EXCLI Journal 2005;4:7-24 – ISSN 1611-2156
received: March 20, 2005, accepted: May 01, 2005, published: June 20, 2005

Original article:

The effects of αB-crystallin on mitochondrial death pathway during hydrogen peroxide induced apoptosis

Shuang Liu, Gonghua Deng, Zizhi Tu, Junli Li, Meidong Liu, Xianzhong Xiao*

Department of Pathophysiology, Xiangya School of Medicine, Central South University, 110, Xiangya Road, Changsha, Hunan, China, 410078, Tel: +86 731 2355022, Fax: +86 731 2355019, email: xianzhongxiao@xysm.net (*corresponding author)

ABSTRACT

αB-crystallin, a major small heat shock protein, has recently been shown to exert inhibitory effects on apoptosis, while the responsible mechanisms remain largely unknown. In the present study, we discovered that αB-crystallin protected mouse myoblast C_2C_{12} cells against oxidative stress-induced apoptosis. During hydrogen peroxide-induced apoptosis, αB-crystallin showed that it decreased the redistribution level of phosphatidylserine (PS), reduced the release of cytochrome C and Smac/Diablo from mitochondria into cytoplasm, and decreased the cleavage of Bid. Interestingly, immunoprecipitation experiments with anti-αB-crystallin and anti-myc-tag antibodies demonstrated respectively an interaction between αB-crystallin and p53 during hydrogen peroxide induced apoptosis. Both the NH_2-terminal and COOH-terminal regions of αB-crystallin could interact with p53, suggesting two domains of αB-crystallin are necessary for the interaction. Electrophoresis mobility shift assay (EMSA) and luciferase assay further demonstrated that αB-crystallin inhibited the upregulation of the DNA-binding, as well as the transactivation activity of p53 induced by hydrogen peroxide. Our results show that αB-crystallin has a protective role in oxidative stress induced apoptosis by interference with the mitochondrial death pathway.

Keywords: αB-crystallin, p53, Cytochrome C, Smac/Diablo, Bid, mitochondria, hydrogen peroxide, apoptosis

INTRODUCTION

Apoptosis is an important cellular process in many physiological and pathologic conditions. This endogenous death event processes via two principal signaling pathways (Zhao et al. 2002; Gill, et al. 2002). One is the death receptor signaling pathway and the other is the mitochondrial death signalling pathway, in which many of the stress stimulus triggers the permeabilization of mitochondrial outer membrane and the release of proapoptotic proteins, such as cytochrome c and Smac/Diablo into the cytoplasm, resulting in the activation of Caspases cascade and the induction of apoptosis (Adrain et al. 2001; Kuwana et al, 2003). In this signalling pathway, the permeabilization of mitochondrial outer membrane (MOMP) is considered to be the key step in the early apoptotic events (Green et al. 2004), and is likely to be controlled and influenced by many factors, including the Bcl-2 family members (Kuana et al. 2003; Harada et al. 2003), transcription factor p53 and nuclear factor- kappaB (NF-κB) (Haupt

et al. 2003; Chipuk, et al. 2004; Baptiste et al. 2004; Karin et al. 2002, Zamora et al. 2004), and some heat shock proteins(HSPs) (Gill et al. 2002, Parcellier et al. 2003). So far, two groups of Bcl-2 family proteins (pro-apoptotic proteins, Bax, Bak, Bad, Bid, Bcl-Xs and anti-apoptotic proteins, Bcl-2, Bcl-X_L) are the most well characterized regulators. The balance between these two functional groups dominates the status of MOMP and the determination of apoptosis (Kuana et al. 2003; Green et al. 2004; Harada et al. 2003). p53, another regulator of apoptosis, works through either the transcription regulation of some Bcl-2 family members, or the direct interaction with these proteins after the translocation to mitochondrial from cytoplasm (Zhao et al. 2002; Haupt et al. 2003; Chipuk et al. 2004, Baptiste et al. 2004).

Recently, several heat shock proteins (HSPs) have been shown to inhibit apoptosis by selectively interfering with mitochondrial death signalling pathway in various cell types (Parcellier et al. 2003). For instance, HSP90 plays an important anti-apoptotic role via binding with Apaf-1 to prevent apoptosome formation (Pandey et al. 2000), interacting with Bid to inhibit its cleavage (Zhao C et al. 2004), and regulating the Bcl-2 expression (Dias et al. 2002). HSP70 inhibits apoptosis through its interaction with apoptosis-inducing factor (AIF) (Gurbuxani et al. 2003) and Apaf-1 (Saleh et al. 2000). HSP70 also reduces the release of cytochrome c from the mitochondria (Tsuchiya et al. 2003). HSP60 suppresses apoptosis by modulating and interacting with Bcl-2 family members (Shan et al. 2003; Gupta et al. 2002). HSP27 negatively regulates apoptosis by sequestering both procaspase 3 and cytochrome C (Bruey et al. 2000; Concannon et al. 2001). HSP27 delays the release of cytochrome c from mitochondria (Paul et al. 2002).

αB-crystallin, a major small heat shock protein, is abundantly expressed in eye lens and striated muscle tissues (heart and skeleton muscle) (Clark et al. 2000). It also shows the resistance to apoptosis induced by different stresses in cells and animals (Morrison et al. 2003, 2004; Mao et al. 2001; Andley et al. 2000, Kamradt et al. 2002), however. But the mechanisms are poorly understood. Recent studies indicate that αB-crystallin negatively regulates TNF-α and DNA damage-induced apoptosis by directly binding to p24, a partially processed caspase-3 intermediate, therefore inhibits the autoproteolytic maturation of caspase-3 (Kamradt et al. 2001). This inhibition of αB-crystallin on caspase-3 activation has been proven in several kinds of cells (Morrison et al. 2003; Kamradt et al. 2002; Alge et al. 2002; Liu et al. 2004; Kamradt et al. 2005). More recently, studies reveal that αB-crystallins protect human lens epithelial cells from Staurosporine-induced apoptosis through binding Bax and Bcl-Xs, which are two pro-apoptotic Bcl-2 family proteins which function in the upstream of mitochondrial death pathway, and by sequestering their translocation into mitochondria (Mao et al. 2004).

In the present study, we discovered that αB-crystallin protected mouse myoblast C_2C_{12} cells against oxidative stress-induced apoptosis. During hydrogen peroxide-induced apoptosis, αB-crystallin in C_2C_{12} cells reduced the redistribution level of phosphatidylserine (PS) and the release of cytochrome C and Smac/Diablo from mitochondria, and decreased the Bid cleavage. Immunoprecipitation experiments with anti-αB-crystallin and anti-myc-tag antibodies demonstrated that an interaction of αB-crystallin with p53 existed during apoptosis. Both the NH_2-terminal (Met1-Ser59) and COOH-terminal (Trp60-Lys175, "α-crystallin domain") regions of αB-crystallin, (Narberhaus et al. 2002) could interact with p53. By electrophoresis mobility shift assay (EMSA) and luciferase assay, we further demonstrated that αB-crystallin inhibited hydrogen peroxide induced the upregulation of DNA-binding and transactivation activities of p53. Our results indicated that an interaction of αB-crystallin and p53 existed, and αB-crystallin

also decreased the p53 transcriptional activity during oxidative stress induced apoptosis.

MATERIALS AND METHODS

1. Plasmid construction

Wild type αB-crystallin and its NH_2-terminal region (Met1-Ser59) and COOH-terminal region (Trp60-Lys175, containing "α-crystallin domain") were constructed into pcDNA3.1-myc-his(-)B(Invitrogen) and pEGFP-C_2(Clontech), respectively. The primers were used as following: for wild type αB-crystallin(1-175): CG GAA TTC GAC ATA GCC ATC CAC CAC CCC (forward) and GG GGT ACC CTT CTT AGG GGC TGC AGT GAC (reverse); for NH_2-terminal region(Met1-Ser59) of αB-crystallin:□GG *GAA TTC* GAC ATA GCC ATC CAC CAC CCC (forward) and GG *GGT ACC* CCA GCT AGG TGC CCG CAG GAA(reverse); for COOH-terminal region (Trp60-Lys175) of αB-crystallin:□GG *GAA TTC* ATT GAC ACT GGG CTC TCA GAG (forward) and GG *GGT ACC* CTT CTT AGG GGC TGC AGT GAC (reverse). *EcoR* I and *Kpn* I sites were created at each end of the peptide by polymerase chain reaction according to the structure of plasmids. The constructs were amplified in DH5α and purified by plasmid purification kit (Qiagen).

2. Culture of mouse myoblast C_2C_{12} cells

Mouse myoblast C_2C_{12} cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% newborn calf serum (Gibco). The medium was prepared in ion-exchanged double-distilled water supplemented with 26mmol/L $NaHCO_3$ and 100U/ml penicillin and streptomycin. The medium were sterilized by filtration through 0.22-μm filters with pH adjusted to 7.2. Cells were kept in 5% CO_2 and 95% air and at 37°C.

3. Stable transfection and analysis

pcDNA3.1-myc-his plasmids and pEGFP-C_2 plasmids expressing wild type αB-crystallin(1-175), NH_2-terminal region(Met1-Ser59) and COOH-terminal region (Trp60-Lys175) of αB-crystallin, were each stably transfected into C_2C_{12} cells using Lipofectamine2000(Invitrogen). The transfected cells were then subjected to 1,000μg/ml of G418 selection for 4-6 weeks before individual clones were obtained and identified by Western blot analysis. All these stable clones were maintained with 100μg/ml of G418.

4. Treatment of hydrogen peroxide (H_2O_2)

Cells were grown in DMEM containing 10% newborn calf serum until confluence. After 2×10^6 cells were plated into a $60cm^2$ culture bottles for 12 h, the medium were replaced with 10ml of DMEM containing 0.5mmol/L of H_2O_2 for the induction of apoptosis. In control groups, cells were treated with DMEM without H_2O_2. Finally, the cells were collected and subjected to the indicated analysis procedures.

5. Protein extraction

For total protein: Whole-cell extracts were prepared essentially as described (Li *et al*. 1998). Cells were incubated with lysis buffer (10mmol/L Tris-HCl, pH 8.0, 1mmol/L EDTA, 2% SDS, 5mmol/L DTT and 10mmol/L PMSF, supplemented with 1 tablet/10ml of protein inhibitor cocktail) for 30min on ice. After sonication, the lysates were centrifuged at 13,000rpm for 10min at 4℃; the supernatants were collected as total soluble protein for further analysis.

For mitochondria protein: Cells were harvested by centrifugation at 850 g for 2 min. Intact mitochondrial fractions were isolated using Optiprep™ mitochondria isolation kit (Pierce) according to the instruction, except that the final centrifugation was taken at 3,000g instead of 12,000g to get purer mitochondrial fraction.

6. Western Blot analysis

After protein quantification by the BCA assay Reagent (Pierce), 100μg aliquots of protein lysate were mixed with sample buffer and boiled for 5 min. The samples were transferred to a Polyvinyldine Difluoride (PVDF) membrane (Millipore) after the separation by electrophoresis on a 10% SDS-

PAGE. The membrane was incubated in blocking buffer (PBS containing 5% skim milk and 0.1% Tween20) for 2 h, followed by incubation with the primary antibody diluted in the same buffer. After washing in PBS for 5 times, the membrane was then incubated with the proper secondary antibody for 1h. All the incubations were performed at room temperature. At last, the membrane were detected by supersignal chemiluminescence system (Pierce) followed by exposure to an auto-radiographic film.

7. Immunoprecipitation

After apoptosis induction, cells were lysed in ELB buffer (140 mmmol/L NaCl, 0.5% NP40, 50 mmol/L Tris-HCl, pH8.0, plus protease and phosphatase inhibitors) at a final concentration of 1×10^6 cells/ml, and incubated on ice for 30min. Lysates were then centrifuged at 12,000rpn at 4℃ for 15min. The supernatant was taken and quantified with BCA assay. Protein G-Sepharose beads (Amersham Biosciences) together with extracts were precleared in 1 ml of lysis buffer at 4℃ for 30 min. After precleared, the supernatant was incubated with 5 μl of rabbit anti-αB-crystallin antibody or rabbit IgG for 1 h at 4℃. After incubation, 50 μl of protein G-sepharose was added into each incubated sample. These samples were then incubated at 4℃overnight while gently shaking. On the following day, beads were washed 5 times with lysis buffer plus inhibitors and boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer for loading onto a gel of 12% SDS-PAGE for Western Blotting assay.

8. Apoptosis Assay and Annexin V-EGFP assays by flow cytometry

For analysis of apoptosis attached and floating cells were harvested, rinsed with PBS by centrifugation, resuspended in 10 mmol/L HEPES pH 7.4, 140 mmol/L NaCl, 5 mmol/L $CaCl_2$, and fixed in 70% ethanol. Cells were stained with propidium iodide (100μg/ml; Molecular Probes) to label dead cells and annexinV- EGFP (BD) to label apoptotic cells before sorting on a FACS flow cytometer (Becton-Dickinson). Apoptosis was determined as the number of cell staining positive for annexinV.

9. Electophoresis mobility shift assay (EMSA)

For EMSA (LightShift TM Chemi-luminescent EMSA Kit, Pierce), 5μg of nuclear extract was incubated with 2mmol/L of the end-labeled with biotin double-stranded oligonucleotide probes in reaction buffer for 20min at room temperature. For supershift reactions, 1μl of p53 antibody was titration into the EMSA reaction mixture to the final concentration of 1mM. Samples were resolved on a nondenaturing 4% polyacrylamide –2% glycerol gel, transferred to Biodyne@ B Nylon membrane, avidin-HRP to probes, and visualized and quantitated with a phosphorImager. The double-stranded probes were synthesized as follows: 5'biotin-TCC TAC AGA ACA TGT CTA AGC ATG CTG GGG ACT G-3'.

10. Luciferase assay

Cell were co-transfected with p53 luciferase reporter DNA andβ-galactosidase reporter plasmids by the Lipofectamine 2000 (Invitrogen). The p53-pGL3 luciferase reporter plasmid, which contained the firefly luciferase gene under the control of p53-reponsive element, was a generous gift of Prof. Yuan ZM (Department of Cancer Cell Biology, Harvard University School of Public Health, USA). After harvest, cells were lysed in Repoter Lysis Buffer (Promega, Madison, WI) after the transfection for 24 h, and assayed immediately, 20μl of cell extracts plus 100μl luciferase assay mix were mixed and assayed for 10s in Lumi-Scint. All luciferase activities were normalized for protein concentration and transfection efficiency using BCA and RSV-β-galactosidase assay respectively. All experiments were performed in triplicate and repeated at least three times.

RESULTS

1. Overexpression of αB-crystallin in C_2C_{12} cells.

To study the mechanisms by which αB-crystallin protects cells from hydrogen peroxide-induced apoptosis, we constructed a fusion protein of αB-crystallin with an epitope tag of Myc-His_6 using the pcDNA3.1-myc-his(-)B vector. The expression construct (pcDNA 3.1-αB) and the vector construct (pcDNA 3.1) were both stably transfected into mouse myoblast C_2C_{12} cells by Lipofectamine 2000. The cells stably expressing high levels of αB-crystallin (pcDNA 3.1-αB- C_2C_{12}) were obtained after the 4-6weeks selection period by G418 (1,000μg/ml). As shown in Figure 1, the fusion protein was highly expressed in C_2C_{12} cells (Clone1 and 2) determined by Western blot analysis using anti-αB-crystallin and anti-His_6 tag antibodies respectively (Figure 1).

2. αB-crystallin prevented hydrogen peroxide-induced phosphotidylserin externalization

αB-crystallin has been shown to prevent apoptosis induced by various agents including staurosporine, etoposide, hydrogen peroxide, TNF, UVA and calcimycin (Kamradt et al. 2005). Recent studies demonstrate that αB-crystallin could bind to caspase-3 intermediate and suppress its activation (Kamradt et al. 2001). However, the roles of αB-crystallin in the early apoptosis stage remains to be unknown. Redistribution of phosphotidylserine(PS) from the inner leaflet to the outer leaflet of the plasma membrane occurs early and is a hallmark of the apoptotic process. Annexin V, a high affinity to PS, has been used to detect early apoptotic cells (van Engeland et al. 1998; Green et al. 2002). Here we used an EGFP labeled Annexin V and flow cytometry to detect the degree of PS Redistribution. As shown in Figure 2, after H_2O_2 (0.5 mmol/L) treatment for 3 h, the ratio of PS positive cells in vector group (pcDNA 3.1) significantly increased (from 0.33% to 6.78%), while in αB-crystallin cells (pcDNA 3.1-αB), the ratio just slightly increased (from 0.23% to 0.96%), suggesting αB-crystallin could obviously prevent hydrogen peroxide induced apoptosis in the early stage.

3. αB-crystallin reduced the release of cytochrome C and Smac/Diablo after the treatment of hydrogen peroxide

The mitochondrial death pathway is a principal mechanism of apoptosis, which the release of cytochrome C and Smac/Diablo from mitochondria into the cytoplasm is a crucial event of early apoptosis (Zhao et al. 2002; Adrain et al. 2001). So, we observed the effect of αB-crystallin on the release of cytochrome C and Smac/Diablo from mitochondria induced by hydrogen peroxide. As data shown in figure 3, the levels of cytochrome C and Smac/Diablo in the cytosol of αB-crystallin transfected and vector transfected cells increased after H_2O_2 (0.5mmol/L) treatment for 1 h, but the release of cytochrome C and Smac/Diablo to the cytoplasm in αB-crystallin transfected cells reduced significantly as compared with the vector transfected cells. The results indicated that αB-crystallin could decrease the release of cytochrome C and Smac/Diablo from mitochondria during hydrogen peroxide induced apoptosis.

4. αB-crystallin decreased hydrogen peroxide-induced Bid cleavage

The Bcl-2 family proteins regulate mitochondrial apoptosis and consist of both anti-apoptotic and pro-apoptotic members. Bid belongs to the BH3-only subgroup of the pro-apoptotic molecules and serves as a sentinel to diverse apoptotic signals (Harada et al. 2003; Esposti et al. 2002; Goonesinghe et al. 2004; Choi et al. 2004; Wang et al. 2004). The full length of Bid becomes the activation form after proteolytic cleavage, especially by apical caspases like caspase 8. Once activated, the truncated Bid (tBid) induces the release of apoptotic factors including cytochrome C and Smac/Diablo after the translocation from cytoplasm into the mitochondria (Esposti et al. 2002;

Goonesinghe et al. 200). Thus Bid activation (tBid formation) is an important event in the upstream of mitochondrial death pathway.

To further determine whether αB-crystallin could protect cells from oxidative stress-induced apoptosis at an earlier stage of apoptosis, we examined Bid activation in αB-crystallin transfected cells and control cells after the treatment of H_2O_2 (0.5mmol/L) for 15, 30, 60 and 180 min. Data in figure 4 demonstrated that hydrogen peroxide promoted tBid formation in both vector transfected cells and αB-crystallin transfected cells, but tBid formation was obviously attenuated in αB-crystallin transfected cells as compared with the vector cells, indicating a protection role of αB-crystallin at the upstream step of mitochondrial signalling pathway.

5. Interaction of p53 and αB-crystallin increased during hydrogen peroxide induced apoptosis

αB-crystallins bind to proapoptotic proteins including p24 intermediate of caspase-3, Bax and Bcl-X_s and prevent their autoproteolytic maturation or translocation during apoptosis in tumor, lens, myofibril cells (Kamradt et al. 2001, Mao et al. 2004). In the present study, we detected the possible interactions between αB-crystallin and some other proteins related to the mitochondrial signalling pathway by immunoprecipitation experiments. In C_2C_{12} cells, data showed that αB-crystallin bound to p53 in the normal conditions. After the treatment of hydrogen peroxide for 1 h, the interaction of p53 and αB-crystallin increased significantly (Figure 5-1). We further confirmed the interaction in αB-crystallin transfected C_2C_{12} cells using anti-myc-tag antibody, we found that the interaction of p53 and αB-crystallin also increased after the treatment of hydrogen peroxide in figure 5-2.

To confirm that which domain of αB-crystallin is responsible for the interaction of p53, we constructed two plasmids expressing NH2-terminal domain of αB-crystallin (1-59 amino acid) and COOH-terminal domain of αB-crystallin (60-175 amino acid, including "α-crystallin" domain of all sHSP family members) respectively and stably transfected in C_2C_{12} cells (figure 5-3). Then we detected the interaction domain of αB-crystallin with p53. Data in figure 5-4 showed that p53 could bind to both domains of αB-crystallin and the interaction of NH2-αB-crystallin with p53 was much stronger than the interaction of COOH-αB-crystallin with p53.

We also observed the distribution of GFP fusion protein of wild type αB-crystallin and its two domains in transfected C_2C_{12} cells. Data in figure 5-5 showed that αB-crystallin and its 60-175 amino acids domain were located in the cytoplasm of C_2C_{12} cells, but the 1-59 amino acids domain of αB-crystallin was distributed in the cytoplasm and nucleus.

6. αB-crystallin inhibited hydrogen peroxide induced p53 DNA-binding and trans-activation activity

Since p53 exerts its proapoptotic function mainly through the regulation of many apoptosis related the transcription of genes, such as Bax, Bid, PUMA, Noxa and apaf-1 (Haupt et al. 2003), we further detected whether αB-crystallin could affect the DNA binding and transactivation activities of p53 induced by hydrogen peroxide using EMSA and luciferase reportor gene analysis respectively. Data in figure 6-1 showed that hydrogen peroxide increased the DNA binding activity of p53 while αB-crystallin decreased the DNA binding activity after the treatment of hydrogen peroxide. Also, data in figure 6-2 demonstrated that hydrogen peroxide increased the transactivation activity of p53 significantly, while αB-crystallin decreased the transactivation activity of p53. The findings indicated an inhibition role of αB-crystallin on the nuclear function of p53 including DNA binding and transactivation activities during hydrogen peroxide induced apoptosis.

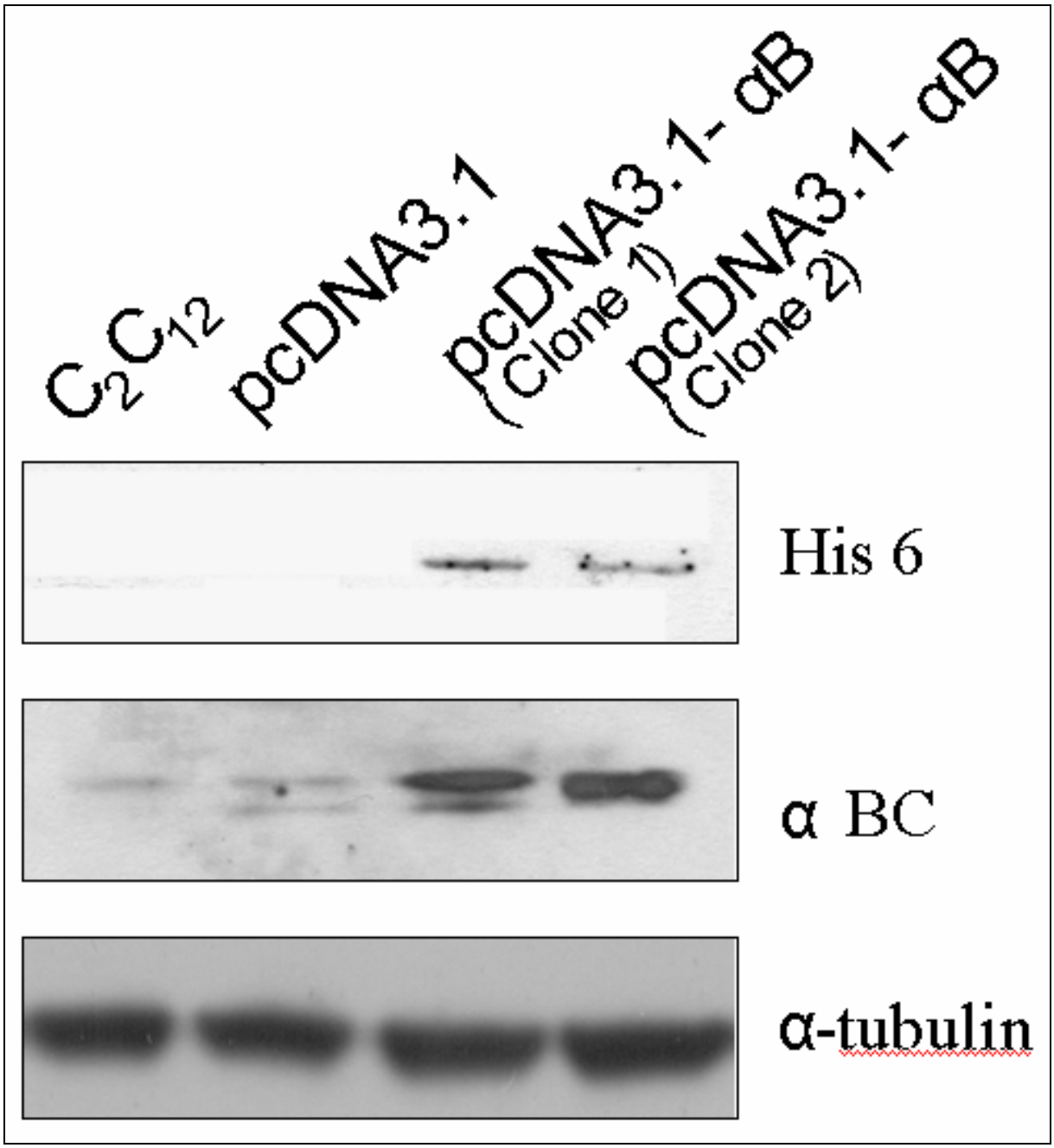

Figure 1: Overexpression of αB-crystallin in mouse myoblast C_2C_{12} cells
Total proteins (100µg) from parental C_2C_{12}, pcDNA3.1-αB transfected C_2C_{12} (clone1,2) and vector transfected C_2C_{12} cells were separated by 10% SDS- PAGE and Western blot analysis using antibodies to penta His (top panel), αB-crystallin (middle panel) and α-tubulin (bottom panel) as control.Data showed that αB-crystallin was significantly increased in αB-crystallin transfected C_2C_{12} cells.

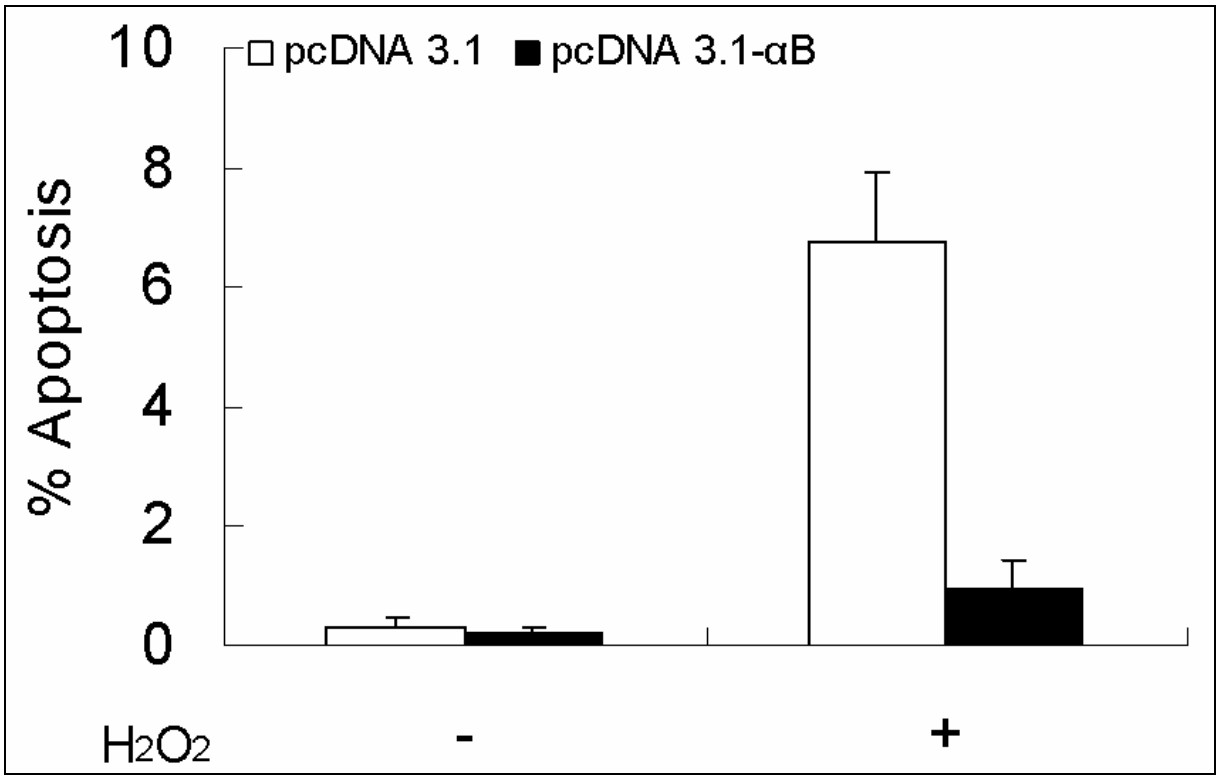

Figure 2: αB-crystallin prevented hydrogen peroxide-induced early apoptosis
2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C_2C_{12} cells were treated with H_2O_2 (0.5mmol/L) for 3 h. Cells were collected and labelled with annexin V-EGFP for indicating phosphatidylserine redistribution. The percent of annexin V positive cells was measured by flow cytometry.

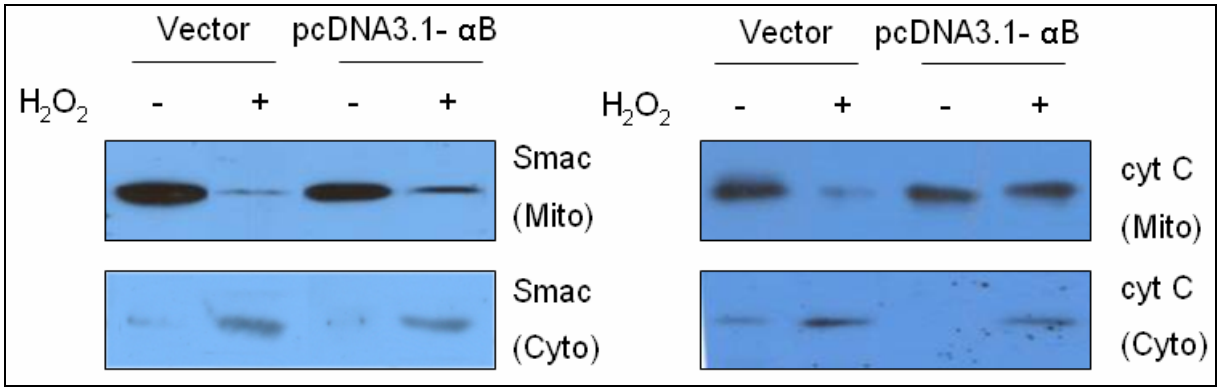

Figure 3: αB-crystallin reduced the release of cytochrome c and Smac/Diablo from mitochondria after treatment with hydrogen peroxide
2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C_2C_{12} cells were treated with 10 ml DMEM containing H_2O_2 (0.5mmol/L) for 1 h. Then the mitochondrial (Mito) and cytosolic (Cyto) fractions were isolated. The distributions of cytochrome c (cyt c) and Smac/Diablo(Smac) were determined by Western blot analysis using the corresponding antibodies.

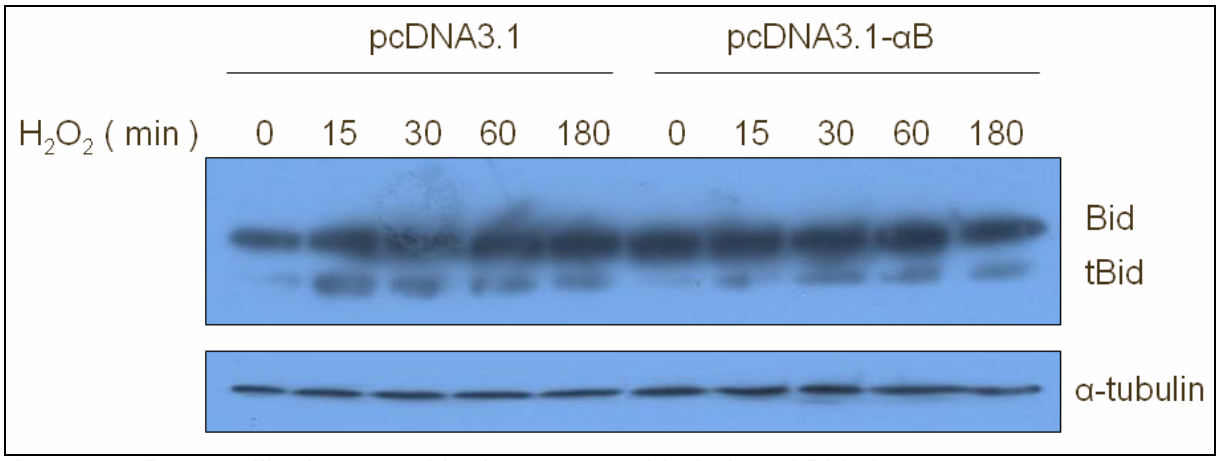

Figure 4: αB-crystallin decreased hydrogen peroxide-induced Bid cleavage
2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C_2C_{12} cells were treated with H_2O_2 (0.5 mmol/L) for 0, 15, 30, 60 and 180 min, respectively. Total proteins were extracted and detected for Bid and tBid by Western blot analysis using the corresponding antibody.

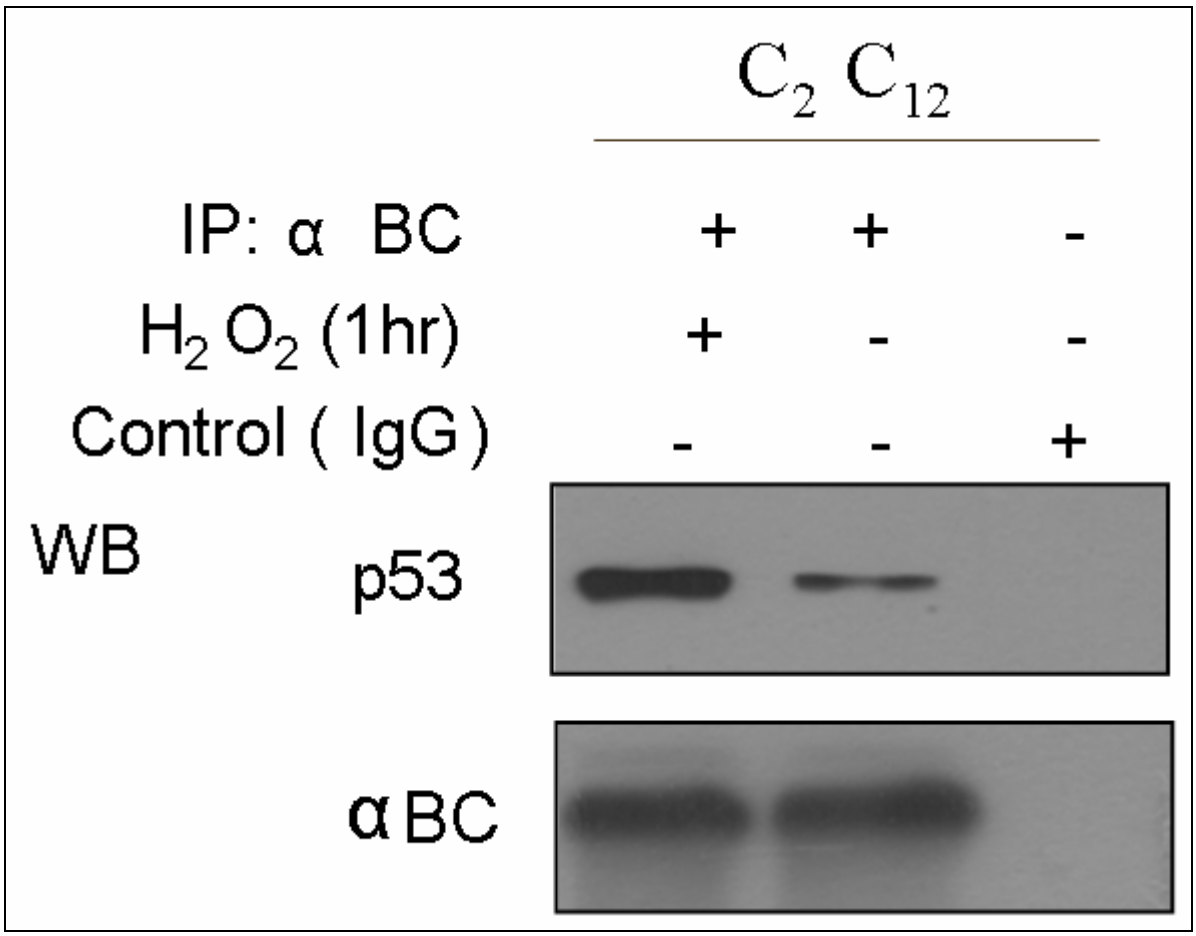

Figure 5-1: αB-crystallin bound to p53 during hydrogen peroxide induced apoptosis
C_2C_{12} cells were treated with H_2O_2 (0.5 mmol/L) for 1h. Total proteins were extracted under natue conditions for immunoprecipitation with antibody against to αB-crystallin. The precipitated samples were then sequentially blotted with anti-p53 antibody.

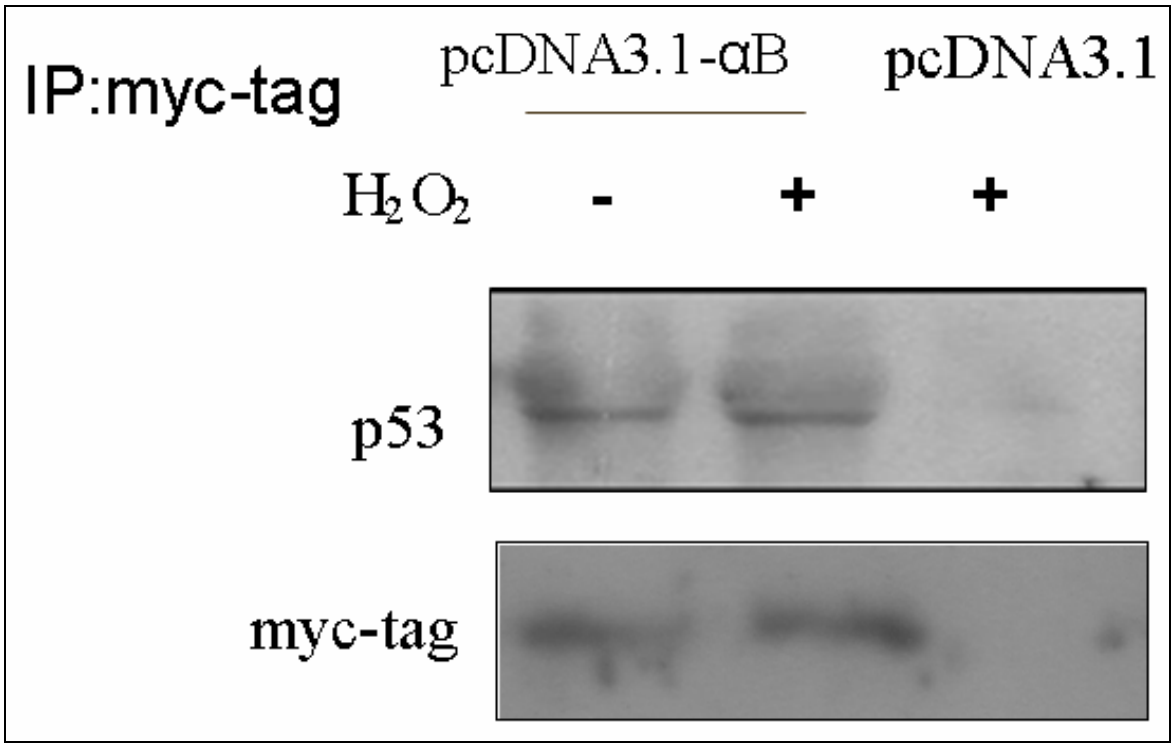

Figure 5-2: αB-crystallin (pcDNA3.1-αB) and vector (pcDNA3.1) transfected C_2C_{12} cells were treated with H_2O_2 (0.5mmol/L) for 1h. Total proteins were extracted under nature conditions for immunoprecipitation with antibody to myc-tag. The precipitated samples were then sequentially blotted with anti-p53 antibody.

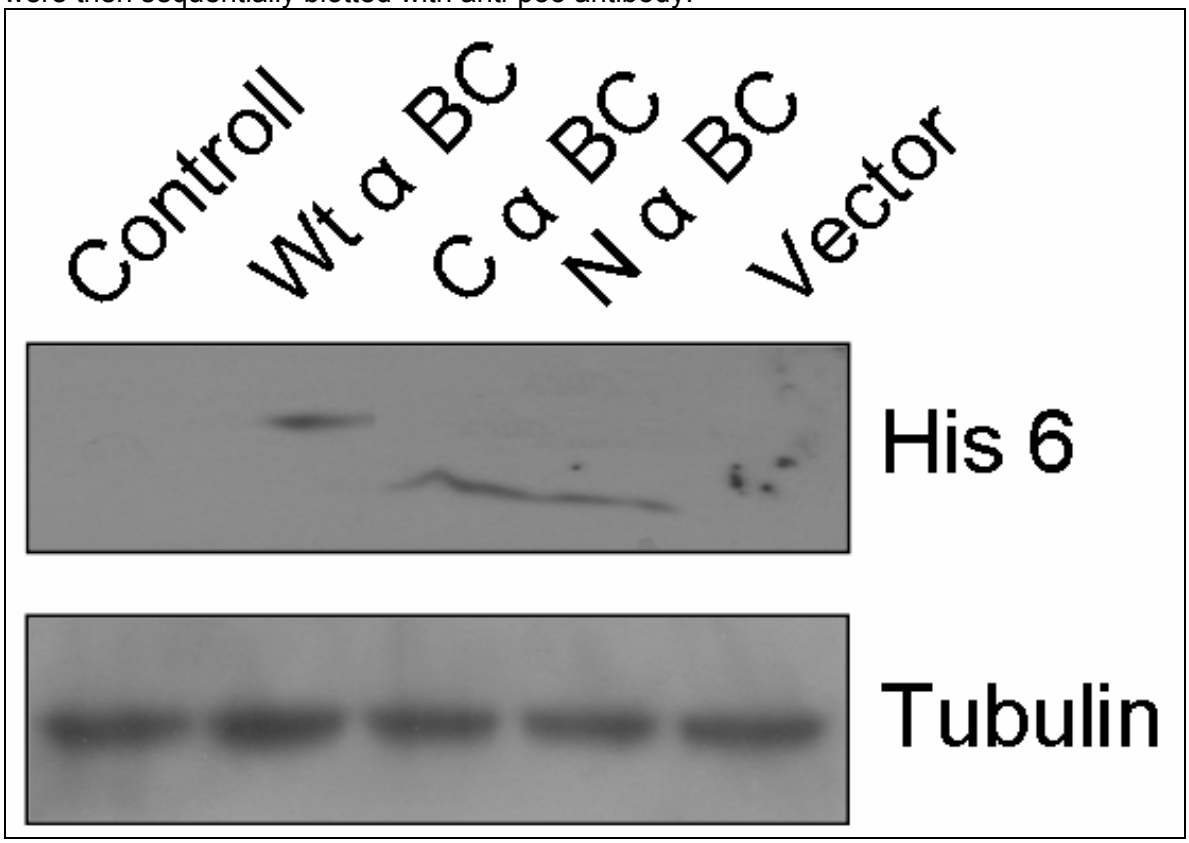

Figure 5-3: αB-crystallin mutants were stably transfected into C_2C_{12} cells. Total proteins 100μg were separated by 12% SDS- PAGE and Western blot analysis was performed using an antibody against penta His. Data showed that the1-59 amino acid domain (NH2) and the 60-175 amino acid domain (COOH) of αB-crystallin have been successfully transfected into C_2C_{12} cells.

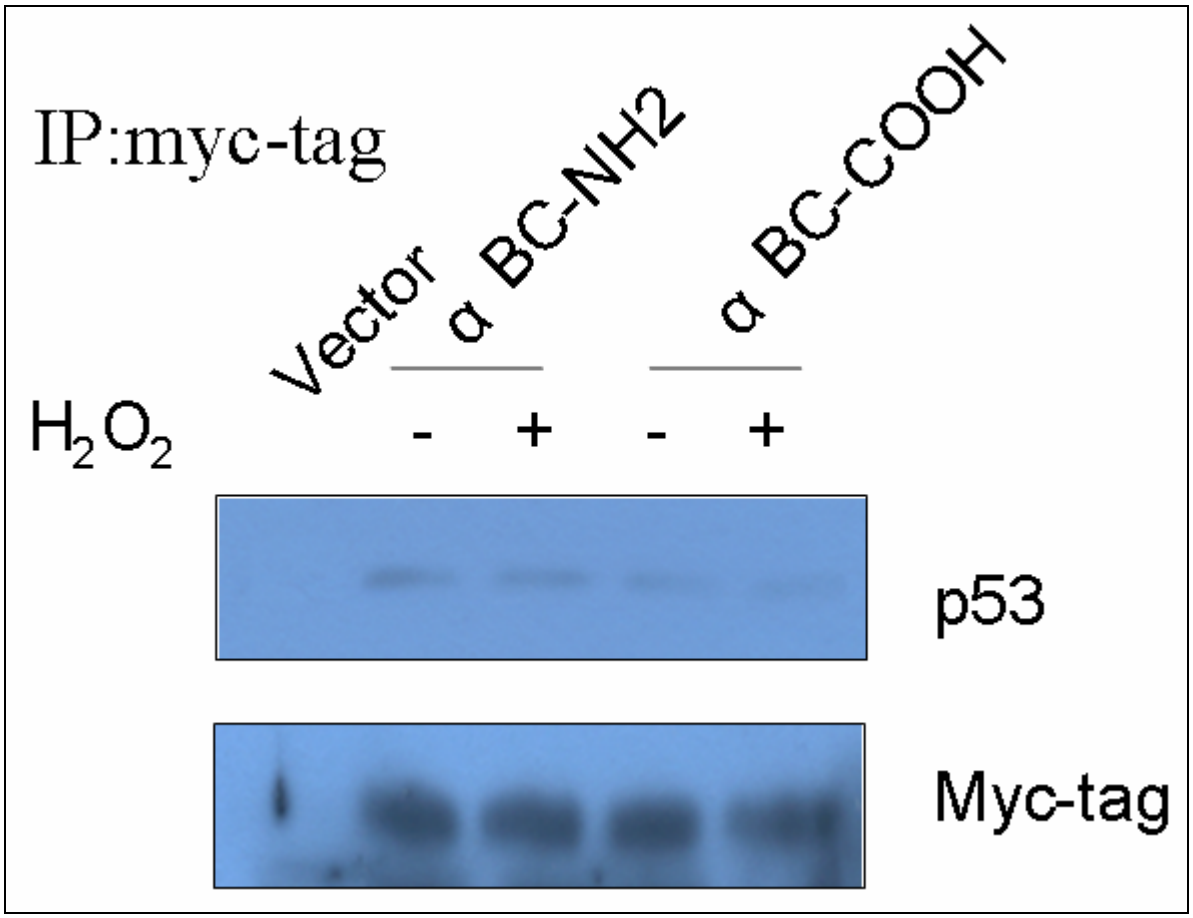

Figure 5-4: Mutants of αB-crystallin transfected C_2C_{12} cells were treated with H_2O_2 (0.5 mmol/L) for 1h. Total proteins were extracted under nature conditions for immunoprecipitation with antibody against to myc-tag. The precipitated samples were then sequentially blotted with anti-p53 antibody.

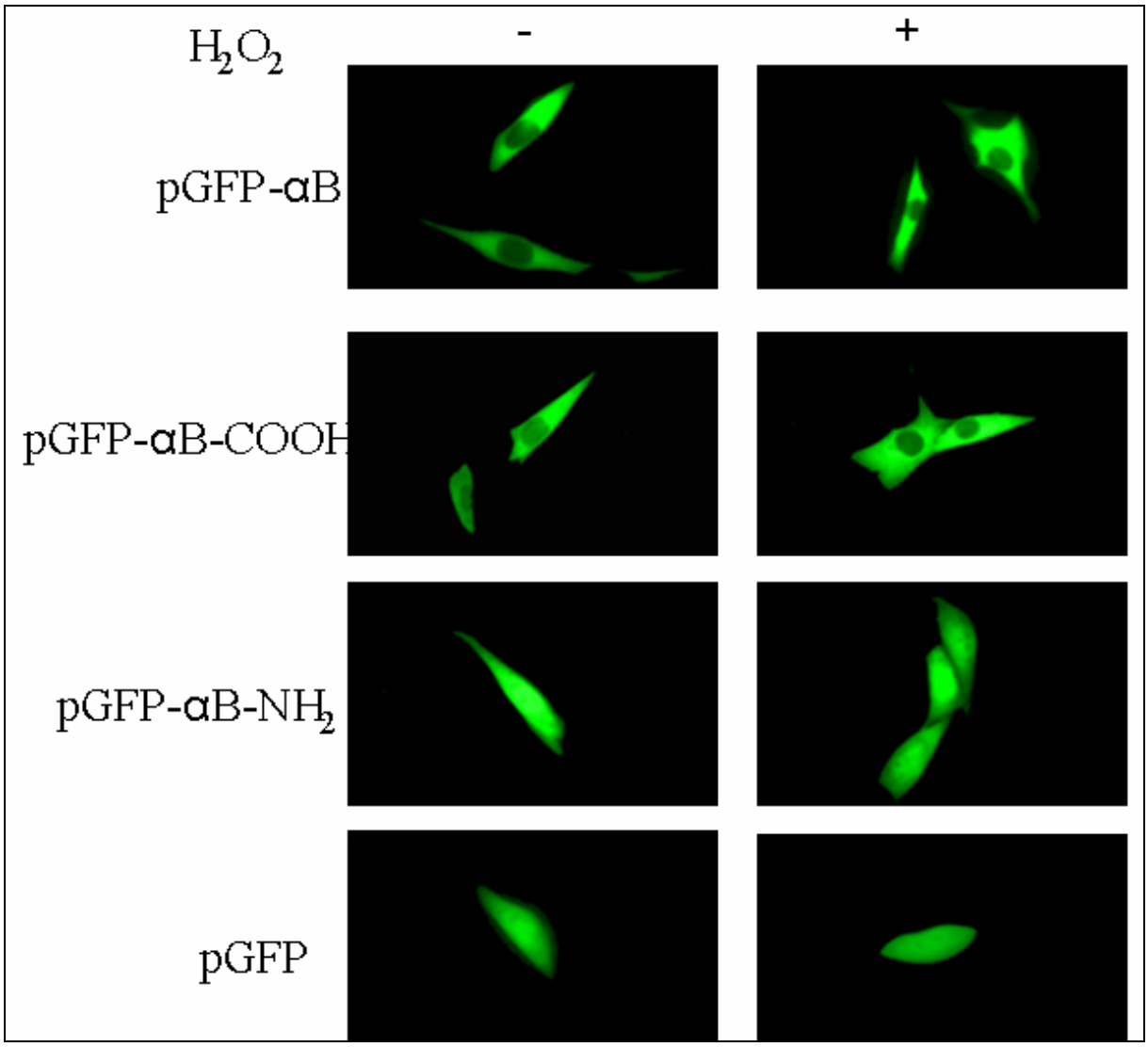

Figure 5-5: Mutants of αB-crystallin fusion to GFP protein transfected C_2C_{12} cells were treated with H_2O_2 (0.5 mmol/L) for 1h. The cells were directly visualized with a fluorescent microscope.

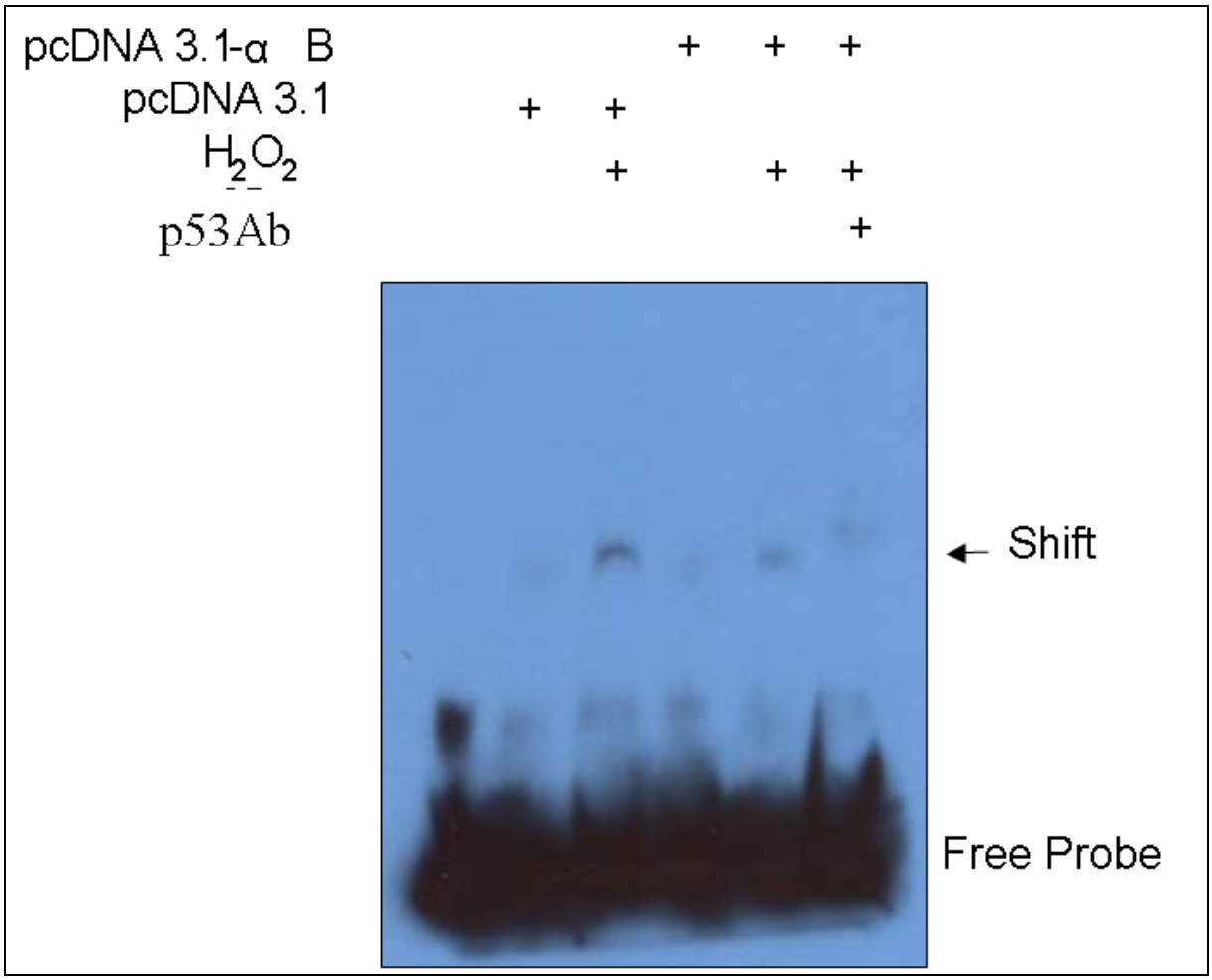

Figure 6-1: αB-crystallin inhibited hydrogen peroxide induced p53 DNA-binding and transactivation activity

αB-crystallin (pcDNA3.1-αB) and vector (pcDNA3.1) transfected C_2C_{12} cells were treated with H_2O_2 (0.5 mmol/L) for 1 h. The binding to biotin-labelled of p53 from the nuclear extracts from the cells was examined by EMSA and supershift in the absence or presence of p53 antibody (DO-1).

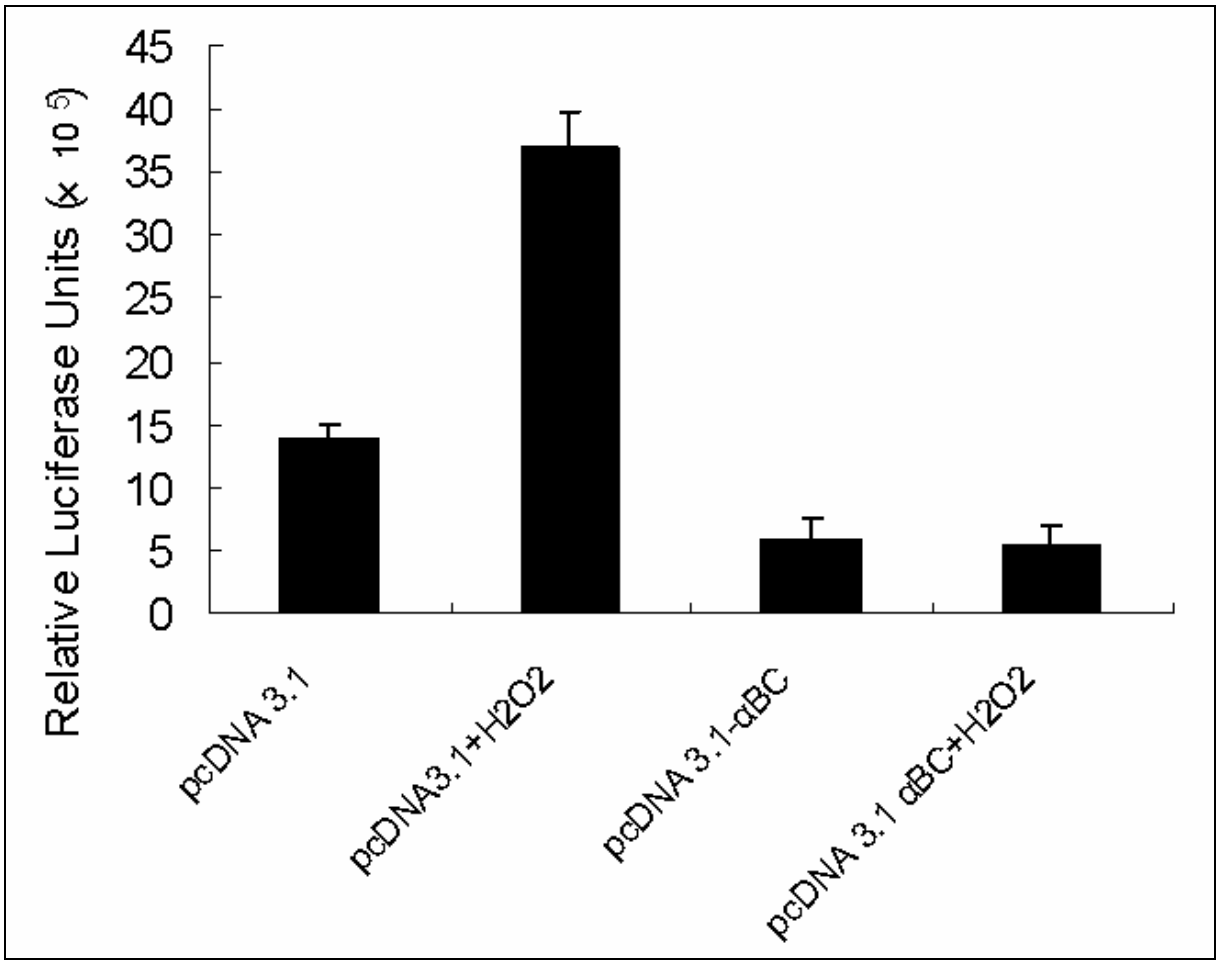

Figure 6-2: αB-crystallin (pcDNA3.1-αB) and vector (pcDNA3.1) transfected C_2C_{12} cells were transiently transfected with constructs driving a p53-reponsive element reporter gene for 24 h, and then the cells were treated with H_2O_2 (0.5mmol/L) for 1 h. Luciferase normalized toβ-galactosidase activity is presented.

DISCUSSION

αB-crystallin, a major small heat shock protein, has recently been found to exert multiple functions (Koh et al. 2004; Kumarapeli et al. 2004; van Rijk et al. 2003; den Engelsman et al. 2003; Gangalum et al. 2004). One of the most important functions is to protect cells from apoptosis induced by a large numbers of stress factors, including oxidative stress. The mitochondrial death pathway of apoptosis is a crucial mechanism in many types of cells including myocardial and myofibril cells (Zhao et al. 2002, Gill et al 2002), while lately quite a number of heat shock proteins protects cells from apoptosis through disruption of this intrinsic apoptotic pathway. αB-crystallin could negatively regulate apoptosis by a distinct mechanism in several kinds of cells, in which it interacts with partially processed procaspase-3 to prevent its activation (Kamradt et al 2001). αB-crystallin also prevents staurosporine-induced apoptosis through interactions with pro-apoptotic Bcl-2 family member protein Bax and Bcl-Xs to sequester their translocation to mitochondria (Mao et al. 2004). Since most heat shock proteins exert apoptosis-regulatory functions in multiple aspects and levels (Parcellier A et al. 2003; Pandey P et al. 2000; Zhao C, et al. 2004; Dias S, et al. 2002; Gurbuxani et al. 2003 Saleh A et al. 2000; Tsuchiya et al. 2003 ; Shan et al. 2003;Gupta et al. 2002; Bruey et al. 2000; Concannon et al. 2001; Paul et al. 2002), it is not yet clarified whether αB-crystallin could protect myoblast C_2C_{12} cells against oxidative-induced apoptosis is not yet clarified.

In the present study, we explored the effects of αB-crystallin on several early stages of mitochondrial death pathways during hydrogen peroxide-induced apoptosis in C_2C_{12} cells. Our findings demonstrated that αB-crystallin effectively inhibited hydrogen peroxide induced apoptosis at early stage indicated by phosphatidylserine(PS) externalization analysis. Also, αB-crystallin reduced the degree of cytochrome C and Smac/Diablo release from mitochondria and attenuated Bid cleavage. These results suggested that αB-crystallin regulated apoptosis through interference in the upstream events of mitochondrial signaling pathway.

Oxidative stress takes place under various conditions in striated muscle tissues (myocardium and skeleton muscles) (Zhao et al. 2002; Kumar et al. 2003), in which αB-crystallin is abundantly expressed. It is obvious that reactive oxygen species (ROS) could activate mitochondrial death signalling pathway and mediate cell apoptosis (Kumar et al. 2003; Zhao et al. 2004). ROS also increases the accumulation of p53 and promotes its transcriptional (Zhao et al. 2002; Haupt et al. 2003). In the present study, we found that αB-crystallin bound to p53 under oxidative stress and inhibited hydrogen peroxide induced DNA-binding and transactivation activities of p53. Both the NH_2-terminal (1-59) and COOH-terminal (60-175) domain of αB-crystallin could bind to p53, indicating that there might be at least two binding sites in αB-crystallin responsible for the interaction. The wild type αB-crystallin and its COOH-terminal domain (60-175) distributed mainly in the cytoplasm of C_2C_{12} cells under oxidative stress, suggesting the interaction of αB-crystallin with p53 occurred in cytoplasm. The interaction of αB-crystallin with p53 might arrest the import of p53 into the nucleus and thus decreased its function.

In myocardial and skeletal muscle tissues, αB-crystallin immediately translocates to the skeleton fractions and structural proteins such as actin, titin and desmin upon oxidative stress (Golenhofen et al. 1999a, 1999b; Clark et al. 2000). The co-localization of αB-crystallin with skeleton proteins might offer a kind of support to the skeleton structure of cells, thus helps them to resist disconstruction during apoptosis. Also, cell skeletons were considered as the channels of signalling molecules transmission and the depository site of them, thus the immediately translocation of αB-crystallin might also afford contact to some functional signalling molecules related to apoptosis. As several structural proteins have been prooven to be targets of αB-crystallin *in vitro* and *in vivo* (Golenhofen et al. 2002; Wang et al. 2003; Arai et al. 1997; Wieske et al. 2001), some signalling molecules including Bax also could also be targeted by αB-crystallin during apoptosis (Kamradt et al. 2001, Mao et al. 2003).

In conclusion, we studied the effects of αB-crystallin on several early stages of apoptosis induced by oxidative stress in C_2C_{12} cells. We revealed the binding of αB-crystallin to p53 and its inhibition of the nuclear function of p53 during apoptosis. The findings indicate that αB-crystallin protects against apoptosis induced by oxidative stress.

ACKNOWLEDGEMENTS

The project was supported by: National Basic Research Program of China (G2000056908), National Nature Science Foundation of China (30270533, 30330280), and Specialized Research Fund for the Doctoral Program of Higher Education of China (20020533032). We are grateful to Dr. Yongguang Tao, Cancer Research Institute, Xiangya School of Medicine, Central South University, China, for the generous helps of the projects and manuscript.

REFERENCES

Adrain C, Martin S,J. The mitochondrial apopytosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci* 2001;26:390-6

Alge CS, Priglinger SG, Neubauer AS, Kampik A, Zillig M, Bloemendal H, Welge-Lussen U. Retinal pigment epithelium is protected against apoptosis by alphaB-crystallin. *Invest Ophthalmol Vis Sci* 2002;43:3575-82

Andley UP, Song Z, Wawrousek EF, Fleming TP, Bassnett S. Differential protective activity of alpha A- and alphaB-crystallin in lens epithelial cells. *J Biol Chem* 2000;275:36823-31

Arai H, Atomi Y. Chaperone activity of alpha B-crystallin suppresses tubulin aggregation through complex formation. *Cell Struct Funct* 1997;22:539-44

Baptiste N, Prives C. p53 in the cytoplasm: a question of overkill? *Cell* 2004;116:487-9

Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, Garrido C. Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol* 2000; 2:645-52

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 2004;303:1010-4

Choi WS, Eom DS, Han BS, Kim WK, Han BH, Choi EJ, Oh TH, Markelonis GJ, Cho JW, Oh YJ. Phosphorylation of p38 MAPK induced by oxidative stress is linked to activation of both caspase-8- and -9-mediated apoptotic pathways in dopaminergic neurons. *J Biol Chem* 2004;279:20451-60

Clark JI, Muchowski PJ. Small heat-shock proteins and their potential role in human disease. *Curr Opin Struct Bio* 2000;10:52-9

Concannon CG, Orrenius S, Samali A. Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. *Gene Expr* 2001;9:195-201

den Engelsman J, Keijsers V, de Jong WW, Boelens WC. The small heat-shock protein alpha B-crystallin promotes FBX4-dependent ubiquitination. *J Biol Chem* 2003;278:4699-704

Dias S, Shmelkov SV, Lam G, Rafii S. VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood* 2002;99:2532-40

Esposti MD. The roles of Bid. *Apoptosis* 2002;7:433-40

Gangalum RK, Schibler MJ, Bhat SP. Small heat shock protein alphaB-crystallin is part of cell cycle-dependent Golgi reorganization. *J Biol Chem* 2004;279:43374-7

Golenhofen N, Arbeiter A, Koob R, Drenckhahn D. Ischemia-induced association of the stress protein alpha B-crystallin with I-band portion of cardiac titin. *J Mol Cell Cardiol* 2002;34:309-19

Golenhofen N, Htun P, Ness W, Koob R, Schaper W, Drenckhahn D. Binding of the stress protein alpha B-crystallin to cardiac myofibrils correlates with the degree of myocardial damage during ischemia/reperfusion in vivo. *J Mol Cell Cardiol* 1999;31:569-80

Golenhofen N, Arbeiter A, Koob R, Drenckhahn D. Ischemia-induced association of the stress protein alpha B-crystallin with I-band portion of cardiac titin. *J Mol Cell Cardiol* 2002;34:309-19

Gill C, Mestril R, Samali A. Losing heart: the role of apoptosis in heart disease. a novel therapeutic target? *FASEB J*,2002,16:135-46

Goonesinghe A, Mundy ES, Smith M, Khosravi-Far R, Martinou JC, Degli Esposti M. Pro-apoptotic Bid induces membrane perturbation by inserting selected lysolipids into the bilayer. *Biochem J* 2004;387(Pt 1):109-18

Green AM, Steinmetz ND. Monitoring apoptosis in real time. *Cancer J*. 2002;8:82-92

Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science*. 2004;305:626-9

Gurbuxani S, Schmitt E, Cande C, Parcellier A, Hammann A, Daugas E, Kouranti I, Spahr C, Pance A, Kroemer G, Garrido C. Heat shock protein 70 binding inhibits the nuclear import of apoptosis-inducing factor. *Oncogene* 2003;22:6669-78

Gupta S, Knowlton AA. Cytosolic heat shock protein 60, hypoxia, and apoptosis. *Circulation* 2002;106:2727-33

Harada H, Grant S. Apoptosis regulators. *Rev Clin Exp Hematol* 2003; 7: 117-38

Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis_the p53 network. *J Cell Sci* 2003;116:4077-85

Kamradt MC, Chen F, Sam S, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation. *J Biol Chem* 2002;277:38731-6

Kamradt MC, Chen F, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem* 2001;276:16059-63

Kamradt MC, Lu M, Werner ME, Kwan T, Chen F, Strohecker A, Oshita S, Wilkinson JC, Yu C, Oliver PG, Duckett CS, Buchsbaum DJ, Lobuglio AF, Jordan VC, Cryns VL. The small heat shock protein alpha B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. *J Biol Chem* 2005;280:11059-66

Karin M, Lin A.NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002;3:221-7

Koh TJ, Escobedo J. Cytoskeletal disruption and small heat shock protein translocation immediately after lengthening contractions. *Am J Physiol Cell Physiol* 2004;286:C713-22

Kumar D, Jugdutt BI. Apoptosis and oxidants in the heart. *J Lab Clin Med* 2003;142:288-97

Kumarapeli AR, Wang X. Genetic modification of the heart: chaperones and the cytoskeleton. *J Mol Cell Cardiol* 2004;37:1097-109

Kuwana T, Newmeyer DD.Bcl-2-family proteins and the role of mitochondria in apoptosis. *Curr Opin Cell Biol* 2003;6:691-9

Li YP, Schwartz RJ, Waddell ID, Holloway BR, Reid MB. Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-kappaB activation in response to tumor necrosis factor alpha. *FASEB J* 1998;12:871-80

Liu B, Bhat M, Nagaraj RH. AlphaB-crystallin inhibits glucose-induced apoptosis in vascular endothelial cells. *Biochem Biophys Res Commun* 2004;321:254-8

Mao YW, Liu JP, xiang H, Li DW. Human aA- and aB-crystallins bind to Bax and Bcl-Xs to sequester their translocation during staurosporine-induced apoptosis. *Cell Death Differ* 2004;11:512-26

Mao YW, Xiang H, Wang J, Korsmeyer S, Reddan J, Li DW. Human bcl-2 gene attenuates the ability of rabbit lens epithelial cells against H2O2-induced apoptosis through down-regulation of the alpha B-crystallin gene. *J Biol Chem* 2001;276: 3435-45

Morrison LE, Hoover HE, Thuerauf DJ, Glembotski CC. Mimicking phosphorylation of alphaB-crystallin on serine-59 is necessary and sufficient to provide maximal protection of cardiac myocytes from apoptosis. *Circ Res* 2003;92:203-11

Morrison LE, Whittaker RJ, Klepper RE, Wawrousek EF, Glembotski CC. Roles for alphaB-crystallin and HSPB2 in protecting the myocardium from ischemia-reperfusion-induced damage in a KO mouse model. *Am J Physiol Heart Circ Physiol*. 2004;286:H847-55

Narberhaus F. Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. Microbiol Mol Biol Rev 2002;66:64-93

Pandey P, Saleh A, Nakazawa A, Kumar S, Srinivasula SM, Kumar V,Weichselbaum R, Nalin C, Alnemri ES, Kufe D, Kharbanda S. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J* 2000;19:4310-22

Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C. Heat shock proteins, cellular

chaperones that modulate mitochondrial cell death pathways. *Biochem Biophys Res Commun*. 2003;304:505-12

Paul C, Manero F, Gonin S, Kretz-Remy C, Virot S, Arrigo AP. Hsp27 as a negative regulator of cytochrome C release. *Mol Cell Biol* 2002;22:816-34

Saleh A, Srinivasula SM, Balkir L, Robbins PD, Alnemri ES. Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat Cell Biol* 2000;2:476-83

Shan YX, Liu TJ, Su HF, Samsamshariat A, Mestril R, Wang PH. Hsp10 and Hsp60 modulate Bcl-2 family and mitochondria apoptosis signaling induced by doxorubicin in cardiac muscle cells. *J Mol Cell Cardiol* 2003;35:1135-43

Tsuchiya D, Hong S, Matsumori Y, Shiina H, Kayama T, Swanson RA, Dillman WH, Liu J, Panter SS, Weinstein PR. Overexpression of rat heat shock protein 70 is associated with reduction of early mitochondrial cytochrome C release and subsequent DNA fragmentation after permanent focal ischemia. *J Cereb Blood Flow Metab* 2003;23:718-27

van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, and Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998;31:1-9

van Rijk AE, Stege GJ, Bennink EJ, May A, Bloemendal H. Nuclear staining for the small heat shock protein alphaB-crystallin colocalizes with splicing factor SC35. *Eur J Cell Biol* 2003;82:361-8

Zamora M, Merono C, Vinas O, Mampel T. Recruitment of NF-kappaB into mitochondria is involved in adenine nucleotide translocase 1 (ANT1)-induced apoptosis. *J Biol Chem* 2004;279:38415-23

Zhao C, Wang E. Heat shock protein 90 suppresses tumor necrosis factor alpha induced apoptosis by preventing the cleavage of Bid in NIH3T3 fibroblasts. *Cell Signal* 2004;16:313-21

Zhao ZQ. Oxidative stress-elicited myocardial apoptosis during reperfusion. *Curr Opin Pharmacol* 2004;4:159-65

Zhao ZQ, Vinten-Johansen J. Myocardial apoptosis and ischemic preconditioning. *Cardiovasc Res* 2002;55:438-55

Wang WH, Gregori G, Hullinger RL, Andrisani OM. Sustained activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase pathways by hepatitis B virus X protein mediates apoptosis via induction of Fas/FasL and tumor necrosis factor (TNF) receptor 1/TNF-alpha expression. *Mol Cell Biol* 2004;24:10352-65

Wang X, Klevitsky R, Huang W, Glasford J, Li F, and Robbins J. AlphaB-crystallin modulates protein aggregation of abnormal desmin. *Circ Res* 2003;93:998-1005

Wieske M, Benndorf R, Behlke J, Dolling R, Grelle G, Bielka H, Lutsch G. Defined sequence segments of the small heat shock proteins HSP25 and alphaB-crystallin inhibit actin polymerization. *Eur J Biochem* 2001;268:2083-90