

# Activation of the unfolded protein response in aged human lenses

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**Abstract.** Cataract formation is a multifactorial disease, induced by a variety of stressors. The endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) is known to produce reactive oxygen species (ROS) leading to apoptosis. The present study aimed to investigate whether activation of the UPR occurs in human lenses, using human lens epithelial cell (HLEC) lines and lenses obtained from an eye bank, from individuals aged between 50 and 90 years. *In vitro* analysis was performed using calcimycin (10  $\mu$ M) as an ER stressor. The level of ER stress was measured by the production of ROS, staining for cell death, detection of binding immunoglobulin proteins (BIP) and levels of other UPR proteins, including inositol-requiring enzyme-1 (IRE), activating transcription factor (ATF) 6 and PKR-like eukaryotic initiation factor 2 $\alpha$  kinase (PERK). These parameters were examined in HLECs exposed to calcimycin for 12, 24, 48 and 72 h. Fluorescent activated cell sorting analysis of the levels of ROS and apoptosis revealed an increase following 24 h calcimycin exposure. The reverse transcription quantitative polymerase chain reaction results demonstrated a gradual increase in the mRNA levels of BIP, IRE1, ATF6 and PERK between 12 and 72 h. A similar effect was observed in the protein levels, which also demonstrated a gradual increase in the levels of endoplasmic oxidoreductin-1-like (Ero1-L)- $\beta$  and protein disulfide isomerase, but a lower level of Ero1-L- $\alpha$ . Activation of the UPR involved the apoptotic pathway, revealed by increased levels of C/EBP homologous protein, ATF4 and caspase-4. Additionally, the antioxidant protein levels were also suppressed. The investigation of aged human lenses revealed a similar increase in the protein expression of UPR. These results indicated that activation of the UPR-induced ROS production suppressed the antioxidant status and triggered the apoptotic pathway, ultimately leading to the formation of age-related cataracts.

## Introduction

The unfolded protein response (UPR) is an adaptive cellular response, which is involved in the attenuation of protein synthesis to prevent distorted protein aggregation and the activation of endoplasmic reticulum (ER)-associated distorted protein degradation to accelerate the elimination of protein aggregates (1-5). Conversely, extended UPR activation causes ER stress, which leads to apoptosis and activation of activating transcription factor (ATF) (6). ATF6 activates ATF4, a central transcriptional activator, which activates the apoptotic factor, C/EBP homologous protein (CHOP) (6). The UPR also activates caspase-12 (7-9) and disrupts the Ca<sup>2+</sup> balance in the ER (10-12). The UPR apoptotic pathway generates reactive oxygen species (ROS), which are derived from the UPR-associated oxidative protein folding machinery in the ER through protein disulfide isomerase (PDI), coenzyme endoplasmic oxidoreductin-1-like (Ero1-L) and decreased levels of glutathione (13-15).

Previous studies have revealed that the UPR is associated with aging and potentially UPR-induced age-related disease, including cataracts (16-18). Age-related cataracts (ARCs), a major type of cataract, are considered to occur as a result of normal aging processes. Aging, combined with environmental and genetic stresses, is considered to be the predominant contributor to the pathogenesis of lens oxidation, crystallin modification and aggregation (19). However, several of the pathogenic mechanisms involved in ARC remain to be elucidated. Oxidative stress is considered to be one of the cellular conditions associated with the development of ARC (20,21). This is induced by a range of factors, including diabetes, malnutrition, systemic and ocular disease processes, pollutants, drugs, heavy metals, ionizing radiation, glucose and changes in the oxygenation of cells (20,21). These stress factors result in the generation of ROS (16,22,23).

Based on emerging evidence of UPR activation and its association with aging, the present study aimed to investigate whether UPR activation occurs in aging human lens cells, which may be involved in the formation of ARCs.

## Materials and methods

**Age-related human lenses and cell culture.** Human lenses aged between 50 and 90 years were obtained from The Eye Bank of Shandong Eye Institute (Qingdao, China) for the detection of UPR proteins in the lens. The human lens epithelial cell (HLEC) line was used for *in vitro* experiments. HLECs

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were previously stored in liquid nitrogen and then maintained in 25 mM glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal calf serum at 37°C in 5% atmospheric oxygen. The HLECs were pre-cultured overnight in 5 mM glucose DMEM in a CO<sub>2</sub> incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for use in the subsequent experiments.

**ROS and apoptosis measurements.** The HLECs were cultured for 12, 24, 48 and 72 h prior to harvesting to determine the levels of ROS and apoptosis. The cells (~5×10<sup>6</sup>) were collected by centrifugation and subsequently resuspended in 250 µl phosphate-buffered saline (PBS; pH 7.4), containing 20 µM dihydrorhodamine. After 30 min incubation at room temperature in the dark, the cells were washed using PBS and resuspended in 500 µl PBS for fluorescence activated cell sorting (FACS) analysis using a BD FACS Aria™ II (BD Biosciences, Franklin Lakes, NJ, USA). To measure the levels of apoptosis, the cells were counterstained with propidium iodide (2 µg/ml) at 4°C. The cells were filtered through a 50 µm nylon mesh (BD Biosciences) to remove cell clumps and were seeded into FACS tubes for FACS analysis to assess levels of apoptosis.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The total RNA was extracted from the HLECs, following treatment with or without varying concentrations of homocysteine using a Quick-RNA™ MicroPrep solution (Zymo Research Corporation, Orange, CA, USA) according to the manufacturer's instructions. Subsequently, the purified total RNA was reverse transcribed using iScript™ Reverse Transcription supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The reverse transcribed RNA was analyzed by RT-qPCR using a SsoFast™ EvaGreen supermix (Bio-Rad Laboratories, Inc.). The primer sequences were designed for optimal design using OligoPerfect™ Designer software (Invitrogen Life Technologies), according to the manufacturer's instructions and were synthesized commercially (Invitrogen Life Technologies). The primer sequences used were as follows: Binding immunoglobulin protein (BIP), forward (5'-GGAAAGAAGGTTACCC ATGC-3') and reverse (5'-TTAGGCCAGCAATAGTTCCA-3'); inositol-requiring enzyme-1 (IRE1), forward (5'-AGACTTTG TCATCGGCCTTT-3') and reverse (5'-TGTATTCTGTTCGC CCAAGA-3'); ATF6 forward (5'-GATTAAAGGCTGCCCTC TCA-3') and reverse (5'-GCCTCTGGTTCTCTGACACA-3'); PKR-like eukaryotic initiation factor 2a kinase (PERK), forward (5'-CACCAGAGAAGTGGCAAGAA-3') and reverse (5'-CATCCATTGGGCTAGGAGAG-3') and β-actin forward (5'-CCAACCGCGAGAAGATGA-3') and reverse (5'-CCAGA GGCGTACAGGGATAG-3'). The PCR conditions included: 2 min at 95°C, 20 sec at 95°C, 20 sec at the annealing temperature, 30 sec at 72°C for 35 cycles and 10 min at 72°C. Each reaction was performed in triplicate in three independent experiments. A standard curve (18S rRNA) was constructed from a dilution series of a reference cDNA sample and was included in each RT-qPCR run to correct for possible variations in product amplification. The relative copy numbers were obtained using the standard curve and were normalized to the values obtained for β-actin, the internal control. The fold

change in expression levels were obtained using the 2<sup>-ΔΔCT</sup> method (24).

**Western blot analysis.** The HLECs were lysed in radioimmunoprecipitation buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). The soluble proteins (10–20 µg/l) were separated using SDS-PAGE and were subsequently blotted onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked using 5% non-fat dry milk for 1 h at room temperature prior to incubation overnight with primary antibodies at 4°C. The primary antibodies included: Mouse monoclonal antibodies against Bip (1:1,000) and IRE1 (1:2,000); rabbit polyclonal ATF6 (1:2,000) and PERK (1:3,000; BD Biosciences); rabbit polyclonal antibodies against ATF4 (1:500) and catalase (1:2,000); mouse monoclonal antibodies against CHOP (1:1,000), Erol-Lα (1:1,000), Erol-Lβ (1:500), PDI (1:2,000) and glutathione reductase (1:2,000), superoxide dismutase (SOD; 1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antibodies against GAPDH (1:4,000) obtained from Novus Biological (Littleton, CO, USA). The membranes were washed and subsequently incubated with secondary antibodies, including horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:5,000) and goat anti-mouse IgG-HRP (1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Luminol reagent (0.1 ml per cm<sup>2</sup> of membrane; Thermo Fisher Scientific) was applied to the membranes and the bands were detected using a Bio-rad XRS system (Bio-Rad Laboratories, Inc.). Antibodies against BIP, IRE1, ATF6, PERK (BD Biosciences), ATF4, CHOP, Erol-Lα, Erol-Lβ, PDI, glutathione reductase (GR), catalase, and superoxide dismutase (SOD), from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA) and GAPDH from Novus Biological, (Littleton, CO, USA) were used. The intensity of each band was normalized to GAPDH.

**Statistical analysis.** The data are expressed as the mean ± standard deviation and the statistical significance was evaluated using Student's t-test with SPSS (version 15.0) software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**ROS production and apoptosis.** Following exposure of the HLECs to calcimycin for 12, 24, 48 and 72 h, a gradual increase was observed in the production of ROS, with a significant increase at 48 h. Similarly, apoptosis was observed after 24 h and a significant increase was observed after 72 h. ROS production and apoptosis were measured using FACS analysis. ROS production was determined by dihydrorhodamine staining and propidium iodide staining was used to measure apoptotic cell population. The percentage of staining with the respective dyes is shown in Fig. 1A.

**mRNA expression levels of UPR-associated genes.** The mRNA expression levels of UPR-associated genes were measured using RT-qPCR. The mRNA expression level of BIP gradually increased after 24 h of exposure, with a significant 10-fold increase after 72 h (Fig. 1B). BIP is considered a marker for UPR activation, which triggers the activation of

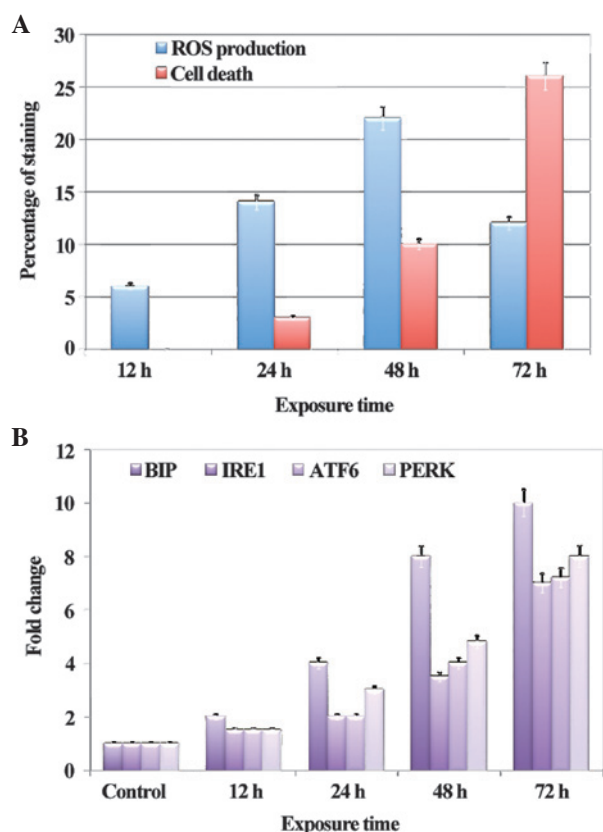


Figure 1. UPR activation in the HLECs. (A) FACS analysis demonstrating the percentage of ROS production and cell death at different time points. (B) Reverse transcription quantitative polymerase chain reaction analysis of the fold change in the mRNA levels of UPR-associated genes. Error bars represent the mean  $\pm$  standard error of the mean. UPR, unfolded protein response; HLECs, human lens epithelial cells; FACS, fluorescent activated cell sorting; ROS, reactive oxygen species; BIP, binding immunoglobulin protein; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor; PERK, PKR-like eukaryotic initiation factor 2a kinase.

other UPR-associated proteins. The mRNA expression levels of UPR-associated genes, including IRE1, ATF6 and PERK, began increasing at 48 h, with a maximum 8-fold increase at 72 h (Fig. 1B), indicating the effect of UPR activation in the HLECs following exposure to calcimycin, the ER stressor.

**Activation of UPR-associated proteins in the HLECs.** The western blot analysis of UPR-associated proteins revealed that the level of BIP gradually increased in the HLECs after 12 h of exposure to calcimycin. A significant increase in the protein level of BIP was identified after 24 h of exposure. The levels of other UPR proteins were elevated after 24 h of exposure, indicating activation of the UPR in the HLECs (Fig. 2A). As the duration of exposure increased, the levels of the UPR proteins also increased, which was correlated with the activation of the UPR observed in aging human lenses. As a result of UPR activation, the levels of the proteins involved in the protein folding machinery, including PDI and Ero1-L $\alpha$ , decreased. Conversely, the protein levels of Ero1-L $\beta$ , a counter regulator for Ero1-L $\alpha$ , increased (Fig. 2B). This confirmed that HLECs exposed to calcimycin led to activation of the UPR.

**Suppression of the antioxidant system and activation of apoptosis.** The protein levels of antioxidant and apoptotic signaling

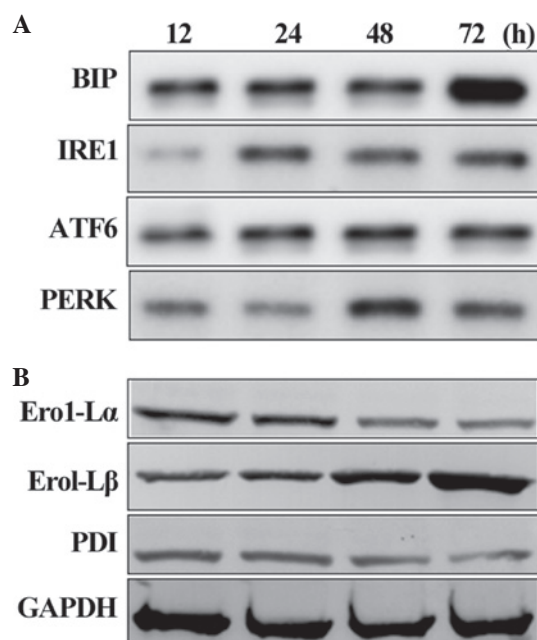


Figure 2. Activation of the UPR in the HLECs. (A) Western blot analysis of the levels of UPR signaling proteins BIP, IRE1, ATF6 and PERK. (B) Western blot analysis of the levels of UPR-associated proteins, which were expressed following UPR activation. UPR, unfolded protein response; HLECs, human lens epithelial cells; ROS, reactive oxygen species; BIP, binding immunoglobulin protein; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor; PERK, PKR-like eukaryotic initiation factor 2a kinase, Ero1-L, endoplasmic oxidoreductin-1-like; PDI, protein disulfide isomerase.

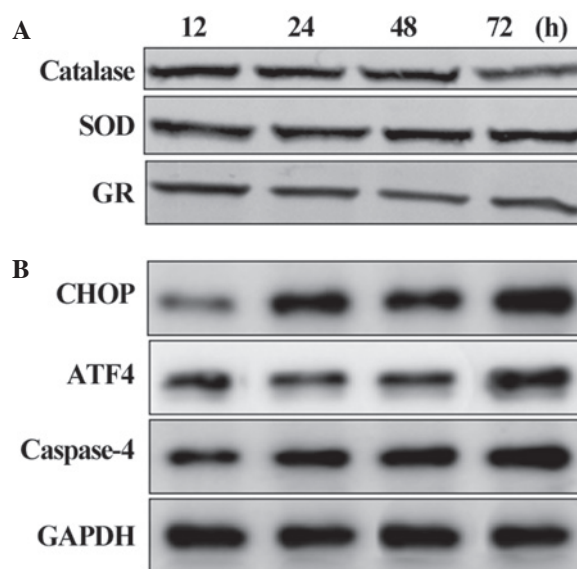


Figure 3. Effect of UPR activation on the (A) antioxidant proteins and (B) signaling proteins involved in the apoptotic pathway. UPR, unfolded protein response; SOD, superoxide dismutase; GR, glutathione reductase; CHOP, C/EBP homologous protein; ATF, activating transcription factor.

proteins were assessed in the HLECs exposed to calcimycin. The protein levels of the antioxidants catalase, SOD and GR decreased after 48 and 72 h of exposure, indicating that UPR activation suppressed the antioxidant level and produced ROS (Fig. 3A). The levels of the CHOP, ATF4 and caspase-4 apoptotic signaling proteins gradually increased after 24 h and increased markedly at 72 h (Fig. 3B). This suggested that the



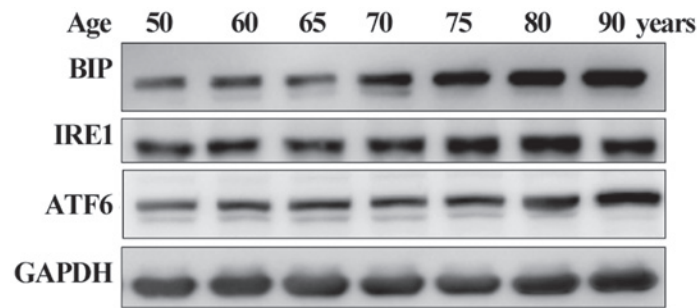


Figure 4. Age-dependent activation of the unfolded protein response in aged human lenses. BIP, binding immunoglobulin protein; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor.

activation of UPR triggers the ER stress-mediated apoptotic signaling pathway in HLECs.

**Age-related activation of the UPR-associated proteins in human lenses.** Based on the *in vitro* results, the present study aimed to confirm whether UPR activation is observed in human lenses aged between 50 and 90 years old. The protein expression of BIP was observed in all the lenses and significantly elevated in lenses aged  $\geq 70$  years. The IRE1 and ATF6 UPR proteins gradually increased with age (Fig. 4). These results confirmed that the UPR was activated in the human lenses. Therefore, activation of the UPR was involved in the production of ROS, decreased antioxidant levels and activation of the apoptotic pathway, ultimately leading to lens oxidation or the formation of cataracts.

## Discussion

Cataract formation is a multifactorial disease occurring predominantly in older individuals based on their exposure to internal or external stressors. Cataractogenic factors are responsible for the generation of ROS and the failure of the antioxidant system in the lens epithelial cells, leading to lens oxidation or cataract formation (25). Generally, ROS are generated in the mitochondria of the cell, however, lens epithelial cells lack mitochondria and the generation of ROS in the lens cells remains to be elucidated (26,27). The present study revealed that ER stress induced the activation of the UPR and that chronic activation of the UPR leads to the generation of ROS and causes apoptosis. At higher levels of calcimycin, misfolded proteins accumulated in the ER, which can then induce the UPR (28,29).

In the present study, HLECs exposed to calcimycin, an ER stressor, for 24 h led to the formation of ROS and, after 48 h, apoptosis occurred. It was hypothesized that this may be due to activation of the UPR. To investigate this hypothesis, gene expression levels were assessed after 12, 24, 48 and 72 h of calcimycin exposure. The initial responses of the UPR are the phosphorylation of three ER-associated transmembrane proteins; PERK, IRE1 and ATF6, which are cleaved and separated from the ER membrane (16). These responses lead to translational and transcriptional arrest and a reduction in protein production in order to recover from the accumulation of unfolded proteins in the ER. However, in the presence of continued ER stress, the apoptotic pathway is activated by the central transcriptional activators, ATF4 and ATF6,

which subsequently activate the apoptotic factor CHOP (30). Consistent with these results, the present study revealed a significant increase in the mRNA and protein levels of IRE1, ATF6, PERK 72 h after exposure. The levels of BIP, which is considered to be an ER stress marker, were increased after 24 h. A similar pattern of protein expression levels were identified in the 50-90 year old lenses, in which an age-dependent increase in UPR-associated protein levels was observed. These results demonstrated that UPR activation is common in aging lenses and may trigger the formation of ARC.

To elucidate whether activation of the UPR is involved in altering the antioxidant system to cause apoptosis, the levels of antioxidants and other UPR-associated proteins involved in apoptosis were assessed. The UPR also activates caspase-4 (caspase-12 in rodents) resulting in apoptosis (29,31) and generates excess levels of ROS, driven by PDI, Ero1-L $\alpha$  and Ero1-L $\beta$  (13-15). The production of ROS decreases the levels of cytosolic glutathione and contributes to an additional source of ROS from the mitochondria (32) resulting in apoptosis. The results from the present study were consistent with these findings. The initial step of UPR activation triggered the survival pathway by increasing the levels of PDI and Ero1-L $\beta$ . Notably, the levels of Ero1-L $\alpha$  decreased during the exposure period. The protein levels of catalase, SOD and GR decreased in the cells exposed for  $>24$  h. Similarly, activation of the UPR induced the apoptotic pathway, which was confirmed by elevated protein levels of ATF4, CHOP and caspase-4.

In conclusion, the present study demonstrated that the UPR was activated in HLECs, which was confirmed in human aged lenses. Furthermore, UPR activation generated excess levels of ROS and altered the antioxidant system. Prolonged exposure to UPR activation triggered apoptotic pathway signaling proteins, leading to lens oxidation or the formation of ARC. Thus, UPR activation is important in the formation of ARC.

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