Heat shock pretreatment inhibited the release of Smac/DIABLO from mitochondria and apoptosis induced by hydrogen peroxide in cardiomyocytes and C₂C₁₂ myogenic cells

Bimei Jiang, Weimin Xiao, Yongzhong Shi, Meidong Liu, and Xianzhong Xiao

Department of Pathophysiology, Xiangya School of Medicine, Central South University, Changsha, Hunan 410078, People's Republic of China

Abstract Oxidative stress may cause apoptosis of cardiomyocytes in ischemia-reperfused myocardium, and heat shock pretreatment is thought to be protective against ischemic injury when cardiac myocytes are subjected to ischemia or simulated ischemia. However, the detailed mechanisms responsible for the protective effect of heat shock pretreatment are currently unclear. The aim of this study was to determine whether heat shock pretreatment exerts a protective effect against hydrogen peroxide (H₂O₂)-induced apoptotic cell death in neonatal rat cardiomyocytes and C₂C₁₂ myogenic cells and whether such protection is associated with decreased release of second mitochondria-derived activator of caspase—direct IAP binding protein with low pI (where IAP is inhibitor of apoptosis protein) (Smac/DIABLO) from mitochondria and the activation of caspase-9 and caspase-3. After heat shock pretreatment (42 ± 0.3°C for 1 hour, recovery for 12 hours), cardiomyocytes and C₂C₁₂ myogenic cells were exposed to H₂O₂ (0.5 mmol/L) for 6, 12, 24, and 36 hours. Apoptosis was evaluated by Hoechst 33258 staining and DNA laddering. Caspase-9 and caspase-3 activities were assayed by caspase colorimetric assay kit and Western analysis. Inducible heat shock proteins (Hsp) were detected using Western analysis. The release of Smac/DIABLO from mitochondria to cytoplasm was observed by Western blot and indirect immunofluorescence analysis. (1) H₂O₂ (0.5 mmol/L) exposure induced apoptosis in neonatal rat cardiomyocytes and C₂C₁₂ myogenic cells, with a marked release of Smac/DIABLO from mitochondria into cytoplasm and activation of caspase-9 and caspase-3, (2) heat shock pretreatment induced expression of Hsp70, Hsp90, and αB-crystallin and inhibited H₂O₂-mediated Smac/DIABLO release from mitochondria, the activation of caspase-9, caspase-3, and subsequent apoptosis. H₂O₂ can induce the release of Smac/DIABLO from mitochondria and apoptosis in cardiomyocytes and C₂C₁₂ myogenic cells. Heat shock pretreatment protects the cells against H₂O₂-induced apoptosis, and its mechanism appears to involve the inhibition of Smac release from mitochondria.

INTRODUCTION

Apoptosis, characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation, is indispensable for embryo development, tissue homeostasis, and regulation of the immune system (Li et al 2000). Dysregulated apoptosis has been implicated in the pathogenesis of cardiovascular diseases and cardiac dysfunction

(Thompson et al 1995; Ekhterae et al 1999). Accumulating evidence from in vitro and in vivo studies strongly suggests that loss of cardiomyocytes by apoptosis is an important cellular mechanism in the development of cardiac failure during injury due to ischemia and reperfusion and myocardial infarction (Ekhterae et al 1999). Therefore, it is important to find molecules that inhibit cardiomyocyte apoptosis to prevent the development of heart failure.

The signaling events leading to apoptosis can be divided into 2 distinct pathways involving either mitochondria

Received 3 April 2005; Revised 19 May 2005; Accepted 26 May 2005.

Correspondence to: Xianzhong Xiao, Tel: 86-731-2355019; E-mail: xianzhongxiao@xysm.net.

or death receptors (Ashkenazi and Dixit 1998; Green and Reed 1998). In the mitochondria pathway, death signals lead to changes in mitochondrial membrane permeability and the subsequent release of proapoptotic factors involved in various aspects of apoptosis (Deng et al 2002). The released factors include cytochrome *c* (Liu et al 1996), apoptosis-inducing factor (AIF) (Susin et al 1999), and endonuclease G (Li et al 2001). Once in the cytoplasm, cytochrome *c* catalyzes the oligomerization of apoptotic protease activating factor-1 (Apaf-1) (Zou et al 1997). This promotes the activation of procaspase-9, which then initiates a caspase cascade involving downstream executioner procaspase-3, -6, and -7 (Li et al 1997a; Srinivasula et al 1998). Concurrent with cytochrome *c* release, another mitochondrial protein, Smac (Du et al 2000) (second mitochondria-derived activator of caspase) or DIABLO (Verhagen et al 2000) (direct IAP binding protein with low pI, where IAP is inhibitor of apoptosis protein), was recently found to be released into the cytosol during apoptosis triggered by UV or irradiation, cytotoxic drugs and DNA damage, as well as by ligation of the CD95 death receptor in human cancer cells. Whereas cytochrome *c* induces multimerization of Apaf-1 to activate procaspase-9 and procaspase-3, Smac/DIABLO promotes apoptosis by binding to the IAPs and preventing them from sequestering caspases (Chai et al 2000; Du et al 2000; Liu et al 2000; Srinivasula et al 2000; Verhagen et al 2000; Wu et al 2000). Furthermore, Smac/DIABLO is highly expressed in myocardium. However, whether Smac/DIABLO plays a role in cardiomyocyte apoptosis induced by myocardial ischemia and reperfusion injury or oxidative stress is still uncertain.

Heat shock pretreatment can induce a conserved heat shock response that protects living organisms against subsequent lethal injury. Mammalian hearts pretreated with heat shock have enhanced resistance to ischemia (Currie et al 1988, 1993; Yellon et al 1992). Moreover, heat shock pretreatment protects cardiomyocytes against apoptosis induced by ischemia and reperfusion injury or hypoxia (Bhara et al 2000; Li et al 2004). However, the mechanisms by which heat shock pretreatment protects cardiomyocytes against apoptosis induced by a variety of stressors remain to be identified. In this study, we report that hydrogen peroxide (H_2O_2) induces the release of Smac/DIABLO from mitochondria, activation of caspase-9 and caspase-3, and apoptosis; and heat shock pretreatment significantly counteracts the release of Smac/DIABLO from mitochondria and inhibits the activation of caspase-9 and caspase-3 and apoptosis induced by H_2O_2 in cardiomyocytes and C_2C_{12} myogenic cells.

MATERIALS AND METHODS

Cell culture and treatment

C_2C_{12} myoblasts were cultured in growth medium (Dulbecco modified Eagle medium [DMEM] supplemented

with 10% heat-inactivated fetal bovine serum [FBS]) at 37°C in the presence of 5% CO_2 under a humidified atmosphere. Primary cultures of neonatal rat cardiomyocytes were cultivated as described previously (Long et al 1997, 1998). In brief, hearts from neonatal Wistar rats (1–3 days after birth) were removed, minced, and trypsinized at 37°C with gentle stirring in D-Hanks buffer containing 0.1% trypsin (GIBCO). Then cells were centrifuged and resuspended in DMEM (GIBCO) containing 15% FBS (Hyclone). After incubation at 37°C for 120 minutes, the unattached cardiomyocytes were seeded at a density of 5×10^5 cells/mL. All cells were cultured in DMEM (GIBCO) containing 15% FBS for 24 hours before the initiation of experiments. 5-Bromo-2'-deoxyuridine (Sigma) (0.1 mM) was added to the culture for 36 hours to inhibit the proliferation of nonmyocytes. H_2O_2 was diluted in phosphate-buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , pH = 7.4) and further diluted in culture medium. The final H_2O_2 concentration in medium was 0.5 mmol/L.

Heat shock treatment

Subconfluent cultured cells in 50-mm dishes were subjected to hyperthermia of $42 \pm 0.3^\circ C$ for 1 hour with a water bath. As a control, cells were cultured under normal conditions without hyperthermia. Cells were then routinely allowed to recover for 12 hours at 37°C in a humidified atmosphere containing 5% CO_2 . After recovery, cells were used for the following in vitro experiments.

Quantitation of apoptotic cells

At the predetermined time point after H_2O_2 exposure, adherent cells were released from tissue culture plates with trypsin–ethylenediamine-tetraacetic acid (EDTA) or cell scrapers, combined with the nonadherent cells, sedimented at $500 \times g$ for 5 minutes, fixed with 4% paraformaldehyde for 30 minutes in room temperature, and then washed once with PBS. Hoechst 33258 (50 ng/mL) was added to the fixed cells, incubated for 30 minutes at room temperature, and washed with PBS. Cells were mounted onto glass slides and examined by fluorescence microscopy. Apoptotic cells were identified by the condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. At minimum, 500 cells were counted for each slide.

Western blot analysis

Cells were washed with $1 \times$ PBS and resuspended with 5 volumes of cold lysis buffer (50 mM Tris-HCl [pH 7.5],

250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Science). The cell lysate was incubated on ice for 30 minutes, then centrifuged for 10 minutes at 4°C. The protein content of the supernatant was determined by the Bradford assay (Bio-Rad). Equal amounts of proteins (10–20 µg) were loaded in each lane and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and stained with Ponceau as a loading control, the resolved proteins were transferred to nitrocellulose membrane. After blocking with 2% albumin in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C, the blot was probed with anti-Smac (Research & Development), anti-anti-cytochrome oxidase subunit II (COX II) (A-6404; Molecular Probes), anti-caspase-9, and anti-caspase-3 (Santa Cruz Biotechnology) antibodies at the appropriate concentration for 2 hours at room temperature. The membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours. The membrane was washed 3 times in TBST and developed by DAB assay kit using the manufacturer's protocol (Boster Biological Technology).

Caspase activity assay

The caspase fluorescent assay kits specific for caspase-3 or caspase-9 (Biovision) were used to detect caspase activation by measuring the cleavage of a synthetic fluorescent substrate. In brief, cells were cultured in 60-mm dishes and treated with 0.5 mmol/L H₂O₂ for the indicated periods. Cell lysates were prepared in the lysis buffer from the assay kits, the lysates were centrifuged at 10 000 × g for 1 minute, and the supernatants were collected. With bovine serum albumin as the standard, equal amounts of proteins from each sample were reacted with the synthetic fluorescent substrates at 37°C for 1.5 hours and read at 405 nm on a microplate reader. Fold-increase in caspase-3 activity was determined by comparing the data of treatment samples with those of control samples.

Preparation of mitochondrial and cytosolic fractions

Untreated and H₂O₂-treated cells were harvested by centrifugation at 1000 × g for 10 minutes at 4°C. Adherent cells were removed by trypsinization in 0.25% trypsin at 37°C for 5 minutes. When cells were treated with H₂O₂, floating cells were also collected in the same tubes, after being washed once with ice-cold PBS, the cell pellet was suspended in 5 volumes of buffer A (20 mM N-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM Na-EDTA, 1 mM Na-ethylene glycol-bis(aminoethylether)-tetraacetic acid, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl

fluoride containing 250 mM sucrose) supplemented with protease inhibitors (5 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 µg/mL aprotinin, and 25 µg/mL *N*-acetyl-leu-leu-norleucine). After incubation on ice for 15 minutes, cell suspension was gently homogenized with a glass Dounce homogenizer (20–30 strokes), and cell lysates were checked by trypan blue staining. After centrifugation twice at 750 × g for 10 minutes at 4°C, the supernatant was collected and centrifuged at 10 000 × g for 15 minutes at 4°C, and the resulting mitochondrial pellets were resuspended in buffer A. The supernatants of the 10 000 × g spin were further centrifuged at 100 000 × g for 1 hour at 4°C, and the resulting supernatants were designated the S-100 cytosolic fraction.

Detection of DNA fragmentation

Floating and adherent cells (5 × 10⁷) from each culture condition were combined, centrifuged, pelleted at 400 × g for 5 minutes, and washed twice with PBS. The pellet was resuspended in 200 µL lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5% Triton X-100, and 0.1 mg/mL ribonuclease [RNase] A). Cells were lysed during incubation for 1 hour at 37°C. The cell lysates were further incubated for 30 minutes at 54°C in the presence of 0.2 mg/mL Protease K. The DNA was extracted by 2 separations, with phenol-chloroform and then with chloroform only. The DNA pellet was then washed in 70% ethanol and resuspended in 1 mM EDTA, 10 mM Tris-HCl (pH 8.0) at a final concentration of 20 µg/mL, electrophoresed on 1.5% agarose gel containing ethidium bromide, and photographed under ultraviolet illumination. GeneRuler 100-bp DNA ladder (MBI Fermentas) was used as DNA size markers.

Immunofluorescence analysis

For immunofluorescence analysis, cells were grown on coverslips and treated with or without 0.5 mmol/L H₂O₂ for the indicated periods. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were then incubated with rabbit anti-human Smac diluted 1:500 in 5% FBS-PBS for 2 hours at room temperature in a humidified chamber. Excess antibody was removed by washing the coverslips 3 times with PBS. Cells, which were protected from light, were then incubated with biotin-labeled goat anti-rabbit IgG diluted 1:100 in 5% FBS-PBS for 1 hour at room temperature. After washing 3 times with PBS, cells were incubated with CY3-streptavidin diluted 1:100 in 5% FBS-PBS for 1 hour. After washing 3 times with PBS, cells on coverslips were examined under a fluorescent microscope and photographed.

Fig 1. Heat shock pretreatment induced heat shock protein expression in cardiomyocytes and C₂C₁₂ myogenic cells. Both cells were subjected to hyperthermia of $42 \pm 0.3^\circ\text{C}$ for 1 hour with a water bath then routinely allowed to recover for the indicated times at 37°C in a humidified atmosphere containing 5% CO₂. Cell extracts were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western immunoblotting with anti-Hsp70, anti-Hsp90, anti-αB-crystallin separately in cardiomyocytes (A) and C₂C₁₂ myogenic cells (B). Ctrl: Control. Ponceau staining was used for loading control. The blot presented is representative of 3 independent experiments. Hsp, heat shock proteins.

Statistical analysis

Data are expressed as mean $\pm$ SEM of the indicated number of separate experiments. Statistical comparison between experimental group and control was performed using unpaired 2-tailed Student's *t*-tests. *P* values less than 0.05 were considered significant.

RESULTS

Heat shock pretreatment–induced expression of heat shock protein

Cardiomyocytes and C₂C₁₂ myogenic cells were subjected to heat shock ($42 \pm 0.3^\circ\text{C}$ for 1 hour) or normal incubation (control) and examined at the indicated times later. No

dead (rounded) cells were found in either group under microscopic observation. Western blotting confirmed a higher level expression of Hsp70, Hsp90, and αB-crystallin in the heat shocked cells compared with control cells (Fig 1).

Heat shock pretreatment protects cardiomyocytes and C₂C₁₂ myogenic cells from H₂O₂-induced apoptosis

We first examined whether heat shock pretreatment protected cardiomyocytes and C₂C₁₂ myogenic cells from H₂O₂-induced apoptosis. Cardiac myocytes and C₂C₁₂ myogenic cells were exposed to H₂O₂ (0.5 mmol/L) for the indicated times, and the cells were stained by Hoechst 33258 and observed by fluorescent microscopy. In the con-

Fig 2. Heat shock pretreatment protects cardiomyocytes and C₂C₁₂ myogenic cells from H₂O₂-induced apoptosis. Cells were stained with Hoechst 33258, and apoptotic cells were measured by observation under a fluorescence microscope. Apoptotic cells, which contained condensed chromatin fragments, were scored and expressed as a percentage of the total cell number counted. Results from 3 independent experiments are shown as means $\pm$ SEM. A and B represent control cardiomyocytes and C₂C₁₂ myogenic cells; C and D, cardiomyocytes and C₂C₁₂ myogenic cells incubated with 0.5 mmol/L H₂O₂ for 24 hours; E and F, cardiomyocytes and C₂C₁₂ myogenic cells incubated with 0.5 mmol/L H₂O₂ for 24 hours after heat shock pretreatment ($42 \pm 0.3^\circ\text{C}$ for 1 hour, recovery for 12 hours at 37°C). Magnification 200 $\times$. G and H represent the percentage of apoptotic cells in cardiomyocytes and C₂C₁₂ myogenic cells treated with 0.5 mmol/L H₂O₂ for the times indicated. At least 10 fields per slide and 4 independent cultures were examined. H₂O₂, hydrogen peroxide.

trol group, ~5% of cardiac myocytes and ~4% of C₂C₁₂ myogenic cells were positive (Fig 2 A,B). When cells were incubated with 0.5 mmol/L H₂O₂ for 24 hours, the number of positive cells was significantly increased (~50%) (Fig 2 C,D). The nuclei of these positive cells were condensed and contracted, suggesting that H₂O₂ induced apoptosis in both cardiomyocytes and C₂C₁₂ myogenic cells. When cardiomyocytes and C₂C₁₂ myogenic cells were pretreated with heat shock ($42 \pm 0.3^\circ\text{C}$ for 1 hour, recovery for 12 hours) before addition of H₂O₂ (0.5 mmol/L for 24 hours), the number of Hoechst 33258-positive cardiomyocytes and C₂C₁₂ myogenic cells was significantly decreased (cardiomyocytes ~27%, C₂C₁₂ myogenic cells ~25%) (Fig 2 E,F)

compared with the cells without heat shock pretreatment and incubated with H₂O₂. Survival curves are presented for the cardiomyocytes and C₂C₁₂ myogenic cells (Fig 2 G,H). These results suggest that heat shock pretreatment protected cardiomyocytes and C₂C₁₂ myogenic cells from oxidative stress-induced apoptosis.

Heat shock pretreatment protected cardiomyocytes and C₂C₁₂ myogenic cells from H₂O₂-induced DNA degradation

During apoptosis, the endonucleases attack the DNA structure, produce DNA fragments (folds of 180–200 bp)

Fig 3. Heat shock pretreatment inhibited DNA fragmentation resulting from H_2O_2 -induced apoptosis in cardiomyocytes and C_2C_{12} myogenic cells. Cells lysates were first incubated with RNase and then with proteinase K. By this method, only cytosolic fragmented DNA was extracted. DNA was separated by electrophoresis in 1.5% agarose gels and stained by ethidium bromide. M, DNA marker; Ctrl, untreated control cells; H_2O_2 , hydrogen peroxide; RNase, ribonuclease.

M, DNA marker;

that are released from nuclei into cytosol. Electrophoresis of cytosolic DNA from apoptotic cells shows characteristic laddering pattern. To confirm that H_2O_2 induced apoptosis in cardiac myocytes and C_2C_{12} myogenic cells, we examined DNA laddering formation by agarose gel electrophoresis. Cells that were cultured in medium for 24 hours showed a faint DNA ladder (Fig 3, lane A). When cardiomyocytes and C_2C_{12} myogenic cells were exposed to 0.5 mmol/L H_2O_2 for 24 and 36 hours, extracted DNA showed a prominent DNA laddering characteristic of apoptosis (Fig 3, lanes B and C). However, DNA degradation was significantly decreased when cardiomyocytes and C_2C_{12} myogenic cells were pretreated with heat shock before exposure to H_2O_2 (Fig 3, lanes D and E).

Heat shock pretreatment suppressed the activation of proteolytic caspase-3 and caspase-9

We next examined whether the morphological features of apoptosis were accompanied by activation of caspase-3 and caspase-9. Caspase-3 is a critical effector caspase of the apoptotic process. Caspase-9 is an initiator of caspase-3 in the mitochondria-dependent pathway. The relative intensities of the cleaved free fluoroprobes of caspase-9 and caspase-3 obtained with different treatments from cardiomyocytes and C_2C_{12} myogenic cells are shown in Figure 4 A,B, respectively. H_2O_2 treatment significantly activated procaspase-9 and procaspase-3 in both cardiomyocytes and C_2C_{12} myogenic cells. Heat shock pretreat-

ment significantly reduced the activation of both caspase-9 and caspase-3 (Fig 4 A,B). Similarly Western analysis also showed heat shock pretreatment suppressed proteolytic caspase-3 and caspase-9 activation. Under normal conditions, only the uncleaved proform of endogenous procaspase-9 and procaspase-3 was detected by immunoblotting (Fig 5 A,B). Exposure of cardiomyocytes and C_2C_{12} myogenic cells to 0.5 mmol/L H_2O_2 induced proteolytic cleavage of caspase-9 and caspase-3, as revealed by the appearance of the characteristic 10 kDa (p10) and 12 kDa (p12) fragments on the Western immunoblot, respectively. The p10 and p12 proteolytic fragments were significantly decreased in lysates from cardiomyocytes and C_2C_{12} myogenic cells pretreated with heat shock before exposure to H_2O_2 (Fig 5 A,B).

Heat shock pretreatment inhibited the release of mitochondrial Smac/DIABLO to the cytosol

To observe whether heat shock pretreatment could suppress the activation of caspase-3 and caspase-9 through interfering with the release of Smac/DIABLO from mitochondria, we examined the amount of Smac/DIABLO in mitochondria-rich and cytosolic fractions isolated from cardiomyocytes and C_2C_{12} myogenic cells and found that under normal conditions, most of Smac/DIABLO existed in the mitochondria and little or no Smac/DIABLO was located in the cytosol. Exposure to H_2O_2 resulted in the release of Smac/DIABLO from the mitochondria into the

A **Cardiomyocyte****B** **C2C12 myogenic cells**

Fig 4. Determination of caspase-9 and caspase-3 activities. Cell lysates from control cells, cells that were treated for 12 hours with H_2O_2 or the cells that were exposed to H_2O_2 for 12 hours after heat shock pretreatment were obtained from cardiomyocytes (A) or C_2C_{12} myogenic cells (B) and incubated with fluorimetric substrates of caspase-3 (Ac-DEVD-PNC) or caspase-9 (Ac-LEHD-PNC) in a reaction buffer at $37^\circ C$ for 1 hour and the caspase activities were determined as described in Materials and Methods. Similar results were observed from 3 independent experiments. H_2O_2 , hydrogen peroxide.

cytosol in cardiomyocytes and C_2C_{12} myogenic cells (Fig 6 A,B). Such release of Smac/DIABLO was alleviated if the cells were pretreated with heat shock before exposure to H_2O_2 (Fig 6 A,B). The mitochondrial inner membrane protein COX II was retained in the mitochondria-rich fractions in all samples, indicating that the heat shock pretreatment and H_2O_2 exposure did not lead to general mitochondrial destruction. Ponceau staining of the same lysates confirmed equal amount of proteins, indicating that the results were not due to differential loading of samples. Similar to Western analysis, the immunofluorescence data also showed that heat shock pretreatment could partly prevent the release of Smac/DIABLO. In untreated control cells, Smac/DIABLO showed more punctual appearance around the nucleus. On H_2O_2 treatment,

Smac/DIABLO showed diffuse immunofluorescence in the cytoplasm indicating their release from mitochondria and heat shock pretreatment inhibited the release of Smac/DIABLO from mitochondria (Data not shown).

DISCUSSION

In this study, H_2O_2 -induced apoptosis in cardiomyocytes and C_2C_{12} myogenic cells was accompanied by the release of Smac/DIABLO from mitochondria, activation of caspase-9 and caspase-3, and DNA fragmentation. Proapoptotic factors, such as growth factor withdrawal, UV irradiation, or chemotherapeutic agents, cause apoptosis via a mitochondrial-dependent release of cytochrome *c*, which induces activation of apoptosome, a complex containing cytochrome *c*, Apaf-1, and caspase-9, and resulted in cleavage and activation of caspase-9 (Green and Reed 1998). Reactive oxygen species (ROS) trigger cytochrome *c* release by increasing cytoplasmic calcium concentration, or decreasing ATP levels, as well as by activating or enhancing levels of proapoptotic proteins, such as p53 or Bax (Long et al 1997; Green and Reed 1998). Smac/DIABLO is a proapoptotic protein that is normally located in mitochondria and proteolytically processed and released during apoptosis along with cytochrome *c* and other proapoptotic factors. Smac/DIABLO in the cytosol can neutralize IAPs, disrupt the ability of the IAPs to inhibit caspases-3, -7, and -9, and play important roles in apoptosis of cancer cells induced by a variety of stressors. For example, Smac is required for cytochrome *c*-induced apoptosis in prostate cancer LNCaP cells (Haunstetter and Izumo 1998); Smac/DIABLO release from mitochondria regulates tumor necrosis factor-related, apoptosis-inducing, ligand-induced apoptosis of human melanoma (Olivetti et al 1997). Here, we show for the first time, that H_2O_2 induces Smac/DIABLO release from the mitochondria and with increased activation of caspase-9 and caspase-3 in cardiomyocytes and C_2C_{12} myogenic cells. Our results also suggest that mitochondria played a pivotal role in ROS-induced apoptosis of cardiomyocytes and myogenic cells.

In this study, we show for the first time, that heat shock pretreatment inhibited Smac/DIABLO release from the mitochondria induced by H_2O_2 in both cardiomyocytes and myogenic cells. However, the mechanism by which heat shock pretreatment interferes with Smac/DIABLO release is not understood. It is possible that heat shock pretreatment targets and inhibits proapoptotic proteins that induce Smac/DIABLO release from mitochondria. It has been demonstrated that death receptor-initiated mitochondrial release of cytochrome *c* and Smac/DIABLO was caspase dependent (Colin et al 2001) and caspase-2 could also induce directly the release of cytochrome *c*, AIF, and Smac from isolated mitochondria independent

Fig 5. Western analysis showing the effect of heat shock pretreatment on caspase-3 and caspase-9 activation induced by H₂O₂ in cardiomyocytes and C₂C₁₂ myogenic cells. Cells were exposed to 0.5 mmol/L H₂O₂ for the indicated times. (A) The activation of caspase-3 (upper) and caspase-9 (lower) in cardiomyocytes. (B) The activation of caspase-3 (upper) and caspase-9 (lower) in C₂C₁₂ myogenic cells. Ponceau staining was used as a loading control. Similar results were observed in 3 independent experiments. H₂O₂, hydrogen peroxide.

C2C12 myogenic cells

of Bid or other cytosolic factors (Guo et al 2002). Whether heat shock pretreatment inhibited Smac/DIABLO release from mitochondria by inhibiting the activation of caspases is still to be clarified. Heat shock pretreatment might also inhibit Smac/DIABLO release from mitochondria directly or indirectly at mitochondrial sites. Several mechanisms have been proposed to explain how cytochrome *c* is released from mitochondria during apoptosis (Li et al 1997; Jurgensmeier et al 1998; Heiden et al 1999). The proposed mechanisms include a cytochrome *c*-specific channel, the opening of the permeability transition pore, and swelling with rupture of the outer mitochondrial

membrane (Heiden et al 1997; Li et al 1997b). Smac/DIABLO and cytochrome *c* release might occur with similar kinetics in death receptor-induced and Bcl-2-regulated apoptosis (Heiden et al 1999). Therefore, it is conceivable that 1 of these mechanisms is inhibited directly or indirectly by heat shock pretreatment.

Heat shock pretreatment induces the synthesis of diverse Hsps that confer a protective effect against a wide range of cellular stresses. Hsps help maintain the metabolic and structural integrity of the cell as a protective response to external stresses. They are known to protect the myocardium from the damaging effects of ischemia

Fig 6. Western analysis demonstrating that heat shock pretreatment inhibited Smac/DIABLO release from mitochondria. Preparation of mitochondrial and cytosolic fractions was as described in Materials and Methods. Forty micrograms of cytosolic and membrane proteins was analyzed by immunoblotting with rabbit anti-human Smac/DIABLO and mitochondrial inner membrane protein cytochrome oxidase subunit II(COXII). (A) Cardiomyocytes. (B) C₂C₁₂ myogenic cells. Smac/DIABLO, second mitochondria-derived activator of caspase-direct IAP binding protein with low pI (where IAP is inhibitor of apoptosis protein); Ctlol, Control; Cyto, cytoplasm; Mit, mitochondria. Ponceau staining was used as a loading control. Similar results were observed in 3 independent experiments.

and reperfusion (Yaglom et al 2003). Induction of Hsp72 and other Hsps was suggested to play an important role in preconditioning, a phenomenon of protection of a heart from strong ischemic insult by previous exposure to mild ischemia or other mild stresses. Our previous studies showed that targeted disruption of heat shock transcription factor-1 abolished inducible expression of Hsps and protection against heat-induced apoptosis (McMillan et al 1998; Xiao et al 1999). However, the molecular mechanism by which Hsps inhibit apoptosis was not well explored. Recent progress in studies on Hsps and apoptosis revealed that Hsps function at key regulatory points in the control of apoptosis by directly interacting with different apoptotic proteins. For example, Hsp27 bound to cytochrome *c* (Bruey et al 2000) or Hsp70 and Hsp90 bound to Apaf-1 (Beere et al 2000; Saleh et al 2000) results in inhibition of caspases activation. Additional negative influence of Hsp27 and Hsp70 on events both

further upstream and downstream have been described (Jaattela et al 1998; Pandey et al 2000a, 2000b; Paul et al 2002). Hsp90 interacts with RIP-1 kinase and Akt and promotes NF- κ B-mediated inhibition of apoptosis (Lewis et al 2000; Sato et al 2000). In addition to these mechanisms of inhibition, our present studies identified that heat shock pretreatment could induce the expression of Hsps, inhibit the release of Smac/DIABLO, inhibit the activation of caspase-9 and caspases-3 and apoptosis in cardiomyocytes and C₂C₁₂ myogenic cells.

We found that heat shock pretreatment increased Hsps expression, inhibited Smac/DIABLO release from the mitochondria, inhibited the activation of caspase-3 and caspase-9, and protected cardiomyocytes and C₂C₁₂ myogenic cells from H₂O₂-induced apoptosis. However, it remains unclear whether it is the newly expressed Hsps or some other aspect of the heat shock pretreatment that is regulating apoptosis upstream of mitochondria in this

model of H₂O₂-induced apoptosis. Additional studies with cardiac myocytes will be necessary to establish whether some Hsps interact directly with Smac/DIABLO and then inhibit the release of Smac/DIABLO from mitochondria.

ACKNOWLEDGMENTS

This study was supported by grants from The National Natural Science Foundation of China (30000069; 30270533), The Special Funds for Major State Basic Research of China (G2000056908), and The Special Funds for PhD training from The Ministry of Education (20020533032). The authors would like to thank Dr Xiaodong Wang (University of Texas Southwestern Medical Center at Dallas) for providing the Smac/DIABLO plasmid and Dr William Currie for critical comments on the study.

REFERENCES

- Ashkenazi A, Dixit VM. 1998. Death receptors: signaling and modulation. *Science* 281: 1305–1308.
- Beere HM, Wolf BB, Cain K, et al. 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol* 2: 469–475.
- Bhora FY, Wise RM, Foster AH. 2000. Heat shock protects cardiomyocytes from hypoxia-mediated apoptosis by attenuation of nitric oxide. *In Vivo* 14(5): 597–602.
- Bruey JM, Ducasse C, Bonniaud P, et al. 2000. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol* 2: 645–652.
- Chai J, Du C, Wu JW, et al. 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 406: 855–862.
- Colin A, Emma M, Seamus J. 2001. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. *J EMBO* 20(23): 6627–6636.
- Currie RW, Karmazyn M, Kloc M, Mailer K. 1988. Heat-shock response is associated with enhanced post-ischemic ventricular recovery. *Circ Res* 63: 543–549.
- Currie RW, Tanguay RM, Kingma JG. 1993. Heat-shock response and limitation of tissue necrosis during occlusion/reperfusion in rabbit hearts. *Circulation* 87: 963–971.
- Deng Y, Lin Y, Wu X. 2002. TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Dev* 16(1): 33–45.
- Du C, Fang M, Li L, et al. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102: 33–42.
- Ekhterae D, Lin ZW, Lundberg MS, et al. 1999. ARC inhibits cytochrome c release from mitochondria and protects against hypoxia-induced apoptosis in heart-derived H9c2 cells. *Circ Res* 85: e70–e75.
- Green DR, Reed JC. 1998. Mitochondria and apoptosis. *Science* 281: 1309–1312.
- Guo Y, Srinivasa M, Srinivasula AD, et al. 2002. Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J Biol Chem* 277(16): 13430–13437.
- Haunstetter A, Izumo S. 1998. Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res* 82(11): 1111–1129.
- Heiden GM, Chandel NS, Schumacker PT, et al. 1999. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell* 3: 159–167.
- Heiden MG, Chandel NS, Williamson EK, et al. 1997. Bcl-xL regulated the membrane potential and volume homeostasis of mitochondria. *Cell* 91: 627–637.
- Jaattela M, Wissing D, Kokholm K, et al. 1998. Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *J EMBO* 17: 6124–6134.
- Jurgensmeier JM, Xie Z, Deveraux Q, et al. 1998. Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* 95: 4997–5002.
- Lewis J, Devin A, Miller A, et al. 2000. Disruption of hsp90 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor- κ B activation. *J Biol Chem* 275: 10519–10526.
- Li CY, Lee JS, Ko YG, et al. 2000. Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J Biol Chem* 275: 25665–25671.
- Li F, Srinivasan A, Wang Y, et al. 1997a. Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-xL has activity independent of cytochrome c release. *J Biol Chem* 272: 30299–30305.
- Li G, Bae S, Zhang L. 2004. Effect of prenatal hypoxia on heat stress-mediated cardioprotection in adult rat heart. *Am J Physiol Heart Circ Physiol* 286(5): H1712–H1719.
- Li LY, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 412: 95–99.
- Li P, Nijhawan D, Budihardjo I, et al. 1997b. Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147–157.
- Liu Z, Sun C, Olejniczak ET, et al. 2000. Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* 408: 1004–1008.
- Long X, Boluyt MO, Hipolito ML. 1997. p53 and hypoxia-induced apoptosis of cultured neonatal cardiomyocytes. *J Clin Invest* 99: 2635–2643.
- Long X, Crow MT, Sollott SJ, et al. 1998. Enhanced expression of p53 and apoptosis induced by blockade of the vacuolar proton ATPase in cardiomyocytes. *J Clin Invest* 101: 1453–1461.
- McMillan DR, Xiao X, Shao L, et al. 1998. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* 273: 7523–7528.
- Olivetti G, Abbi R, Quaini F, et al. 1997. Apoptosis in the failing human heart. *N Engl J Med* 336(16): 1131–1141.
- Pandey P, Farber R, Nakazawa A, et al. 2000a. Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3. *Oncogene* 19: 1975–1981.
- Pandey P, Saleh A, Nakazawa A, et al. 2000b. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *J EMBO* 19: 4310–4322.
- Paul C, Manero F, Gonin S, et al. 2002. Hsp27 as a negative regulator of cytochrome c release. *Mol Cell Biol* 22: 816–834.
- Saleh A, Srinivasula SM, Balkir L, et al. 2000. Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol* 2: 476–483.
- Sato S, Fujita N, Tsuruo T. 2000. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A* 97: 10832–10837.
- Srinivasula SM, Ahmad M, Fernandes T, et al. 1998. Autoactivation

- of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1: 949–957.
- Srinivasula SM, Datta P, Fan XJ, et al. 2000. Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J Biol Chem* 275: 36152–36157.
- Susin SA, Lorenzo HK, Zamzami N, et al. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397: 441–446.
- Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1465–1467.
- Verhagen AM, Ekert PG, Pakusch M, et al. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 102: 43–53.
- Wu G, Chai J, Suber TL, et al. 2000. Structural basis of IAP recognition by Smac/DIABLO. *Nature* 408: 1008–1012.
- Xiao X, Zuo X, Davis AA, et al. 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *J EMBO* 18: 5943–5952.
- Yaglom JA, Ekhterae D, Gabai VL, et al. 2003. Regulation of necrosis of H9c2 myogenic cells upon transient energy deprivation. *J Biol Chem* 278(50): 50483–50496.
- Yellon DM, Pasini E, Cargnoni A, et al. 1992. The protective role of heat stress in the ischaemic and reperfused rabbit myocardium. *J Mol Cell Cardiol* 24: 895–907.
- Zou H, Henzel WJ, Liu X, et al. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90: 405–413.