

Downregulation of Smac attenuates H₂O₂-induced apoptosis via endoplasmic reticulum stress in human lens epithelial cells

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Abstract

Background: Second mitochondria-derived activator of caspases (Smac) is reported to promote apoptosis. Given the important role of apoptosis in cataract development, the aim of this study was to investigate whether Smac induces human lens epithelial cell (HLEC) apoptosis via endoplasmic reticulum stress (ERS).

Methods: Smac expression was examined by immunohistochemistry in anterior lens capsules from 157 patients with age-related cataracts and 5 normal controls. The role of Smac in hydrogen peroxide (H₂O₂)-induced ERS and apoptosis was further evaluated using small interfering RNA knockdown in an HLEC line.

Results: Notably, Smac expression was significantly higher in patients with cataracts than in controls, but showed no association with cataract severity. Cell survival was inversely correlated with H₂O₂ concentration, and was most significantly affected at 200 μmol/L. Moreover, flow cytometry revealed that Smac knockdown attenuated H₂O₂-induced apoptosis and enhanced apoptotic- and endoplasmic reticulum-related marker expression—including that of glucose-regulated protein 78, C/EBP homologous protein, caspase 3, B-cell chronic lymphocytic leukemia/lymphoma 2-associated X, and BCL2—at the gene and protein level.

Conclusion: Collectively, these results indicate that Smac plays an important role in ERS-induced apoptosis in HLECs, suggesting its close association with cataract development.

Abbreviations: BAX = Bcl-2-associated X, Bcl-2 = B-cell chronic lymphocytic leukemia/lymphoma 2, CASP3 = caspase 3, CCK-8 = cell counting Kit-8, CHOP = C/EBP homologous protein, ERS = endoplasmic reticulum stress, GRP78 = glucose-regulated protein 78, H₂O₂ = hydrogen peroxide, HLEC = human lens epithelial cell, IAP = inhibitor of apoptosis protein, PBS = phosphate-buffered saline, siRNA = small interfering RNA, Smac = second mitochondria-derived activator of caspases.

Keywords: cell apoptosis, endoplasmic reticulum stress, Smac, small interfering RNA

1. Introduction

Cataracts are the main cause of blindness-related diseases worldwide and bring serious economic losses for society.^[1] The prevalence of cataracts has increased with the expanding elderly population. In China, cataracts are routinely removed by surgery to restore patient sight; however, this surgery is often expensive and the medical conditions in remote communities are subpar, leaving many rural citizens untreated.^[2] For this

reason, pharmacological therapies are an urgent desire for many doctors.

The endoplasmic reticulum (ER) plays an important role in metabolic regulation in humans. When the body is stimulated by environmental stressors, endoplasmic reticulum stress (ERS) is activated to restore homeostasis, but strong ERS elicits cell apoptosis.^[3,4] Recent studies have identified an association between ERS and cataract pathogenesis.^[5] Moreover, second mitochondria-derived activator of caspases (Smac) could promote cell apoptosis in the mitochondria-induced pathway.^[6,7] Smac interacts with inhibitors of apoptosis proteins (IAPs) to inhibit their activity. The IAP family consists of 3 key members: cellular IAP1 (c-IAP1), cellular IAP2 (c-IAP2), and X-linked IAP (XIAP).^[8] cIAP1 localizes to the ER membrane^[9] and is involved in metabolic activity and ERS-induced apoptosis. Notably, cIAP1 can attenuate C/EBP homologous protein (CHOP) expression in response to ERS, thereby hindering GADD34 (growth arrest and DNA damage-inducible protein) expression. Thus, decreased Smac expression may play an important role in ERS marker expression in human lens epithelial cells (HLECs); however, this has not been demonstrated experimentally. As such, the present study examined the relationship between Smac and age-related cataracts, and analyzed the significance of Smac protein expression on ERS in HLECs.

This experiment is mainly divided into 2 parts to study, on the one hand, Smac and age-related cataract lens opacity the correlation between them, on the other hand, influence on HLECs by downregulation of Smac expression by ERS.

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2. Materials and methods

2.1. Patients

Patients who underwent surgery from November 2015 to December 2016 at our hospital were enrolled in this study. A total of 152 patients who met the inclusion criteria were selected and classified according to the Emery system (64 ± 2 years old; range, 55–85 years) (Table 1). At the same time, corneas were collected from 5 organ donors for use as controls. All protocols were performed in accordance with the Institutional Review Board of our hospital and the guidelines of the Declaration of Helsinki, and all patients provided informed consent for participation in the study. Patients with malignant tumors, ocular trauma, and long-term exposure to hazardous substances were excluded.

2.2. HLEC culture

The HLE-B3 cell line was purchased from Genechem Co, Ltd. (Shanghai, China) and cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), gentamycin, 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, Grand Island, NY and Burlington, VT) in a 6-well plates at 37°C in a 95% humidified atmosphere with 5% CO_2 . The culture medium was replaced with fresh medium on the second day. Cells were harvested for experiments at logarithmic growth phase (~60% confluency).

2.3. Smac siRNA plasmid transfection

Four small interfering RNA (siRNA) sequences targeting Smac (Smac1–4) and negative control (NC) siRNA were purchased from Sangon Biotech Co., Ltd (Shanghai, China) (Table 2). Cells transfected with the Smac siRNA plasmid or empty plasmid, or left untransfected were utilized as the experimental, vehicle control, and blank control groups, respectively. For this, approximately 2×10^5 cells/L were inoculated into each well, cultured for 24 hours, and then transfected in triplicate with 7 μL of Smac siRNA and Lipofectamine2000 (Invitrogen; Thermo Fisher Scientific (Chinese) Co. Ltd, Shanghai, China) according to the manufacturer's instructions. Cell transfections were confirmed by Western blotting 24 hours after transfection for treatment studies, cells were treated with 200 $\mu\text{g}/\text{mL}$ hydrogen peroxide (H_2O_2) (Sigma, St. Louis, MO) for 12 hours to elicit ERS.

2.4. Immunohistochemistry

Paraffin-embedded lens anterior capsular tissue sections (3- μm thick) were subjected to xylene dewaxing, gradient ethanol hydration, dehydration in graded alcohol. Antigens were revived in citric acid buffer for 3 hours and each section was incubated with anti-Smac, anti-B-cell chronic lymphocytic leukemia/

Table 1
Patient characteristics according to Emery classification.

| Emery classification | | | | |
|----------------------|------------------------------------|-----------|----|------------|
| Nuclear hardness | Classification | Groups | N | Age, y |
| I | Transparent, soft core | Control | 5 | 54 \pm 2 |
| II | Nuclear white or yellow, soft core | Mild | 38 | 59 \pm 2 |
| III | Dark yellow, medium hardness | Moderate | 38 | 60 \pm 3 |
| IV | Nuclear brown or amber, hard core | Severe | 38 | 60 \pm 5 |
| V | Brown or black, very hard core | All white | 38 | 62 \pm 4 |

N = number of patients.

Table 2
Sequences of Smac-specific siRNA molecules.

| siRNA | Sequence (5'→3') |
|-------------------------|-----------------------------|
| siRNA _{Smac1} | 5'-CCAGACATCAGATGACTTCAA-3' |
| siRNA _{Smac2} | 5'-GTAACGCAATGTACAAGTTTC-3' |
| siRNA _{Smac3} | 5'-GCTGTGCTTTTCGTAACACAT-3' |
| siRNA _{Smac4} | 5'-CGTAAATGCCTGATGCATGCA-3' |
| siRNA _{SmacNC} | 5'-TTACCAACGCTGCAATGCGTA-3' |

Smac = second mitochondria-derived activator of caspases, siRNA = small interfering RNA.

lymphoma 2-associated X (BAX), anti-BCL2, anti-caspase 3, anti-GPR78, anti-CHOP, or β -actin (Abcam, Cambridge, MA) at 4°C for 12 hours, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hours. For visualization, the sections were stained with DAB and hematoxylin, cleared with xylene, dehydrated in graded alcohol, and mounted on slides for imaging.

2.5. Cell counting Kit-8 assay

Cell counting Kit-8 (CCK-8) assays (Sigma) were performed to determine the effect of Smac and ER stress on cell proliferation. For this, cells were cultured in a 96-well plate and treated with H_2O_2 at 200, 400, 600, or 800 $\mu\text{mol}/\text{L}$ for 24 hours. Sample absorbance (A) was measured at 450 nm, with 5 replicates per sample. We ultimately selected 200 $\mu\text{mol}/\text{L}$ H_2O_2 for subsequent experiments, given that the drug action time was 12 hours. Cell viability was calculated as follows: $[(A_{\text{control}} - A_{\text{control blank}}) - (A_{\text{sample}} - A_{\text{sample blank}})] / (A_{\text{control}} - A_{\text{control blank}}) \times 100\%$.

2.6. Apoptosis assay

Cell apoptosis was assessed after H_2O_2 treatment. Briefly, cells were harvested with EDTA trypsin and the cell concentration adjusted to about 1×10^5 cells/L. In order to collect cells, the suspension was centrifuged for 5 minutes at 2000 rpm, and washed 2 times with phosphate-buffered saline (PBS). The samples were then incubated with 5 μL Annexin V-APC and 5 μL PI dye mix in the dark for 10 minutes, and then analyzed by flow cytometry.

2.7. RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from HLE-B3 cells with Trizol reagent and used to synthesize cDNA, using SY Green and a PrimeScript RT-PCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Gene expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) with specific primers (Sangon Biotech Co., Ltd., Shanghai, China; Table 3). Reactions were performed in a thermocycler (Eppendorf, Hamburg, Germany) with 30 cycles of 95°C denaturation for 30 seconds, 60°C annealing for 30 seconds, and 72°C primer extension for 1 minute. The mRNA expression levels of SMAC, GRP78, CHOP, BCL2, and BAX were calculated relative to that of β -actin.

2.8. Western blot analysis

Proteins were extracted with RIPA buffer following the manufacturer's instructions and quantified with a butyleanoacrylate protein assay kit (Sigma). Proteins were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride or nitrocellulose membranes according to the molecular weight of the protein.

Table 3**Primer sequences used for reverse transcription-polymerase chain reaction.**

| Gene | Primer sequence | Product, bp |
|--------------|---|-------------|
| <i>CASP3</i> | F: 5'-CTGGAATATCCCTGGACAAC-3' R: 5'-CTTTAGAAAACATCACGCATC-3' | 143 |
| <i>GRP78</i> | F: 5'-CATCACGCCGTCTATGTCG-3' R: 5'-CGTCAAAGACCGTGTCTCG-3' | 104 |
| <i>CHOP</i> | F: 5'-CAGACTGATCCAACCTGCA-3' R: 5'-GACTGGAATCTGGAGAGTG-3' | 280 |
| <i>BCL2</i> | F: 5'-GGTGGGGTCATGTGTGG-3' R: 5'-CGGTTCAAGTACTCAGTCATCC-3' | 348 |
| <i>BAX</i> | F: 5'-CCCGAGAGGTCTTTTCCGAG-3' R: 5'-CCAGCCCATGATGGTTCTGAT-3' | 155 |
| <i>GAPDH</i> | F: 5'-GGAGCGAGATCCCTCAAAT-3' R: 5'-GGCTGTTGCATACTTCTCATGG-3' | 197 |

BAX = Bcl-2-associated X, BCL2 = B-cell chronic lymphocytic leukemia/lymphoma 2, Bp = base pairs, CASP3 = caspase 3, CHOP = C/EBP homologous protein, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, GRP78 = glucose-regulated protein 78.

The membranes were blocked in 5% nonfat milk and then incubated for 10 hours at 4°C with primary antibodies for Smac (ab32023), glucose-regulated protein 78 (GRP78) (ab21685), CHOP (ab11419), cleaved caspase 3 (CASP3) (ab32042), BAX (ab32503), BCL2 (ab32124), or β -actin (ab7817). The probed membranes were washed and incubated with secondary antibody (ab6721) for 1 hour. Immunoreactive bands were detected by ECL (Emitter-Coupled Logic) and analyzed using Image J software (National Institutes of Health, Bethesda, MD).

2.9. Statistical analysis

Data are presented as the means \pm standard deviation of the results of 3 independent experiments. Multiple groups were compared by 1-way analysis of variance. In cases where the variance was equal, results were subjected to post hoc pairwise comparisons with Dunnett *t* test; otherwise, Dunnett T3 test was conducted. The

means of 2 groups were compared using Student *t* test. All tests were performed using SPSS 21.0 software (SPSS Statistics, Inc., Chicago, IL). $P < .05$ was considered statistically significant.

3. Results

3.1. Smac expression in the anterior capsule of the human lens

As shown in Fig. 1A–D, Smac expression was increased in the samples obtained from the 157 cataract patients recruited in this study. Interestingly, mild and severe cataract samples (N=38 for both) showed intermediate expression, whereas moderate and severe mature samples (N=38 for both) displayed low and high Smac expression, respectively (Fig. 1). Analysis of control samples showed a complete absence of immunostaining, demonstrating weak Smac expression. While the correlation between Smac expression and mild and moderate cataract lens opacity was not statistically significant ($P = .68$), there was a positive correlation between the expression level of Smac and severe (IV) and moderate (III) cataracts ($P < .05$). These results suggested that Smac might be involved in cataract development, but in a manner independent of expression level.

3.2. Downregulation of Smac expression by RNA interference in HLEC cells

Western blotting was performed to confirm siRNA-mediated Smac protein knockdown in HLE-B3 cells. As shown in Fig. 2, siRNA-transfected cells showed a ~70% decrease in Smac expression compared to the control group (NC). This result demonstrated that Smac expression was successfully reduced by RNA interference.

3.3. Effect of Smac knockdown on HLEC viability

We selected the optimal Smac plasmid-3, which exhibited the highest with a transfection ratio of ($\geq 70\%$), to further

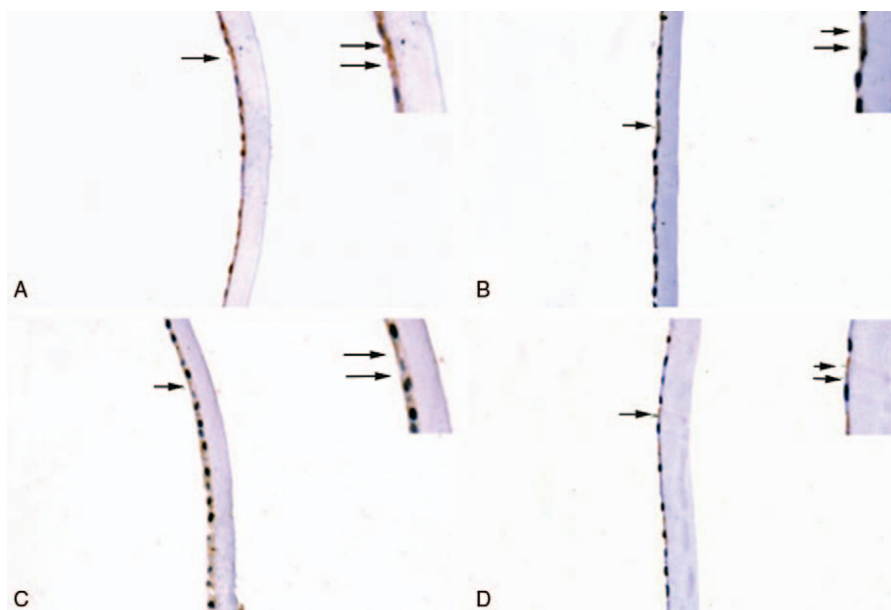


Figure 1. Second mitochondria-derived activator of caspases expression in human corneal lenses of patients with or without cataracts. (A) Severe (IV), (B) moderate (III), and (C) mild (II) disease. (D) Normal control (I). Magnification, 200 \times (Inset, 400 \times). Smac, second mitochondria-derived activator of caspases.

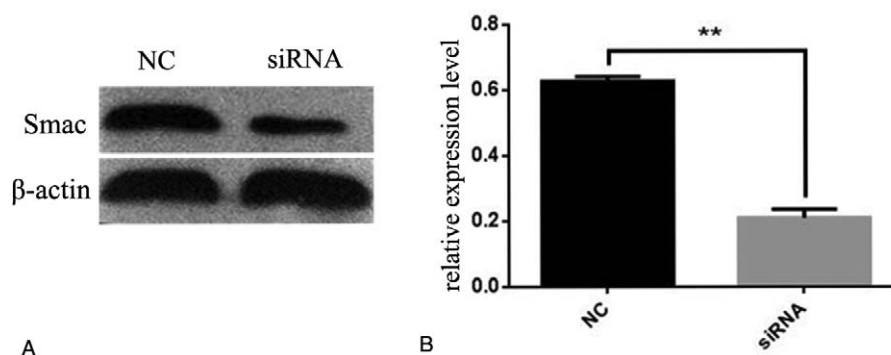


Figure 2. Confirmation of siRNA-mediated second mitochondria-derived activator of caspases (Smac) knockdown. (A) Smac expression was assessed 24 hours after transfection. (B) Graphic depiction of the relative expression level of Smac. siRNA, cells transfected with siRNA-Smac3; NC, normal cells. Smac, second mitochondria-derived activator of caspases.

investigate the effect of Smac knockdown on H_2O_2 -induced apoptosis in HLECs. As shown in Table 4, when the concentration of H_2O_2 was 200 mol/L, the inhibition rate was nearly half. As shown in Table 5, when the concentration of H_2O_2 was 200 μ mol/L, cell viability of the 4 groups was $97.6 \pm 0.3\%$, $44.6 \pm 2.7\%$, $46.5 \pm 1.5\%$, and $58.4 \pm 2.7\%$, respectively. Notably, there was a statistically significant difference between the viability of the siRNA-Smac+ H_2O_2 and the H_2O_2 alone groups. However, at higher concentrations of H_2O_2 , the levels of HLEC cell death increased in all groups, and there were no significant differences in the levels of putrescence between groups. Therefore, the 200 μ mol/L concentration was selected for follow-up experiments, and the optimal incubation time was 24 hours.

3.4. Smac siRNA attenuates H_2O_2 -induced cell apoptosis

Flow cytometry analysis was performed to assess apoptosis in HLE-B3 cells treated with 200 μ mol/L H_2O_2 for 24 hours. As shown in Figs. 3 and 4, apoptosis was lowest in PBS-untreated control cells ($1.7 \pm 1.2\%$; represented as Annexin V-positive cells) and substantially increased following H_2O_2 treatment,

with the highest rates observed in untransfected treated cells, siRNA_{NC}-transfected, and siRNA_{Smac}-transfected cells ($39.1 \pm 3.9\%$, $42.3 \pm 4.4\%$, and $26.5 \pm 2.8\%$, respectively). Notably, Smac knockdown significantly reduced the levels of H_2O_2 -induced apoptosis (D group compared to B group), suggesting that Smac knockdown could block HLEC apoptosis to some extent.

3.5. Smac siRNA knockdown downregulates molecules in the ERS-related signaling pathway by suppressing H_2O_2 -induced apoptosis

BCL2, BAX, GRP78, CHOP, and CASP3 expression were measured to determine the effect of H_2O_2 treatment (Table 6). Western blotting and RT-PCR analysis showed that Smac, GRP78, CHOP, and BAX expression were clearly increased in untransfected cells as compared to that in the Smac siRNA-transfected group after H_2O_2 treatment (Fig. 5), cleaved CASP3 is delined (Fig. 6), whereas the BCL2 expression was relatively low. Collectively, these results demonstrated that H_2O_2 could induce ERS, and further supported that Smac downregulation attenuates the H_2O_2 -induced apoptosis.

Table 4

Cell viability under different concentration of H_2O_2 .

| Treatment | N | Concentration of H_2O_2 | | | |
|-----------|----|---------------------------|-----------------|-----------------|-----------------|
| | | 200 μ mol/L | 400 μ mol/L | 600 μ mol/L | 800 μ mol/L |
| PBS | 20 | 98.6 ± 1.2 | 98.7 ± 0.4 | 98.4 ± 1.7 | 99.3 ± 0.3 |
| H_2O_2 | 20 | 45.7 ± 2.4 | 61.1 ± 0.3 | 75.2 ± 3.1 | 82.7 ± 1.1 |

Compared with the 0 mol/L H_2O_2 value in each group, $P < .01$. The inhibition rate of was up to half when the concentration was close to 200 μ mol/L. PBS = phosphate-buffered saline.

Table 5

Effects of Smac on the cell viability.

| Treatment | N | Concentration of H_2O_2 | | | |
|----------------------------------|----|---------------------------|-----------------|-----------------|-----------------|
| | | 200 μ mol/L | 400 μ mol/L | 600 μ mol/L | 800 μ mol/L |
| PBS | 16 | 97.6 ± 0.8 | 98.1 ± 0.1 | 97.2 ± 1.5 | 98.1 ± 0.2 |
| H_2O_2 | 16 | 44.6 ± 2.7 | 45.1 ± 1.2 | 36.3 ± 2.2 | 28.5 ± 2.1 |
| siRNA _{NC} + H_2O_2 | 16 | 46.5 ± 1.5 | 40.5 ± 3.2 | 38.1 ± 0.2 | 31.8 ± 2.4 |
| siRNA _{Smac} + H_2O_2 | 16 | 58.4 ± 2.7 | 48.4 ± 2.7 | 36.4 ± 1.7 | 28.4 ± 0.2 |

Compared with the PBS group, the 3 groups were statistically significant ($P < .01$), compared with the H_2O_2 group, the siRNA-Smac + H_2O_2 group was statistically significant ($P < .05$). However, with increasing concentrations of H_2O_2 , these differences disappeared. PBS = phosphate-buffered saline, siRNA = small interfering RNA.

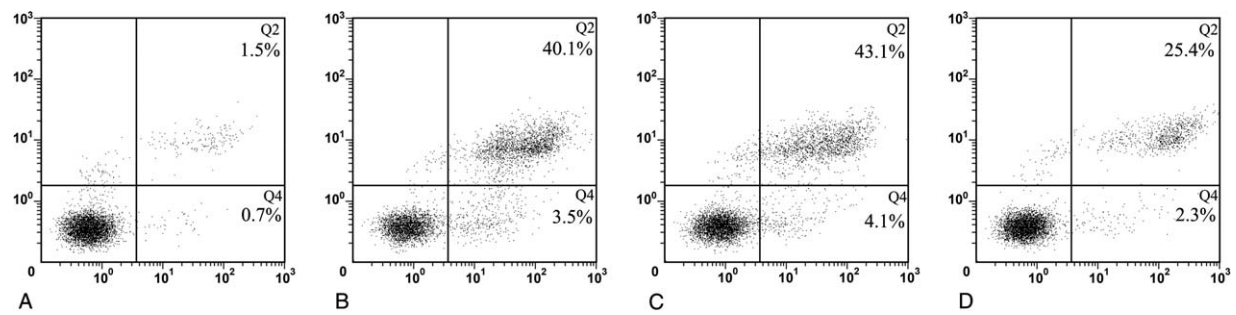


Figure 3. Second mitochondria-derived activator of caspases (Smac) knockdown attenuates H_2O_2 -induced HLEC apoptosis. Early and late apoptotic cells localize to Q4 and Q2, respectively. (A–D) phosphate-buffered saline group, untransfected treated cells, siRNA_{NC}-transfected group, and siRNA_{Smac}-transfected group, respectively. Smac, second mitochondria-derived activator of caspases; HLEC, human lens epithelial cells.

4. Discussion

Visual impairment is an important health issue affecting nearly 300 million people worldwide.^[10] In particular, an estimated 39 million individuals are currently blind, most often because of cataracts.^[11] Preventing blindness is a difficult task in China because of the high prevalence of risk factors associated with age-related cataract development, such as old age, ultraviolet radiation, hormonal disorders, smoking, alcohol abuse, unhealthy diet, radiation, glaucoma, and diabetes.^[11,12]

The mitochondrion is the primary source of reactive oxygen species (ROS), and mitochondrial dysfunction results in excessive ROS production that can be harmful to cells.^[13] HLECs are the only cells that are capable of aerobic metabolism in the crystal. Increased ROS levels have been associated with tumor cell apoptosis and TRAIL pathway activation.^[14] Studies have shown that the Smac antagonists XIAP, c-IAP1, and c-IAP2 can increase intracellular ROS levels and promote tumor cell death, a process that causes the release of Smac analogs and cytochrome C.^[15] ERS is associated with cataract development, as it incites the release of Ca^{2+} release into the cytoplasm of lens epithelial cells, resulting in calpain activation, and subsequent CASP1 and

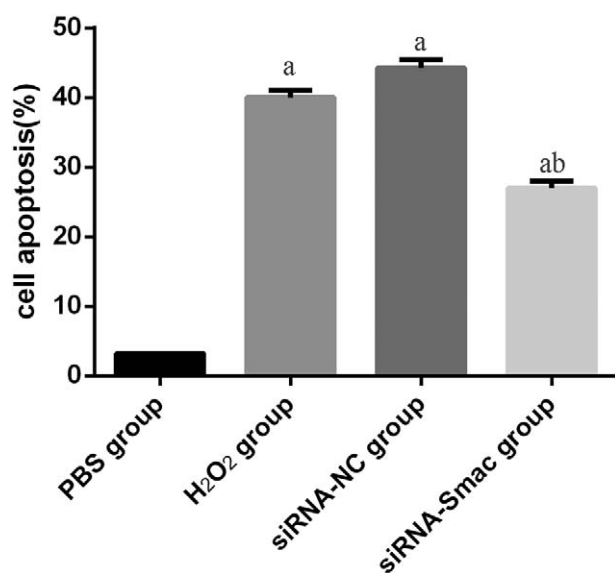


Figure 4. Apoptosis rates in H_2O_2 -treated second mitochondria-derived activator of caspases (Smac) siRNA and control groups. H_2O_2 ($^aP < .05$), siRNA-NC + H_2O_2 ($^bP < .01$), siRNA-Smac + H_2O_2 ($^cP < .05$), $^bP < .05$. Smac, second mitochondria-derived activator of caspases.

CASP3 activity^[16]; thus, Smac overexpression could induce GRP78 upregulation.

In this study, we first assessed Smac expression semiquantitatively in 156 clinical specimens and found a relationship between the expression of this protein and the degree of lens opacity. In mild (II) and moderate (III) cataracts, Smac expression was observed in some parts of anterior lens capsule, and there was no statistically significant difference between the groups. However, a statistically significant increase was observed among the severe (IV) and mature severe (V) groups. On the basis of these data, we theorize that Smac interacts with IAP family members—particularly XIAP—to facilitate CASP3/9 activation and thereby promote HLEC apoptosis.

H_2O_2 elicits ERS and ROS production,^[17] and oxidative stress and ERS are known to cause lens fiber degeneration and crystal protein damage. Studies show that PERK (PKR-like endoplasmic Reticulum Kinase) and eIF2 α exert a protective effect against ERS in mammals. During early ERS, eIF2 α is phosphorylated to block its transcriptional activity and attenuate PERK-mediated gene expression, especially in response to errors in protein folding and synthesis. Alternatively, CHOP is activated in response to severe ERS, which is involved in the destruction. Several reports indicate that caspases are involved in the ERS response, wherein PERK activation can stimulate caspase-12 expression to initiate the apoptotic cascade or elicit ATF4 pathway activity that can lead to serious injury of cells.^[18] Here, CCK-8 analysis revealed that Smac knockdown increased cell viability following H_2O_2 treatment, but that this viability steadily decreased with increasing H_2O_2 concentrations.

In addition, RT-PCR and Western blotting showed that H_2O_2 -induced ERS upregulated GRP78 expression, which is an abundant ER chaperone protein. Under normal physiological circumstances, GRP78 binds PERK to activate ATF6 and IRE1, promotes protein transport, and protects the ER barrier from injury. During long-term ERS, the PERK pathway is advantageous. Activation of IRE1 by XBP1 temporarily plays a role.^[19] When cellular homeostasis cannot be recovered, ATF6 and IRE1 signaling will be gradually decay, guiding apoptosis. Studies have shown that termination of IRE1 expression is an important factor in causing cell death. ATF6 and XBP1s have the ability to regulate lipid biosynthesis and promote the expansion of rough endoplasmic reticulum.^[19] GRP78 overexpression is closely related to many diseases such as Alzheimer and Parkinson disease. In the early ERS, GRP78 elicits a protective response to maintain homeostasis,^[20] but when acute and persistent ERS will be induced apoptosis, activated GRP78 dissociates from PERK and phosphorylated eIF2 α to prevent mRNA translation.^[21] p-eIF2 can increase the expression of transcription factor 4 (ATF4),

Table 6**Effect of Smac knockdown on GRP78, CHOP, BAX/BCL2, CASP3 mRNA expression.**

| Group | GRP78 | CHOP | BAX | BCL2 | CASP3 |
|---|---------------|---------------|---------------|---------------|---------------|
| PBS | 0.201 ± 0.093 | 0.275 ± 0.201 | 0.119 ± 0.093 | 0.598 ± 0.204 | 0.125 ± 0.201 |
| H ₂ O ₂ | 0.517 ± 0.123 | 0.527 ± 0.056 | 0.418 ± 0.123 | 0.247 ± 0.101 | 0.477 ± 0.056 |
| siRNA _{NC} + H ₂ O ₂ | 0.577 ± 0.197 | 0.541 ± 0.113 | 0.513 ± 0.107 | 0.197 ± 0.007 | 0.511 ± 0.113 |
| siRNA _{Smac} + H ₂ O ₂ | 0.342 ± 0.103 | 0.351 ± 0.143 | 0.292 ± 0.203 | 0.417 ± 0.091 | 0.290 ± 0.043 |

BAX = Bcl-2-associated X, BCL2 = B-cell chronic lymphocytic leukemia/lymphoma 2, CASP3 = caspase 3, CHOP = C/EBP homologous protein, GRP78 = glucose-regulated protein 78, PBS = phosphate-buffered saline, siRNA = small interfering RNA.

and ATF4 can promote the expression of GRP78, resulting in rapid increases in expression of this protein^[22]. At this time, we detected the expression of apoptosis protein CASP3, which showed strong positive expression. Notably, both GRP78 and CASP3 expression were decreased in Smac knockdown cells, which may explain its effect on apoptosis. At the same time, we found that CHOP was upregulated at the mRNA and protein level. Activation of the CHOP pathway indicates that the ERS has entered a stage during which the cell structure is damaged. In particular, researchers have found that the bZIP structure functions as an important mediator of apoptosis in the CHOP pathway. A recent study showed that CHOP can increase ROS levels in mouse models of diabetes, and also that ERS is related to oxidative stress, and that the 2 mutually aggravate apoptosis.^[23] Western blotting also showed that upon Smac knockdown, apoptotic protein BAX expression declines and BCL2 expression

increases. Consistent with these findings, previous studies found that expression of Smac can affect the expression of BAX/BCL2 in tumor cells.^[24] Here, we established an ERS model. After downregulation of Smac, we also observed the changes in expression of BAX/BCL2. The antiapoptotic protein BCL2 is widely expressed in various cells; BCL2 is not only distributed in the outer membrane of mitochondria, but also in the cell membrane and nuclear membrane and ER.^[25] BCL2 family proteins are recognized as being involved in the regulation of the ER pathway, and ER and mitochondrial membrane interaction between the 3 is believed to be an important inducing factor that control ER and Ca²⁺ homeostasis and mediate release of cytochrome C from mitochondria to the cytoplasm.^[26] Bax plays a key role in regulating intracellular homeostasis during apoptosis, which can impact changes in Ca²⁺ and mitochondrial transport.^[27] We analyzed the dual characteristics of siRNA-

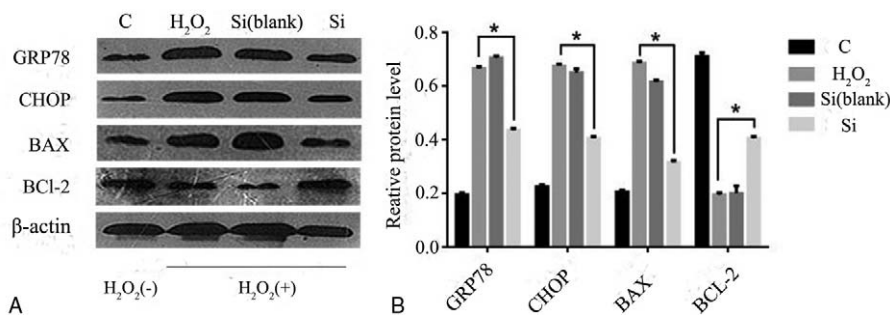


Figure 5. Down regulation of second mitochondria-derived activator of caspases expression could result in decreased expression of glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and Bcl-2-associated X (BAX). (A) Western blot showing the expression levels of GRP78, CHOP, BAX, and BCL2. (B) Results showed relative protein levels ($P < .05$). Smac, second mitochondria-derived activator of caspases; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; BAX, Bcl-2-associated X; Bcl-2, B-cell chronic lymphocytic leukemia/lymphoma 2.

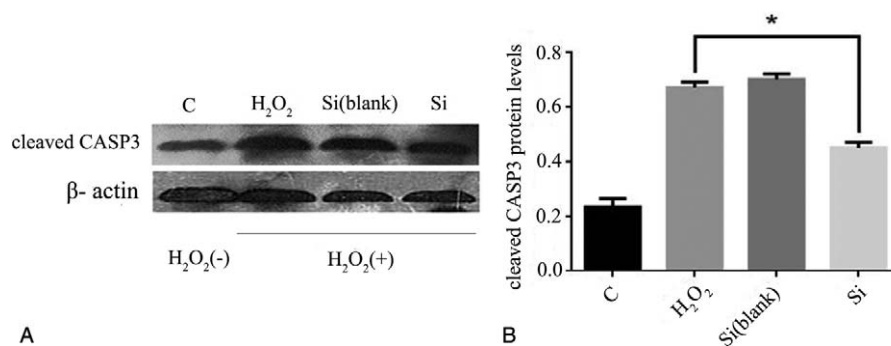


Figure 6. Downregulation of second mitochondria-derived activator of caspases (Smac) downregulated resulted in reduced HLEC apoptosis and CASP3 cleavage. (A) Western blot showing the levels of cleaved CASP3. (B) Levels of cleaved CASP3 are expressed in terms of the band density. Effect of Smac downregulation on ERS protein marker expression ($P < .05$). Smac, second mitochondria-derived activator of caspases; HLEC, human lens epithelial cells; CASP3, caspase 3; ERS, endoplasmic reticulum stress.

Smac, inhibiting cell proliferation and arresting cell apoptosis, which is the cytological basis to prevent cataract formation.

Previous studies have shown that Smac mediates the production of ERS in tumor cells.^[28] According to our study, Smac can also cause ERS and cell damage in LECs, and thus is worthy of further studies that can help regulate the production of many apoptotic factors in various apoptotic pathways. Most importantly, this research furthers our understanding of apoptosis signaling. In the process of studying how Smac that is involved in the regulation of cell homeostasis and apoptosis, we found that the expression of intracellular apoptotic signal was abnormal. Under these circumstances, our results show that Smac can promote HLEC apoptosis via the ERS pathway. However, this is a preliminary study; the specific interaction mechanism is complex. Whether the cell apoptosis observed in this study is affected by other pathways is worthy of further study.

5. Conclusion

The present study showed that Smac knockdown by siRNA can prevent the H₂O₂-induced cell apoptosis in HLE-B3 cells via ERS, which was further supported by the decrease in GRP78 protein expression and increased cell viability. Furthermore, Smac expression varied in the anterior capsule of the lens between cataract patients and controls, implying a role of Smac in cataract formation. These results suggest that the protective role of Smac against H₂O₂-induced apoptosis in HLE-B3 cells operates by inhibiting acute ERS.

References

- [1] Brian G, Taylor H. Cataract blindness—challenges for the 21st century. *Bull World Health Organ* 2001;79:249–56.
- [2] Zhu M, Zhu J, Lu L, et al. Four-year analysis of cataract surgery rates in Shanghai, China: a retrospective cross-sectional study. *BMC Ophthalmol* 2014;14:3.
- [3] Elanchezian R, Palsamy P, Madson CJ, et al. Age-related cataracts: homocysteine coupled endoplasmic reticulum stress and suppression of Nrf2-dependent antioxidant protection. *Chem Biol Interact* 2012;200:1–0.
- [4] Watson GW, Andley UP. Activation of the unfolded protein response by a cataract-associated AlphaA-crystallin mutation. *Biochem Biophys Res Commun* 2010;401:192–6.
- [5] Lyu L, Whitcomb EA, Jiang S, et al. Unfolded-protein response-associated stabilization of p27 (Cdkn1b) interferes with lens fiber cell denucleation, leading to cataract. *FASEB J* 2016;30:1087–95.
- [6] Ng H, Smith DJ, Nagley P. Application of flow cytometry to determine differential redistribution of cytochrome c and Smac/DIABLO from mitochondria during cell death signaling. *PLoS One* 2012;7:e42298.
- [7] Li J, Li Y, Qin D, et al. Mitochondrial fission leads to Smac/DIABLO release quenched by ARC. *Apoptosis* 2010;15:1187–96.
- [8] Hikami S, Shiozaki A, Kitagawa-Juge M, et al. The role of cIAP1 and XIAP in apoptosis induced by tumor necrosis factor alpha in esophageal squamous cell carcinoma cells. *Dig Dis Sci* 2017;62:652–9.
- [9] Qi Y, Xia P. Cellular inhibitor of apoptosis protein-1 (cIAP1) plays a critical role in beta-cell survival under endoplasmic reticulum stress: promoting ubiquitination and degradation of C/EBP homologous protein (CHOP). *J Biol Chem* 2012;287:32236–45.
- [10] Chan CH, Trope GE, Badley EM, et al. The impact of lack of government-insured routine eye examinations on the incidence of self-reported glaucoma, cataracts, and vision loss. *Invest Ophthalmol Vis Sci* 2014;55:8544–9.
- [11] Indaram M, Agron E, Clemons TE, et al. Changes in lens opacities on the age-related eye disease study grading scale predict progression to cataract surgery and vision loss: age-related eye disease study report no. 34. *Ophthalmology* 2015;122:888–96.
- [12] Mukesh BN, Le A, Dimitrov PN, et al. Development of cataract and associated risk factors: the visual impairment project. *Arch Ophthalmol* 2006;124:79–85.
- [13] Korge P, Calmettes G, Weiss JN. Reactive oxygen species production in cardiac mitochondria after complex I inhibition: modulation by substrate-dependent regulation of the NADH/NAD(+) ratio. *Free Radic Biol Med* 2016;96:22–33.
- [14] Seyfrid M, Marschall V, Fulda S. Reactive oxygen species contribute toward Smac mimetic/temozolomide-induced cell death in glioblastoma cells. *Anticancer Drugs* 2016;27:953–9.
- [15] Varfolomeev E, Blankenship JW, Wayson SM, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007;131:669–81.
- [16] Morishima N, Nakanishi K, Takenouchi H, et al. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002;277:34287–94.
- [17] Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 2007;9:2277–93.
- [18] Huang CL, Lee YC, Yang YC, et al. Minocycline prevents paraquat-induced cell death through attenuating endoplasmic reticulum stress and mitochondrial dysfunction. *Toxicol Lett* 2012;209:203–10.
- [19] Lin JH, Li H, Yasumura D, et al. IRE1 signaling affects cell fate during the unfolded protein response. *Science* 2007;318:944–9.
- [20] Pfaffenbach KT, Lee AS. The critical role of GRP78 in physiologic and pathologic stress. *Curr Opin Cell Biol* 2011;23:150–6.
- [21] Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 2011;334:1081–6.
- [22] Jing X, Shi Q, Bi W, et al. Rifampicin protects PC12 cells from rotenone-induced cytotoxicity by activating GRP78 via PERK-eIF2α-ATF4 pathway. *PLoS One* 2014;9:1–9.
- [23] Bommasamy H, Back SH, Fagone P, et al. ATF6alpha induces XBP1-independent expansion of the endoplasmic reticulum. *J Cell Sci* 2009;122(Pt 10):1626–36.
- [24] Carriere A, Carmona MC, Fernandez Y, et al. Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect. *J Biol Chem* 2004;279:40462–9.
- [25] Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002;2:647–56.
- [26] Lam M, DUBYAK G, Chen L, et al. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca²⁺ fluxes. *Proc Natl Acad Sci U S A* 1994;91:6569–73.
- [27] Zou XJ, Yang L, Yao SL. Endoplasmic reticulum stress and C/EBP homologous protein-induced Bax translocation are involved in angiotensin II-induced apoptosis in cultured neonatal rat cardiomyocytes. *Exp Biol Med* (Maywood) 2012;237:1341–9.
- [28] Greer RM, Peyton M, Larsen JE, et al. SMAC mimetic (JP1201) sensitizes non-small cell lung cancers to multiple chemotherapy agents in an IAP-dependent but TNF-α-independent manner. *Cancer Res* 2011;71:7640–8.