Increased stability of Bcl-2 in HSP70-mediated protection against apoptosis induced by oxidative stress

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Received: 16 July 2010 / Revised: 7 September 2010 / Accepted: 8 September 2010 / Published online: 3 October 2010
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Abstract We have previously shown that heat shock protein 70 (HSP70) markedly inhibits H₂O₂-induced apoptosis in mouse C2C12 myogenic cells by reducing the release of Smac. However, the molecular mechanism by which HSP70 interferes with Smac release during oxidative stress-induced apoptosis is not understood. In the current study, we showed that HSP70 increased the stability of Bcl-2 during oxidative stress. An antisense phosphorothioate oligonucleotide against Bcl-2 caused selective inhibition of Bcl-2 protein expression induced by HSP70 and significantly attenuated HSP70-mediated cell protection against H₂O₂-induced release of Smac and apoptosis. Taken together, our results indicate that there are important relationships among HSP70, Bcl-2, release of Smac, and induction of apoptosis by oxidative stress.

Keywords Apoptosis · Heat shock protein 70 · Hydrogen peroxide · Smac · Bcl-2 · C2C12 myogenic cells

Abbreviations

HSP70	Heat shock protein 70
H ₂ O ₂	Hydrogen peroxide
IAPs	Inhibitors of apoptosis proteins
Smac	The second mitochondria-derived activator of caspases

COX II	Cytochrome oxidase subunit II
DTT	Dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
PBS	Phosphate-buffered saline
Apaf-1	Apoptotic protease activating factor-1

Introduction

Apoptosis is a genetically conserved form of programmed cell death required for normal development and homeostasis of multicellular organisms. As such, it plays a major role in embryonic development, tissue homeostasis, and regulation of the immune system (Danial and Korsmeyer 2004). Malfunctions of apoptosis have been implicated in human diseases including myocardial infarction, neurodegenerative diseases, cancer, and ischemic stroke (Jacobson et al. 1997; Price et al. 1998). Several factors, including ATP depletion, calcium fluxes, and reactive oxygen species, are usually induced through the intrinsic mitochondrial cell death pathway. In this pathway, loss of mitochondrial integrity results in cytochrome *c* release and subsequent activation of a series of apoptotic proteases known as caspases, through the formation of the apoptosome, a protein complex composed of the apoptotic protease activating factor-1, caspase-9, and cytochrome *c* (Danial and Korsmeyer 2004).

It has recently been shown that a novel mitochondrial protein, second mitochondria-derived activator of caspase (Smac, also known as DIABLO), is released into the cytosol in response to apoptotic stimuli (Du et al. 2000; Verhagen et al. 2000). UVB-irradiation, etoposide, or glucocorticoids are shown to promote caspase activation by eliminating IAP inhibition of caspases (Du et al. 2000; Verhagen et al. 2000). Smac is known as a new important regulator of apoptosis in

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a variety of cancer cells. Our previous study has also shown a vital role for Smac in apoptosis of myocytes (such as C2C12 myogenic cells and cardiomyocytes) induced by oxidative stress (Jiang et al. 2005a, b).

Heat shock protein 70 (HSP70), a major stress-inducible heat shock protein, has been shown to protect cells from a number of apoptotic stimuli, including heat shock, tumor necrosis factor, growth factor withdrawal, oxidative stress, and radiation (Arya et al. 2007; Zhao et al. 2007). Furthermore, HSP70 has been suggested as a promising molecule for controlling apoptosis. Oxidative stress down-regulates HSP70 expression and overexpression of HSP70 can inhibit the release of Smac from mitochondria, activation of caspase-3 and caspase-9, and apoptosis induced by hydrogen peroxide (H_2O_2) in C2C12 myogenic cells (Jiang et al. 2009). However, the mechanisms by which HSP70 inhibited the release of Smac from mitochondria and apoptosis induced by H_2O_2 remain to be identified.

The Bcl-2 family proteins are important in regulating the integrity of mitochondria. Bcl-2 family members share the Bcl-2 homology (BH) domains and can be divided into three subfamilies (Packham and Stevenson 2005; Strasser 2005), including the anti-apoptotic subfamily members such as Bcl-2, Bcl-xL, and Mcl-1, the pro-apoptotic Bax- and Bak-like proteins, and the pro-apoptotic BH3-only subfamily members. In living cells, anti-apoptotic proteins bind to and sequester pro-apoptotic Bax and Bak, thereby inhibiting apoptosis (Chao and Korsmeyer 1998). In this study, we found that HSP70 overexpression increased the stability of Bcl-2 during oxidative stress and that Bcl-2 ablation inhibited HSP70-mediated protection against the release of Smac and apoptosis induced by oxidative stress.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), G418, and fetal-calf serum were purchased from Gibco BRL (Berlin, Germany). The anti-GAPDH antibody was from StressGen Biotechnologies (Victoria, BC, Canada). The anti-Bcl-2 and anti-Smac antibodies were from BD Pharmingen (Heidelberg, Germany). The Hsp70+Hsc70 antibody (ab69412), Hsc70 antibody (ab1427), and Hsp70 antibody (ab47455) were from Abcam (Japan). The anti-mouse IgG was from Amersham Biosciences (Braunschweig, Germany). The anti-rabbit IgG was from Promega (Mannheim, Germany). The chemical 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma (St. Louis, MO). The Cpp32/caspase-3 colorimetric protease assay kit was obtained from Medical and Biological Laboratories Co. (Nagoya, Japan). The secondary antibodies, goat anti-rabbit

or anti-mouse IgG conjugated to horseradish peroxidase, were obtained from Pierce. Cycloheximide was from Sigma Chemical Co. (St. Louis, MO).

Cell culture and treatment

Mouse C2C12 myogenic cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2 under a humidified atmosphere. H_2O_2 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) was used in the medium at a final concentration of 0.5 mM.

Cell viability assay

To determine the cell viability, MTT (0.5 mg) was added to 1 ml of cell suspension (1×10^6 cells/ml in 24-well plates) for 4 h. After three washes with PBS (pH 7.4), the insoluble formazan product was dissolved in DMSO and the optical density (OD) of each culture well was measured using a microplate reader (Titertek Multiskan, Flow Laboratories) at 490 nm. The OD of formazan in control cells was taken as 100% of viability.

Flow cytometric analyses

Both adherent and floating cells were collected after treatment, washed with ice-cold PBS, and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, Franklin Lakes, NJ) and PI for 20 min at room temperature in the dark. The stained cells were then analyzed by a flow cytometer (Beckman Coulter). FITC-conjugated annexin V binds to surface phosphatidylserine translocated from the intra- to the extracellular plasma membrane early in apoptosis. Cells were simultaneously stained with PI to discriminate membrane-permeable necrotic cells from FITC-labeled apoptotic cells. Apoptotic cells were identified as those with annexin V-FITC staining only, and the results were expressed as the proportion of these cells among the total number of cells analyzed.

Western blot analyses

Cells were washed with PBS and resuspended with 100 μl of cold lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 0.5% Nonidet P-40) containing protease inhibitor mixture (Roche Applied Science). The cell lysate was incubated on ice for 30 min and centrifuged at $10,000 \times g$ for 10 min 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

and separated by SDS-PAGE, and the separated proteins were electrotransferred to Hybond-PVDF membrane (Amersham Pharmacia Biotech, Aylesbury, UK), which was soaked in a blocking solution [5% (w/v) nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Tween-20)] for 1 h at room temperature. The soaked PVDF membrane was then incubated with MAb against Bcl-2 [diluted 1:5,000 in 5% (w/v) nonfat milk in TBST] for 2 h at room temperature, washed with TBST buffer three times for 15 min each, incubated at room temperature for 1 h in horseradish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2,000 in TBST buffer), and finally washed three times with TBST for 15 min each. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction (ECL, Amersham). GAPDH was used as a reference protein.

Caspase activity assay

Caspase-3 and caspase-9 activities were measured according to the manufacturer's protocol. Briefly, cells cultured in 60 mm dishes were treated with 0.5 mM H_2O_2 for the indicated periods. The cells (5×10^6) were lysed with 250 μl of chilled cell lysis buffer on ice for 10 min. After microcentrifugation (10,000 $\times g$, 1 min, 4°C), the protein concentration in the supernatant was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Supernatants containing equal amounts of protein (corresponding to $\sim 5 \times 10^5$ cells) used for caspase-3 or caspase-9 colorimetric protease assays. After 1.5 h incubation at 37°C, the absorbance at 405 nm was measured by a microtiter plate reader (Molecular Devices). Fold increases in caspase-3 or caspase-9 activities over their controls were determined.

Preparation of mitochondrial and cytosolic fractions

Untreated and H_2O_2 -treated cells were harvested by centrifugation at 1,000 $\times g$ for 10 min at 4°C. Adherent cells were removed by trypsinization with 0.25% trypsin at 37°C for 5 min. When cells were treated with H_2O_2 , floating cells were also collected into the same tubes. After being washed once with ice-cold PBS, the cell pellet was suspended in five volumes of buffer A (20 mM HEPES-KOH at pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride containing 250 mM sucrose) supplemented with protease inhibitors (5 $\mu\text{g/ml}$ pepstatin A, 10 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, and 25 $\mu\text{g/ml}$ *N*-acetyl-leu-leu-norleucine). After incubation on ice for 15 min, the cell suspension was gently homogenized with a glass Dounce homogenizer (20–30 strokes) and cell lysates were examined by trypan blue staining. After centrifugation twice at 750 $\times g$

for 10 min at 4°C, the supernatant was collected and centrifuged at 10,000 $\times g$ for 15 min at 4°C. The resulting mitochondrial pellets were resuspended in buffer A. S-100 cytosolic fractions were prepared by further centrifugation at 100,000 $\times g$ at 4°C for 1 h.

Lipofectamine-mediated gene transfer

Transfection of C2C12 myogenic cells was carried out following the manufacturer's instruction (LIPOFECTAMINE 2000™, Invitrogen). Briefly, approximately 5×10^5 cells in 5 ml appropriate complete growth medium per bottle were grown at 37°C in a CO_2 incubator until the cells reached 70% to 80% confluent (24 h). After the cells were rinsed with serum-free and antibiotic-free medium, the cells were transfected with pcDNA3.1-Hsp70 (HspA1A, donated by Dr. Ivor Benjamin, University of Utah Health Sciences Center; experimental group) or pcDNA3.1 (vector control) at 8 μg DNA per 20 μl Lipofectamine, followed by incubation at 37°C in a CO_2 incubator for 6 h. The medium was replaced by DMEM culture medium containing 20% FBS. Stably transfected cell lines were selected using G418 (500 $\mu\text{g/ml}$).

Bcl-2 antisense oligodeoxynucleotide

Phosphorothioate oligodeoxynucleotides used in this study were manufactured by Bioasia Biotech (Shanghai, China). The antisense sequence of Bcl-2 oligodeoxynucleotide (As-Bcl2) was 5'-TCTCCCGGCTTGCGCCAT-3'. The scrambled sequence of Bcl-2 oligodeoxynucleotide (Scr-Bcl2) was 5'-TCCCTCACCGTTGCGCTG-3'. The oligodeoxynucleotides were diluted in 10 mM Tris (pH 7.4) and 1 mM EDTA and kept at -20°C.

Transfection of oligodeoxynucleotide using Lipofectamine 2000

Cells were seeded at a density of 3×10^5 per well in 1.0 ml of serum-deprived DMEM. Bcl-2 antisense or reverse oligonucleotide (20 μM) and 8 μl of Lipofectamine reagents in serum-deprived DMEM were mixed gently and incubated for 20 min at room temperature. The mixture was then added to cells and incubated in serum-deprived medium for 48 h at 37°C in a CO_2 incubator.

Statistical analyses

Data are presented as means $\pm$ SEM of the indicated number of independent experiments. Differences between two groups were analyzed by unpaired Student's *t* test. Differences between three or more groups were analyzed by one-way ANOVA followed by Student–Newman–Keuls

post hoc test. p values less than 0.05 were considered statistically significant.

Results

More drastic decrease of Bcl-2 in pcDNA3.1-transfected cells than in HSP70-transfected cells during the apoptosis induced by oxidative stress

Two clones, termed Hsp70-1 and HSP70-2, showing different levels of HSP70 proteins by immunoblot

analysis were selected for further study (Fig. 1c). To determine whether HSP70 overexpression in C2C12 myogenic cells inhibited H_2O_2 -induced apoptosis, the caspase-3 activity and the cell viability of pcDNA3.1-transfected and HSP70-transfected cells under oxidative stress were examined (Fig. 1a, b). After 12 h of exposure to hydrogen peroxide, there was a 3.09-fold increase of caspase-3 activity in control cells. Ectopic overexpression of HSP70 significantly inhibited H_2O_2 -induced activation of caspase-3. The protective effects of HSP70 were correlated with the level of HSP70 expression because the clone with higher HSP70 expression demonstrated a

Fig. 1 Bcl-2 protein analysis in pcDNA3.1-transfected and HSP70-transfected cells during apoptosis induced by H_2O_2 . The effect HSP70 stable overexpression on **a** caspase-3 activity or **b** cell viability after pcDNA3.1-transfected and HSP70-transfected cells were treated with H_2O_2 for different time. C2C12 cells overexpressing HSP70 (HSP70-1, HSP70-2) were analyzed. Data are expressed as means $\pm$ SEM. * p < 0.01 vs Ctrl, # p < 0.01 vs Ctrl + H_2O_2 , ** n = 3. **c** The Bcl-2 protein expression. Total cellular proteins were isolated and separated by 12% SDS-PAGE and analyzed by immunoblotting with anti-HSP70 and anti-Bcl-2. Immunoblot analyses with anti-GAPDH monoclonal antibody were used as an internal marker of gel loading. The ratio of HSP70 or Bcl-2

band density vs GAPDH density in Western blot analyses was corrected to that in the controls (set as 1.0). Data are expressed as means $\pm$ SEM. * p < 0.01 vs pcDNA3.1, n = 3. **d** pcDNA3.1-transfected and HSP70-transfected cells (HSP70-2) were under H_2O_2 exposure for various times (0–36 h). Cells were harvested and washed, and the lysates were centrifuged at 12,000 $\times g$ for 30 min at 4°C. Total cellular proteins (10 μ g) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-Bcl-2, monoclonal anti-HSP70, or monoclonal anti-GAPDH antibody for 2 h at room temperature. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction

more significant inhibition of the caspase-3 activity. Also concomitant with the activation of caspase-3, there was a decrease in cell viability in control and pcDNA3.1-transfected cells after H_2O_2 exposure as determined by MTT assay, however, ectopic overexpression of HSP70 significantly inhibited the decrease in cell viability induced by H_2O_2 .

The steady-state Bcl-2 protein level in these cells during hydrogen peroxide exposure (0–36 h) was also determined. Bcl-2 decreased in both pcDNA3.1-transfected and HSP70-transfected cells (HSP70-2) during hydrogen peroxide exposure (Fig. 1d). However, it decreased much more drastically in pcDNA3.1-transfected cells than in HSP70-transfected cells (Fig. 1d). In parallel, hydrogen peroxide resulted in little change in the protein level of HSP70 in HSP70-transfected cells. HSP70 was barely detected in pcDNA3.1-transfected cells.

To compare the stability of Bcl-2 protein in pcDNA3.1-transfected and HSP70-transfected cells (HSP70-2) during hydrogen peroxide exposure, the half life of Bcl-2 was measured by incubating H_2O_2 -treated (for 12 h) cells with cycloheximide (1 μ g/ml) for up to 24 h. Bcl-2 in both pcDNA3.1-transfected and HSP70-transfected cells was stable and its half life was longer than 12 h (Fig. 2a). Bcl-2 in H_2O_2 -treated pcDNA3.1-transfected cells, however, was found to be highly unstable, with a half life of less than 4 h. In contrast, Bcl-2 in H_2O_2 -treated HSP70-transfected cells (HSP70-2) was as stable as that in H_2O_2 -untreated pcDNA3.1-transfected and HSP70-transfected cells (Fig. 2b).

Transfection of Bcl-2 antisense oligodeoxynucleotide inhibited the upregulation of Bcl-2 induced by HSP70

Our finding that Bcl-2 levels correlated with the anti-apoptotic activity of HSP70 raised the possibility that Bcl-2 levels may be the intermediate between HSP70 and apoptosis inhibition. To test this possibility, we investigated the effect of antisense oligonucleotide-mediated Bcl-2 knockdown on the cytoprotective function of HSP70. Using Lipofectamine 2000 as a transfection reagent, the anti-Bcl-2 oligodeoxynucleotide (As-Bcl2) was successfully transferred into cultured C2C12 cells (the transfection efficacy was about 80%). Western blot analyses demonstrated that As-Bcl2 selectively inhibited Bcl-2 protein content by more than 75% after 36 h compared to the GAPDH, whereas gene transfer of the scrambled control oligonucleotides (Scr-Bcl2) did not alter Bcl-2 expression (Fig. 3a). We further examined the effect of As-Bcl2 on the expression of Bcl-2 induced by HSP70. As shown in Fig. 3b, compared with the control Scr-Bcl2, downregulation of Bcl-2 with As-Bcl2 significantly inhibited Bcl-2 upregulation induced by HSP70. We

Fig. 2 Bcl-2 protein is more stable in HSP70-transfected cells than Bcl-2 in pcDNA3.1-transfected cells during H_2O_2 exposure. pcDNA3.1-transfected (pcDNA3.1) or HSP70-transfected (HSP70-2) cells were **a** untreated with 0.5 mM H_2O_2 or **b** treated with 0.5 mM H_2O_2 for 4 h. Cycloheximide (CHX; 1 mg/ml) was added to the medium and the cells were further incubated for various times (0–8 h). Cells were harvested and washed, and the lysates were centrifuged at $12,000\times g$ for 30 min at $4^\circ C$. Total cellular proteins (10 μ g) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-Bcl-2 or monoclonal anti- β -actin antibody for 2 h at room temperature. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction. Data are expressed as means $\pm$ SEM. * $p < 0.01$ vs pcDNA3.1, $n = 3$

further examined the effect of As-Bcl2 on HSP70 expression. Western blot analyses demonstrated Bcl-2 silencing has no significant effect on expression of both inducible HSP70 and constitutive HSP70 (Hsc70, Fig. 3c).

Fig. 3 Western blot analyses showing the effect of Bcl-2 antisense oligodeoxynucleotide (As-Bcl2) on the expression of Bcl-2 proteins in C2C12 cells. **a** Inhibition of Bcl-2 protein by the antisense Bcl-2 oligodeoxynucleotide (As-Bcl2) in C2C12 cells. After incubation for 24 h, cells were harvested, lysed, and Western blotted with the corresponding antibodies indicated on the right. As-Bcl2 was able to block Bcl-2 expression by about 75% compared to the control Scr-Bcl2. The ratio of Bcl-2 band density to GAPDH control band density was calculated and analyzed statistically. * $p < 0.01$ vs Scr-Bcl2, $n = 3$. **b**

As-Bcl2 inhibited the Bcl-2 expression in HSP70-overexpressed cells (HSP70-2). C2C12 cells overexpressing HSP70 (HSP70-2) were transfected with Bcl-2 antisense oligodeoxynucleotide (As-Bcl2) or with the scrambled control (Scr-Bcl2) as described in the “Materials and methods” section. The ratio of Bcl-2 band density to GAPDH control band density was calculated and analyzed statistically. * $p < 0.01$ vs Scr-Bcl2 group, $n = 3$. **c** The effect of As-Bcl2 on HSP70 expression. Hsc constitutive HSP70

Bcl-2 antisense oligodeoxynucleotide inhibited the HSP70-mediated protection against oxidative stress-induced release of Smac

Having established that As-Bcl2 effectively inhibited the upregulation of Bcl-2 induced by HSP70, we next sought to determine the effects of Bcl-2 downregulation on HSP70-mediated inhibition of Smac release induced by oxidative stress. The C2C12 cells transfected with As-Bcl2 or control Scr-Bcl2 were cultured in the presence or absence of H_2O_2 (0.5 mM) for 2 h. As shown in Fig. 4a, under normal

conditions, most of Smac/DIABLO existed in the mitochondria and little or no Smac was located in the cytosol. Exposure to H_2O_2 resulted in the release of Smac from the mitochondria into the cytosol, and HSP70 overexpression could inhibit the release of Smac. However, in contrast to the protective effect of overexpressing HSP70 seen in the control Scr-Bcl2-treated C2C12 myogenic cells, HSP70 overexpression with no Bcl-2 upregulation was no longer able to inhibit oxidative stress-induced Smac release, as indicated by Smac in mitochondrial and cytosolic fractions by Western blot analyses ($p < 0.05$). Furthermore, we found

that Bcl-2 ablation only was not associated with the release of Smac (Fig. 4b).

The mitochondrial inner membrane protein cytochrome oxidase subunit II (COX II) was retained in the mitochondrial-rich fractions in all samples, indicating that the H₂O₂ exposure did not lead to general mitochondrial destruction.

Bcl-2-specific antisense oligodeoxynucleotide inhibited the HSP70-mediated protection against oxidative stress-induced caspase activation

Caspase activation is believed to be a key factor during apoptosis. Caspase-9 is a proenzyme in the cells and can be processed to smaller fragments during apoptosis mainly through the mitochondrial apoptosis pathway. Caspase-3, which can also be processed into smaller fragments when activated, is a biochemical hallmark of apoptosis. To

investigate the effects of Bcl-2 on HSP70-mediated protection against oxidative stress-induced activation of caspase-9 and caspase-3, HSP70 overexpressing C2C12 myogenic cells were transfected with Bcl-2 antisense, As-Bcl2, or the control Scr-Bcl2 oligodeoxynucleotides, and then exposed to H₂O₂ (0.5 mM) for 12 h. The activities of caspase-9 and caspase-3 were analyzed by an in vitro substrate-cleaving reaction. As shown in Fig. 5a, b, the HSP70-mediated protection against oxidative stress-induced activation of caspase-9 and caspase-3 was inhibited significantly in cells with As-Bcl2 compared with those with the control Scr-Bcl2.

Bcl-2-specific antisense oligodeoxynucleotide inhibited HSP70-mediated protection against oxidative stress-induced apoptosis

We next sought to determine the effects of Bcl-2 suppression on HSP70-mediated protection against apoptosis induced by H₂O₂. For these studies, Bcl-2-specific As-Bcl2 or the control Scr-Bcl2 oligodeoxynucleotides were introduced into HSP70-overexpressing C2C12 myogenic cells for 24 h before the cells were exposed to H₂O₂. As shown in Fig. 5c, d, downregulation of Bcl-2 significantly inhibited HSP70-mediated protection against oxidative stress-induced apoptosis, as indicated by cell viability ($p < 0.05$) and the percentage of apoptotic cells ($p < 0.05$). We found that, in contrast to the protective effect of HSP70 seen in the control Scr-Bcl2-treated C2C12 myogenic cells, HSP70 overexpression in Bcl-2-depleted C2C12 cells was no longer able to inhibit oxidative stress-induced apoptosis.

Fig. 4 Effects of antisense oligodeoxynucleotide against Bcl-2 on HSP70-mediated protection against oxidative stress-induced release of Smac. **a** C2C12 cells overexpressing HSP70 (HSP70-2) were transfected with Bcl-2 antisense oligodeoxynucleotide (As-Bcl2) or the scrambled control (Scr-Bcl2) as described in the “Materials and methods” section. Twenty-four hours after transfection, the cells were treated with 0.5 mM H₂O₂ for 2 h. **b** C2C12 cells were transfected with Bcl-2 antisense oligodeoxynucleotide (As-Bcl2) or the scrambled control (Scr-Bcl2). Cytosolic (Cyto) and mitochondrial (Mit) extracts were separated by 12% SDS-PAGE and analyzed by immunoblotting with anti-Smac, anti-COX II, and GAPDH antibodies. Cytochrome oxidase subunit II (COX II) was used as a marker of mitochondrial protein and GAPDH was used as a marker of cytosolic protein. Data were obtained from four independent experiments. The means of triplicate samples ± SEM are shown. * $p < 0.05$ vs pcDNA3.1+H₂O₂ group; # $p < 0.05$ vs HSP70+Scr-Bcl+H₂O₂ group

Discussion

Heat shock response induces the synthesis of diverse heat shock proteins that confer a protective effect against a wide range of cellular stresses. Induction of HSP70 was suggested to play an important role in preconditioning, a phenomenon of protection of an organ from strong ischemic insult by prior exposure to mild ischemia or other mild stresses. Although it is clear that HSP70 has a general anti-apoptotic function, the mechanism is not known. Some studies have shown that HSP70 affects processes that regulate apoptotic signaling, effector molecule activation, events downstream of caspase activation, release of cathepsins, cytochrome *c*, apoptosis-inducing factor (AIF), and even the death-associated permeabilization of lysosomes (Feng et al. 2006; Lui and Kong 2007; Bivik et al. 2007; Matsumori et al. 2005; Nylandsted et al. 2004). Mitochondria harbor three key modulators of apoptosis: cytochrome *c*, AIF, and Smac (Kroemer and Reed 2000). During stress, these mitochondrial proteins are released into

Fig. 5 Effects of antisense oligodeoxynucleotide against Bcl-2 on HSP70-mediated protection against oxidative stress-induced activation of caspase-3, caspase-9, and apoptosis. C2C12 cells overexpressing HSP70 (HSP70-2) were transfected with Bcl-2 antisense As-Bcl2 or the scrambled control Scr-Bcl2, and the cells were treated with or without 0.5 mM of H₂O₂ for 8 or 24 h. The cells were harvested, and cell lysates were assayed for protease activity of either **a** caspase-3 or

b caspase-9 by using the caspase colorimetric assay kits. **c** The viability of C2C12 cells were determined by the MTT assay. **d** Quantitation of apoptotic cells based on flow cytometry analyses. Data were obtained from four independent experiments. The means of triplicate samples $\pm$ SEM are shown. * $p < 0.05$ vs pcDNA3.1+H₂O₂ group; # $p < 0.05$ vs Scr-Bc

the cytosol where they activate the caspase cascade and subsequently cause cell death. HSP70 inhibits the release of cytochrome *c* and AIF from the mitochondria and prevents apoptosis of these cells (Didelot et al. 2006; Ravagnan et al. 2001). Our previous study found that HSP70 overexpression could inhibit H₂O₂-mediated release of Smac, activation of caspase-3 and caspase-9, and apoptosis in C2C12 myogenic cells (Jiang et al. 2009). In the present study, we found that HSP70 increased Bcl-2 expression. These data suggested that HSP70 inhibited apoptosis at multiple points and by a wide range of mechanisms.

Smac, a mitochondrial protein (Du et al. 2000), is released into the cytosol during apoptosis upon UV- or γ -irradiation, exposure to cytotoxic drugs that induce DNA damage, or ligation of the CD95 death receptor in human cancer cells. Furthermore, Smac/DIABLO is highly

expressed in myocardium. Our previous study found that heat shock pretreatment and HSP70 overexpression could inhibit the release of Smac (Jiang et al. 2005a, b, 2009). However, the molecular mechanism by which HSP70 interferes with Smac release and apoptosis induced by oxidative stress is not well understood. Bcl-2, an anti-apoptotic protein, is localized at the cellular membranes, particularly in mitochondria, where it stabilizes transmembrane potential, reduces membrane permeability, and inhibits the release of substances such as cytochrome *c*, Smac, procaspase-3, and apoptosis-inducing factors (Kim 2005). Bcl-2 is the prototype member of the Bcl-2 family of proteins and is localized to mitochondria, endoplasmic reticulum, and nuclear envelope, with a well-established role in protecting against a variety of apoptotic stimuli, mainly acting at the mitochondrial level (Scorrano and

Korsmeyer 2003). The three-dimensional structure of a related family member, Bcl-xL, as well as the subsequent functional studies, suggest that Bcl-2 may function as a pore for either ions or small molecules (Schendel et al. 1997). Thus, HSP70 may suppress Smac release and apoptosis by regulating the expression of these pro-apoptotic or anti-apoptotic Bcl-2 family proteins. Stankiewicz et al. demonstrate that HSP70 inhibits heat-induced apoptosis by preventing Bax translocation (Stankiewicz et al. 2005). Jacobs et al. found that heat shock factor 1 attenuates 4-hydroxynonenal-mediated apoptosis by upregulating HSP70 expression and stabilizing Bcl-xL (Jacobs and Marnett 2007). Viral vector-mediated HSP72 overexpression is found to be associated with increased levels of Bcl-2 protein in brain cells (Kelly et al. 2002). In the present study, we have established that there is a strong relationship between Bcl-2 and the anti-apoptotic effect of HSP70. Bcl-2 level in HSP70 transfected cells is increased more significantly than that in parental pcDNA3.1 transfected cells during H₂O₂ exposure. HSP70 transfected cells, having maintained a higher level of Bcl-2 during H₂O₂ exposure, are less susceptible to apoptotic death. Bcl-2 antisense oligonucleotide, on the other hand, enables HSP70-transfected cells to be responsive to induction release of Smac and apoptosis in C2C12 cells. These results provide evidence supporting an important role of Bcl-2 as one of the key elements in the protective role of HSP70 against Smac release and apoptosis induced by oxidative stress in C2C12 cells.

Our findings and those from others support a role of Bcl-2 as a mediator for HSP70, but how does HSP70 regulate Bcl-2 levels? HSP70, a highly conserved protein, has the function of molecular chaperones. The activities of chaperones in both housekeeping and stress response are based on their ability to interact with unfolded (nascent) and misfolded (denatured) proteins. They transiently associate with short peptide segments of the folding intermediates to prevent their aggregation by shielding exposed hydrophobic patches (Gosslau et al. 2001). Some studies found that overexpression of HSP70 in hippocampal CA1 neurons reduces evidence of protein aggregation under conditions where neuronal survival is increased. These data suggest that HSP70 may regulate Bcl-2 protein expression by reducing its protein degradation. Here, we also found that HSP70 overexpression decreased Bcl-2 protein degradation, indicating increased stability of Bcl-2 protein. If a HSP70 mutant, which is unable to assist proteins and stabilize them, has no protection against Smac release and apoptosis induced by oxidative stress, it would be helpful to the proposal that this is partly the mechanism of Bcl-2 stabilization.

We demonstrated that HSP70 increased stability of Bcl-2 protein and downregulation of Bcl-2 expression significantly inhibited the HSP70-mediated protection

against oxidative stress-induced release of Smac, activation of caspase-9 and caspase-3, and apoptosis. These data demonstrated that Bcl-2 played important roles in HSP70-mediated protection against oxidative-stress-induced release of Smac and apoptosis. Given that abnormalities in the mitochondrial pathway of caspase activation are known to occur during myocardial ischemia-reperfusion injury and cardiomyocytes possess abundant mitochondria, we suggest that the upregulation of Bcl-2 and inhibition of Smac release from mitochondria may have genuine potential in the treatment of diseases.

Acknowledgments This study was supported by the grants from National Basic Research Program of China (2007CB512007) and The National Natural Science Foundation of China (30700290).

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