ to induce thrombus formation in the femoral artery and the injured artery was characterized by endothelial disruption, extensive platelet and red blood cell clumps and also interspersed fribrin similar to the report of Kurtz *et al*. (11). The morphological change of the thrombus was also similar to the thrombi in the coronary arteries of patients with acute myocardial ischemia (6). $FeCl_3$ causes oxidative injury to the endothelium which facilitates platelet deposition to the subendothelial layes leading to femoral arterial TTO rapidly (5-8 min). We found that TP or PTP significantly delayed the femoral arterial TTO in a similar degree. The application of muscarinic cholinergic blocker, α- and β-adrenergic blockers did not affect TP- or PTP-induced cardiovascular protection. These data consistently suggest that PTP induced protection is temperature dependent.

In conclusion, we have developed a three-step (5 min each) progressive thermal preconditioning method to prevent 42°C whole-body immersion-induced α-adrenergic receptor mediated hypertension and β-adrenergic receptor-mediated tachycardia in the rat. Twenty-four hours after the application of PTP significantly delayed the $FeCl_3$-induced femoral TTO by enhancement of heat shock protein 70 expression. Progressive thermal preconditioning can achieve cardiovascular protection and attenuate the adverse effect during whole-body hyperthermia.

Acknowledgments

This work was supported in part by the Depart-

ment of Medical Research in NTUH and from grant NTUH98-S1141, partly by the National Science Council of the Republic of China (NSC 92-2320-B-002-078 and 95-2320-B-002-022) and Kuang-Tien General Hospital.

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