Heat-shock response protects peripheral blood mononuclear cells (PBMCs) from hydrogen peroxide-induced mitochondrial disturbance

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Abstract The present study was designed to investigate ex vivo the protective mechanisms of heat-shock response against H₂O₂-induced oxidative stress in peripheral blood mononuclear cells (PBMCs) of rats. Twenty-four hours later, heat-shock treatment was executed in vivo; rat PBMCs were collected and treated with H₂O₂. The accumulation of reactive oxygen species and the mitochondrial membrane potential were evaluated by intracellular fluorescent dHE and JC-1 dye staining, respectively, and expression of HSP72 and cytochrome *c* was detected by Western blot analysis. Cellular apoptosis was assayed by TUNEL staining and double staining of Annexin V and PI. The results showed that H₂O₂-induced oxidative stress leads to intracellular superoxide accumulation and collapse of the mitochondrial membrane potential in rat PBMCs. Moreover, cellular apoptosis was detected after H₂O₂ treatment, and the release of mitochondrial cytochrome *c* from mitochondria to cytosol was significantly enhanced. Heat-shock pretreatment decreases the accumulation of intracellular superoxide in PBMCs during H₂O₂-induced

oxidative stress. Moreover, heat-shock treatment prevents the collapse of the mitochondrial membrane potential and cytochrome *c* release from mitochondria during H₂O₂-induced oxidative stress. In conclusion, mitochondria are critical organelles of the protective effects of heat-shock treatment. Cellular apoptosis during H₂O₂-induced oxidative stress is decreased by heat-shock treatment through a decrease in superoxide induction and preservation of the mitochondrial membrane potential.

Keywords Heat-shock response · Rat · Superoxide · Oxidative stress · Mitochondrion

Abbreviation

DAB	diaminobenzidine
dHE	dihydroethidium
HSP	heat-shock protein
$\Delta\psi$	mitochondrial membrane potential
PBMC	peripheral blood mononuclear cell
ROS	Reactive oxygen species
PI	propidium iodine
TUNEL	TdT-mediated dUTP nick end-labeling assay
TdT	terminal deoxynucleotidyl transferase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide

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Introduction

Reactive oxygen species (ROS) are the by-products of normal cell metabolism during enzymatic electron-transporting processes such as mitochondrial respiration, and there is an array of antioxidant systems to maintain the redox balance (Kakkar and Singh 2007). However, excessive accumulation

of ROS can result in the development of oxidative stress. Hydrogen peroxide (H_2O_2) is typically a central oxygen metabolite during the complete reduction of oxygen to H_2O ; it is a biologically important oxidant in its ability to generate the highly reactive hydroxyl radical, which is an extremely potent radical (Clarkson and Thompson 2000; Ji 1999). The half life of hydrogen peroxide is longer than those of other ROS and is usually utilized to induce oxidative stress in vitro (Fatokun et al. 2006). Moreover, it is also an important signaling molecule that induces some genes related to oxidative stress (Ji 1999). An oxidative-stress-induced inflammatory event leading to cellular or tissue injury is considered as a unifying mechanism of injury in many types of disease processes, such as renal, cardiovascular, neoplastic, and neurodegenerative diseases, and aging (Narula et al. 2006; Onyango and Khan 2006; Wardle 2005).

Mitochondria are the critical organelle to generate numerous ROS and also stand in the breach for ROS damage (Kakkar and Singh 2007). Nowadays, the role of mitochondria is understood to be not only that of a powerhouse, but also as an important pivot correlated with cellular survival. Disturbances of mitochondrial function, such as the collapse of the mitochondrial membrane potential, induction of mitochondrial permeability transition and releasing of proapoptotic factors, contribute in evoking the pathway of the death signal transduction (Gogvadze and Zhivotovsky 2007). Among the released proapoptotic factors, cytochrome *c* is one of most typical factors in initiating the cell death signal (Gogvadze and Zhivotovsky 2007; Kakkar and Singh 2007). However, the mitochondrial events responsible for critical oxidative-stress-mediated cell death (toxic oxidative stress) are yet to be defined.

Prevention of ROS formation early in the disease process may prove beneficial. Heat-shock pretreatment contributes to the induction of a series of conserved proteins called heat-shock proteins (HSPs), which can protect living organisms against subsequent lethal injury (Soti et al. 2005). Heat-shock proteins, the appearance of which is one of the major characteristics of heat-shock response, act as molecular chaperones and are regarded as endogenous cytoprotective molecules against deleterious stresses (Beere 2005; Ohtsuka et al. 2005). In our previous studies, it was shown that heat-shock response contributes to the prevention of mitochondria from sepsis-induced structural and functional destruction (Chen et al. 2003; Chen et al. 2004). Moreover, heat-shock pretreatment protects cells against apoptosis induced by ischemia and reperfusion injury or hypoxia (Beere 2005; Jiang et al. 2005). However, the mechanisms by which heat-shock response protects cells from oxidative-stress-induced damage remain to be identified. The aim of the present study is to investigate the

protective effect of heat-shock response on hydrogen peroxide-induced oxidative stress through the mitochondrial pathway in rat PBMCs.

Materials and methods

Animals

Experiments were performed on adult male Sprague–Dawley rats (weighing from 270–350 g) obtained from the National Experimental Animal Center (Nan-Kang, Taipei, Taiwan). The experiments conducted in this study were approved by the Animal Care and Treatment Committee of Kaohsiung Medical University, and the authors have adhered to the National Institute of Health guidelines for the use of experimental animals. Animals were divided into two groups, a non-heated group ($n=8$) and a heated group ($n=8$).

Heat-shock treatment

Rats in the heated group received whole-body heating with an electric pad after anesthesia by pentobarbital injection (Chen et al. 2004; Chen et al. 2005). When the rectal temperature reached 41°C , it was then maintained between 41°C and 42°C for 15 min. The heated rats were then put back in their cages to recover for 24 h. The rats in the non-heated group were also anesthetized, but were not heated.

PBMCs isolation and oxidative-stress induction

PBMCs were isolated from whole blood of non-heated and heated animals 24 h post-heating using Ficoll-Paque™ Plus solution (Amersham Biosciences Corp., Piscataway, NJ, USA). PBS diluted blood sample were overlaid onto the Ficoll-Paque™ Plus solution and centrifuged at $525\times g$ at room temperature for 30 min. After centrifugation, the PBMC layers (found at the interface between the plasma and the Ficoll-Paque™ Plus solution) were collected and washed twice in PBS and centrifuged at $525\times g$ for 5 min. Cells were counted in a counting chamber and cell viability was determined by the trypan blue exclusion method. Cells viability was greater than 95%.

The PBMCs were then resuspended at a concentration of 1×10^6 cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. H_2O_2 was applied as an inducer of oxidative stress. The PBMCs were treated with 100 μM hydrogen peroxide (H_2O_2) at 37°C for 1 h. Each test contained four groups: (1) PBMCs isolated from non-heated animals without H_2O_2 treatment (non-heated control group); (2) PBMCs isolated from non-heated animals and then treated

with H₂O₂ (oxidative-stress non-heated group); (3) PBMCs isolated from heated animals without H₂O₂ treatment (heated control group); (4) PBMCs isolated from heated animals and then treated with H₂O₂ (oxidative-stress heated group).

Preparation of subcellular fractions

The mitochondrial and cytosolic fractions were separated. PBMCs pellets were resuspended in 100 µl digitonin lysis buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 190 µg/ml digitonin) and lysed by 20 passages through a 26-gauge syringe. After centrifugation at 6,000×g and 4°C for 5 min, the pellets were harvested as the mitochondrial fraction. The supernatants were centrifuged at 100,000×g again at 4°C for 30 min and the supernatant harvested as the cytosolic fraction.

Detection of apoptosis

Apoptosis was detected by Annexin V-FITC assay (BD Biosciences, San Jose, CA, USA) and the TdT-mediated dUTP nick end-labeling (TUNEL) assay (Oncogene Research Products, Cambridge, MA, USA).

Annexin V-FITC assay After treatment, PBMCs were resuspended at a concentration of 1×10^6 cells/ml in binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂; 1 mM MgCl₂). Aliquot cells (100 µl) were placed into FACS tubes and stained with Annexin V-FITC and propidium iodide (PI; 2 µl of a 50 µg/ml stock solution) in binding buffer for 15 min at room temperature in the dark. A total of 10,000 cells were analyzed by FACS.

TUNEL assay The TUNEL method identifies apoptotic cells by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the strand breaks of cleaved DNA. The biotin-labeled cleavage sites are then detected by reaction with HRP-conjugated streptavidin and visualized by diaminobenzidine (DAB), showing as a brown color. The reaction was carried out as described by the manufacturer. PBMCs were fixed by 4% paraformaldehyde and immobilized onto glass slides. The specimens were incubated with proteinase K buffer and the endogenous peroxidase was quenched with 3% H₂O₂. After washing, specimens were incubated with 50 µl TUNEL reaction mixture in a humidified chamber at 37°C for 90 min in the dark. After treatment with peroxidase-conjugated streptavidin solution, the color was developed with DAB solution and counterstained with methyl green.

Detection of mitochondrial membrane potential ($\Delta\psi_m$)

The mitochondrial membrane potential, $\Delta\psi_m$, was measured using the lipophilic cation JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; Molecular Probes, Invitrogen, Karlsruhe, Germany). JC-1 stains mitochondria in cells with high mitochondrial potentials by forming orange-red fluorescent J-aggregates that emit at 590 nm after excitation at 490 nm. However, JC-1 in cells with depolarized or damaged mitochondria forms a monomer that emits at 525 nm after excitation at 490 nm. For staining, PBMCs were incubated with JC-1 (5 µg/ml) for 30 min at room temperature in the dark. Cells were then analyzed immediately by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). A total of 10,000 cells were analyzed for green fluorescence with a 525-nm filter and for orange fluorescence with a 590-nm filter. All data were analyzed, within 1 h of staining, using BD CellQuest Pro Software (BD Biosciences, San Jose, CA, USA). The complete depletion of the mitochondrial membrane potential in the presence of the mitochondrial uncoupler CCCP was utilized as a control probe in JC-1 staining.

Detection of intracellular superoxide anions

Free radicals were detected using dihydroethidium (dHE; Molecular Probes, Invitrogen, Karlsruhe, Germany) at a concentration of 10 mM. dHE is oxidized on reaction with superoxide to ethidium bromide (EB), which is trapped by intercalation with DNA in the nucleus and fluoresces red (excitation 475 nm/emission 610 nm). A total of 1×10^4 cells were analyzed within 1 h of staining by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). Ethidium-derived fluorescence was analyzed for orange fluorescence with a 590-nm filter.

Western blot analysis

Equal amounts (10 µg) of protein extract were loaded and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA, USA). Hsp72 (StressGen Biotechnologies, Victoria, BC, Canada), actin (Chemicon, Temecula, CA, USA), and cytochrome *c* (BD Biosciences, San Jose, CA, USA) were used as the primary antibodies, while horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as the secondary antibody. The target protein was detected by enhanced chemiluminescence; actin was detected simultaneously and acts as an internal control. The results were quantified by densitometer and

analysis software (Bio-1D V.97 software, Vilber Lourmat, France).

Statistical analysis

All data are expressed as a mean $\pm$ SD. Two-way analysis of variance, followed by the Newman–Keuls test, were used to analyze the data, with a 95% confidence limit accepted as indicating statistical significance.

Results

Successful heat-shock response and Hsp72 induction *in vivo*

Induction of heat-shock response was executed *in vivo* and Hsp72 was detected as an indicator of successful heat-shock treatment. Twenty-four hours after heat-shock treatment, PBMCs were collected and Hsp72 expression was detected by Western blot analysis (Fig. 1). Hsp72 expression was significantly induced after heat-shock treatment in rat PBMCs and its expression was sustained over the time taken for the hydrogen peroxide treatment process to be completed. Hsp72 was not detected in the non-heated group.

Heat-shock response prevents hydrogen peroxide-induced apoptosis

Hydrogen peroxide-induced cellular apoptosis was detected by Annexin-V/PI staining assay and by TUNEL assay. PI

staining can be used as the viability marker. Annexin V has high affinity for phosphatidylserine (PS) and Annexin-V-mediated detection of PS is important for monitoring cells under apoptosis. The results of the Annexin-V/PI staining assay showed that the percentages of early apoptosis were $10.2\pm0.5\%$ and $8.3\pm0.9\%$ individually and the percentages of late apoptosis/necrosis were $23.7\pm2.6\%$ and $9.7\pm3.7\%$ for the non-heated and heated oxidative-stress groups, respectively (Fig. 2a,b). The early and late apoptotic populations are both significantly increased after hydrogen peroxide treatment in the non-heated and heated groups ($p<0.05$); however, the apoptotic population in the heated group during H_2O_2 -induced oxidative stress was significantly decreased as compared with the non-heated group ($p<0.05$). Heat-shock response contributes to a significant decrease in the early and late apoptotic populations after hydrogen peroxide treatment.

After TUNEL assay staining, apoptotic cells could be discriminated morphologically from non-apoptotic cells by the presence of condensed brown nuclei (Fig. 2c). The TUNEL assay enables detection of the nuclear DNA strand breaks in the late stages of apoptosis. Under high magnification (400 $\times$), the percentages of apoptotic cells in the controls of the non-heated and heated groups were calculated as $5.4\pm1.9\%$ and $7.1\pm1.7\%$, respectively. After the induction of oxidative stress using hydrogen peroxide, the percentages of apoptotic cells in the oxidative-stress non-heated and heated groups were found to be about $42.4\pm4\%$ and $26.1\pm8.5\%$, respectively, showing a significant increase. Despite the fact that cellular apoptosis is still observed, the amount of apoptosis is significantly decreased in the oxidative-stress-heated group compared with the oxidative-stress non-heated group ($p<0.05$). Heat-shock response contributes to the prevention of hydrogen peroxide-induced cellular apoptosis.

Heat-shock response reverses the hydrogen peroxide exposure-induced collapse of the mitochondrial membrane potential

The mitochondrial membrane potential of PBMCs was estimated by JC-1 staining and analyzed by flow cytometry, and the results showed that the cell populations in both the non-heated and heated control groups were localized in the higher mitochondrial membrane potential (high $\Delta\psi$) region, in which there was increased J-aggregate formation (Fig. 3a). After hydrogen peroxide exposure, the cellular dots detected by flow cytometry were found to have shifted to a lower mitochondrial membrane potential (low $\Delta\psi$) region, in which there were more JC-1 monomers in existence in the oxidative-stress non-heated group. The shift from high $\Delta\psi$ to low $\Delta\psi$ is less apparent in the heated group than in the non-heated group during hydrogen peroxide-induced oxidative stress.

Fig. 1 Hsp72 expression induced by heat-shock treatment *in vivo*. Actin was detected simultaneously and acts as the internal control. *NH* non-heated group, *H* heated group; H_2O_2 treatment signed as positive. * $p<0.05$ vs. each other as indicated

Fig. 2 Apoptosis detection.

a Apoptosis detected by Annexin-V FITC/PI staining assay. One representative expression out of five is shown. The horizontal and vertical axes represent labeling with Annexin V and PI, respectively. Early and late apoptotic cells were localized in the lower right (LR) and upper right (UR) quadrant of a dot-plot graph, respectively. **b** Statistical analysis of cellular populations in early and late apoptosis. Cellular apoptosis was quantified by flow cytometry with Annexin V-FITC/PI staining assay. The percentage of early and late apoptosis is represented, respectively. * $p < 0.05$ vs. each other as indicated. **c** Apoptosis detected by TUNEL assay. One representative expression out of four is shown. Nuclei with condensed dark staining were observed by light microscopy (400 $\times$)

Fig. 3 Detection of mitochondrial membrane potential. **a** Mitochondrial membrane potentials were evaluated by staining with the potential sensor JC-1 and analyzing by flow cytometry. One representative dot-plot out of five is shown. Cell populations with higher and lower JC-1-aggregated staining are gated as high $\Delta\psi$ and low $\Delta\psi$, respectively. **b** Overlay of histograms of J-aggregates orange fluorescence and JC-1 monomer green fluorescence. The *horizontal axis* shows the relative fluorescence intensity and the *vertical axis* shows the number of cells. *Black block* non-heated group with/without H_2O_2 treatment as indicated. *Gray line* heated group with/without H_2O_2 treatment as indicated

In the histogram of FL1 (JC-1 monomer) and FL2 (JC-1 aggregation; Fig. 3b), it can be seen that there is a dramatic reduction in J-aggregate orange fluorescence in the oxidative-stress non-heated group, accompanied by a complementary increase in green emission, owing to the prevalence of the monomeric form of the dye with respect to J-aggregates. Decreased J-aggregate orange fluorescence and increased JC-1 green fluorescence is indicative of mitochondrial membrane depolarization. Heat-shock response can reverse those effects by preventing hydrogen peroxide-induced depolarization of the mitochondrial membrane potential in PBMCs.

Heat-shock response decreases the accumulation of superoxide in PBMCs during hydrogen peroxide treatment

The intracellular level of the reactive oxygen species superoxide anion was estimated by application of dHE dye, which binds to nuclear DNA when oxidized by superoxide and emits red fluorescence. The basal levels of dHE-derived fluorescence in PBMCs were found to be no different in the non-heated and heated control groups. After hydrogen peroxide treatment, the dHE-derived fluorescence in PBMCs was significantly increased in the oxidative-

stress non-heated group, indicating that the intracellular superoxide anion level is enhanced (Fig. 4). The shifting of the dHE-derived fluorescent histogram showed a decreased with statistical significance in the oxidative-stress-heated group compared with the oxidative-stress non-heated group.

Heat-shock response prevents hydrogen peroxide-induced cytochrome *c* release from mitochondria

Before the localization of cytochrome *c* in the mitochondria and cytoplasm of PBMCs were detected by Western blotting, cross-contamination between the mitochondrial and cytosolic fractions had to be excluded. Cox IV and catenin were used as individual markers for each fraction. Cox IV was undetectable in the cytosolic fraction, confirming that the cytosolic fraction was not contaminated by the mitochondrial fraction. Likewise, catenin was detected only in the cytosolic fraction, confirming that no mitochondrial contamination had occurred (Fig. 5a).

Expression of cytochrome *c* in the cytosolic fraction could be detected by Western blotting. Cytochrome *c* was not detected in the cytosolic fractions of either the non-heated and heated control group, while the amount of cytochrome *c* is dramatically enhanced after hydrogen

Fig. 4 Detection of superoxide anion levels. Superoxide generation was monitored by dHE and analyzed by flow cytometry. One representative expression out of five is shown. The horizontal axis shows the relative fluorescence intensity and the vertical axis shows the number of cells. Cell populations with higher and lower levels of dHE staining are margined as M1 and M2, respectively

Fig. 5 Cytochrome *c* expression in the cytosolic and mitochondrial fractions. **a** Purity of mitochondrial and cytosolic fractions monitored by Western blotting. One representative expression out of five is shown. Cox IV and catenin were used as individual markers to exclude cross-contamination between the mitochondrial and cytosolic fractions. Actin was detected simultaneously and acts as the internal standard. **b** and **c** Cytochrome *c* detection in the mitochondrial and cytosolic fractions. Cytochrome *c* expression was detected by Western blotting and actin was detected simultaneously and acts as the internal standard. One representative expression of immunostaining out of five is shown in the upper panels and the statistical analyses of relative content are shown in the lower panels. * $p < 0.05$ vs. each other as indicated

peroxide treatment in the oxidative-stress non-heated group. Despite the fact that cytochrome *c* also can be detected in the cytosolic fraction of the oxidative-stress heated group, the amount is significantly decreased as compared with that of the oxidative-stress non-heated group ($p < 0.05$; Fig. 5b). In short, cytochrome *c* can be detected in abundance in the mitochondrial fractions of all groups; however, the amount of cytochrome *c* in the mitochondrial fraction of the oxidative-stress non-heated group is significantly decreased as compared with that of the oxidative-stress-heated group (Fig. 5c).

Discussions

In the present study, hydrogen peroxide was utilized to evoke oxidative stress in rat PBMCs. After hydrogen peroxide treatment, the intracellular superoxide was promptly accumulated and cytochrome *c* was leaked from mitochondria to cytoplasm. The cell death population was found to contain early- and late-cellular apoptosis and was significantly increased in PBMCs. We suggest that the loss of mitochondrial membrane potential is a critical characteristic of oxidative-stress-induced mitochondrial functional perturbation, and the signaling of cell death is then initiated in PDMCs. The induction of heat-shock response contributes to a decrease in the accumulation of intracellular superoxide and prevents the depolarization of the mitochondrial membrane in PBMCs during hydrogen peroxide-induced oxidative stress. Moreover, heat-shock response prevents proapoptotic protein, cytochrome *c*, leaking from mitochondria, and the evolution of cell death signaling transduction is then blocked. Heat-shock response can protect cells from oxidative-stress-induced cell death through the mitochondrial pathway.

Under normal conditions, it is unavoidable for oxygen to be transformed into highly reactive forms, called ROS, such as hydrogen peroxide, superoxide anions and hydroxyl radicals (Andreyev et al. 2005). Small physiological amounts of ROS are required in signaling pathways for

cellular activation (Hansen et al. 2006); however, a large amount of ROS formation when cells are receiving stress signals can lead to an imbalance of oxidants and antioxidants, causing oxidative stress. Related effects of oxidative stress have been noted and have been implicated in a variety of clinical conditions, including infectious diseases (Sakaguchi and Furusawa 2006), aging (Csiszar et al. 2007), and autoimmune diseases (Nicolls et al. 2007). Hydrogen peroxide is a small and diffusible molecule, and it can act as a second messenger of metabolic oxidative stress and induces oxidative damage to many cellular components indiscriminately (Clarkson and Thompson 2000; Ji 1999; Song et al. 2005). Indeed, the accumulation of intracellular superoxide radicals was observed after hydrogen peroxide treatment. The superoxide radical is the most well-known oxygen-derived free radical and can result in the formation of perhydroxyl radicals which is a much stronger radical than the superoxide radical (Clarkson and Thompson 2000). We suggest that oxidative stress is successfully induced by hydrogen peroxide treatment *in vitro*.

Besides inducing superoxide accumulation, the collapse of the mitochondrial membrane potential was observed by JC-1 staining, and the release of cytochrome *c* from the mitochondrial fraction to the cytosolic fraction was detected in PBMCs during hydrogen-peroxide-induced oxidative stress. Mitochondria form one of the major sites of ROS generation and are also the major target in attack by ROS, and eventually become effector organelles for cell death (Kakkar and Singh 2007). Mitochondrial membrane potential is generated through the electrochemical gradient of membrane, which is created during the processes of mitochondrial electron transport chain. A collapse in the mitochondrial membrane potential is one of the early events in and causes of programmed cell death. Following collapse of the mitochondrial membrane potential and mitochondrial function failure, the proapoptotic factor, cytochrome *c*, is leaked from mitochondria and the population of apoptosis increases significantly after hydrogen-peroxide-induced oxidative stress. Cytochrome *c* leaking into the cytoplasm is an important sign in the initiation of apoptotic signals. The release of cytochrome *c* has been shown to be correlated with the activation of caspase-dependent apoptotic signals and the induction of nuclear apoptosis (Liu et al. 1996). We suggest that mitochondria may act as biosensors of high sensitivity during oxidative stress and the detection of mitochondrial membrane potential could be a convenient method for evaluating the oxidative-stress-induced cell perturbation.

ROS generation is constantly occurring in living organisms by endogenous bio-metabolism or exogenous promotion. The implication of ROS-induced oxidative stress in various clinical pathogenesis has been highlighted

in many *in vitro* and *in vivo* studies (Kakkar and Singh 2007). Indeed, antioxidant supplementation has now been tried and applied as a therapeutic agent for various pathological conditions; however, the effects of external supplementation of antioxidants still remain to be investigated. Heat-shock proteins (HSPs), a series of endogenous proteins synthesized concomitantly with the induction of heat-shock response, are regarded as multi-functional proteins that protect living organisms against subsequent lethal injury (Arya et al. 2007). Heat-shock proteins act as molecular chaperones; they contribute to peptide folding, re-naturing and transport between different organelles. Heat-shock treatment, inducing heat-shock protein synthesis, contributes to the prevention of mitochondrial structural and functional damage in the heart and liver during sepsis (Chen et al. 2003; Chen et al. 2004). The expression and activity of the respiratory chain-associated enzymatic complex can be preserved by heat-shock proteins during sepsis (Chen et al. 2003; Chen et al. 2004). Moreover, heat-shock treatment protects the thymus against sepsis-induced apoptosis (Chen et al. 2000) and the delivery of HSPs enhances resistance to ischemia/reperfusion heart injury (Kwon et al. 2007). However, the mechanisms by which heat-shock treatment protects organisms against apoptosis induced by oxidative stress remain to be identified.

HSPs have been reported that may play a critical role in decreasing the accumulation of ROS. HSP70 contributes to decrease ROS accumulation by increasing glutathione peroxidase (GPx) and glutathione reductase (GR) activities during ischemic stress (Guo et al. 2007). GPx and GR are both the major regulating enzymes in the glutathione antioxidant system. Superoxide dismutase activities were significantly decreased in hsp 70.1 knock-out mice than in the wild-type littermates (Choi et al. 2005). The present study showed that hydrogen peroxide-induced superoxide accumulation is decreased by heat-shock response. The mitochondrial membrane potential is also preserved and mitochondrial function is maintained. We believe that the inhibition of superoxide radical induction and the prevention of mitochondrial membrane potential collapse may be the main effects of heat-shock response in the prevention of oxidative-stress-induced cellular damage. After cytochrome *c* is released from mitochondria, Hsp can inhibit the formation of a functional apoptosome complex and prevent late caspase-dependent events (Pandey et al. 2000; Saleh et al. 2000). We suggest that the heat-shock response, inducing heat-shock protein synthesis, can offer pluripotent protective effects in mitochondria to decrease oxidative-stress-induced cellular damage.

Blood cells can be collected and utilized conveniently to evaluate the status of oxidative-stress and anti-oxidative action (Cases et al. 2006). PBMCs are more sensitive than neutrophils in intense exercise-induced oxidative damage

(Sureda et al. 2005) and are useful tools as markers reflecting the systemic symptoms of oxidative stress under physical and mental stimulation (Aslan et al. 2006; Fedeli et al. 2007; Zalata et al. 2007; Zheng and Ariizumi 2007). PBMCs are utilized in the present study; they are sensitive to hydrogen-peroxide-induced oxidative stress and are also highly susceptible in their response to heat-shock-response-associated protective effects. We suggest that PBMCs are a convenient study target in that they reflect the level of oxidative stress and can be used to evaluate the effects of defense against oxidative stress.

In conclusion, mitochondria are critical organelles in the protective effects of heat-shock treatment. Cellular apoptosis by hydrogen-peroxide-induced oxidative stress is decreased by heat-shock treatment through maintaining the mitochondrial membrane potential and decreasing the leaking of cytochrome *c* from mitochondria to cytoplasm.

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