Original Paper

Inhibition of Cartilage Acidic Protein 1 Reduces Ultraviolet B Irradiation Induced-Apoptosis through P38 Mitogen-Activated Protein Kinase and Jun Amino-Terminal Kinase Pathways

Yinghong Ji^a Xianfang Rong^a Dan Li^a Lei Cai^a Jun Rao^b Yi Lu^a

^aDepartment of Ophthalmology, Eye & ENT Hospital of Fudan University, Key Laboratory of Myopia of State Health Ministry, and Key Laboratory of Visual Impairment and Restoration of Shanghai, Shanghai,

^bJiangxi Cancer Hospital, Nanchang, China

Key Words

Cataract • UVB irradiation induced-apoptosis • CRTAC1 • p38 • JNK1/2

Abstract

Background/Aims: Ultraviolet B (UVB) irradiation can easily induce apoptosis in human lens epithelial cells (HLECs) and further lead to various eye diseases including cataract. Here for the first time, we investigated the role of cartilage acidic protein 1 (CRTAC1) gene in UVB irradiation induced-apoptosis in HLECs. **Methods:** Three groups of HLECs were employed including model group, empty vector group, and CRTAC1 interference group. **Results:** After UVB irradiation, the percentage of primary apoptotic cells was obviously fewer in CRTAC1 interference group. Meanwhile, inhibition of CRTAC1 also reduced both reactive oxygen species (ROS) production and intracellular Ca^{2+} concentration, but the level of mitochondrial membrane potential ($\Delta\Psi_m$) was increased in HLECs. Further studies indicated that superoxide dismutase (SOD) activity and total antioxidative (T-AOC) level were significantly increased in CRTAC1-inhibited cells, while the levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH) were significantly decreased. ELISA analysis of CRTAC1-inhibited cells showed that the concentrations of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were significantly decreased, but the concentration of interleukin-10 (IL-10) was significantly increased. Western blot analyses of eight apoptosis-associated proteins including Bax, Bcl-2, p38, phospho-p38 (p-p38), Jun amino-terminal kinases (JNK1/2), phospho-JNK1/2 (p-JNK1/2), calcium-sensing receptor (CaSR), and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) indicated that the inhibition of CRTAC1 alleviated oxidative stress and inflammation response, inactivated calcium-signaling pathway, p38 and JNK1/2 signal pathways, and eventually reduced UVB irradiation induced-apoptosis in HLECs. **Conclusion:** These results provided new insights into the mechanism of cataract development, and demonstrated that CRTAC1 could be a potentially novel target for cataract treatment.

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Yi Lu M.D., Ph.D.,

Department of Ophthalmology, Eye & ENT Hospital of Fudan University, Key Laboratory of Myopia of State Health Ministry, and Key Laboratory of Visual Impairment and Restoration of Shanghai, No. 83 Fenyang Road, Shanghai, 200031 (China)
Tel. +86-21-64377134-407, Fax +86-21-64377134-217; E-Mail luyieent@126.com

Introduction

Cataract is one of the most common eye diseases and can eventually lead to blindness [1, 2]. It is believed that cataract can be caused by some major risk factors including aging, UVB irradiation and hydrogen peroxide (H_2O_2) [3-5]. Among them, UVB irradiation is one of the most important factors for cataract development, which may cause abnormal DNA synthesis, DNA damage, decrease of glutathione, and inactivation of a number of important metabolic enzymes [6]. Previous study by Wölflé et al. revealed that UVB irradiation generated reactive oxygen species (ROS) which would damage lens DNA and proteins and further induced loss of transparency [7]. Li et al. concluded that UVB-induced cataract initiates with damages to HLECs, and apoptosis is an early event during the damage [8].

Apoptosis is a natural biological process involving complex molecular mechanisms and a number of signaling pathways [9]. UVB irradiation-induced apoptosis initiates a series of molecular processes such as inducing inflammation response and activating mitochondria-initiated cell death pathways [10]. Mitochondrial dysfunctions caused by the apoptosis include change of mitochondrial membrane potential, production of ROS, increased permeability of the transition pore, and release of cytochrome C. Ricci et al. revealed that mitochondria rapidly lost transmembrane potential and generated ROS during apoptosis [11]. Wu et al. also discovered that after exposure of human lens epithelial cells (HLE B-3) cells to UVB, $\Delta\Psi_m$ was decreased and the level of ROS was increased [1]. To counter the toxic damages of ROS, lenses have evolved antioxidant systems including both antioxidants and antioxidant enzymes, such as reduced glutathione (GSH), T-AOC, SOD, catalase (CAT), glutathione S-transferase (GST), and glutathione reductase/peroxidase (GR/Gpx) [12-14]. For example, during ursodeoxycholic acid treatment, the changes of MDA and T-AOC levels were determined to be contributing to the prevention of selenite-induced oxidative stress and alleviation of cataract formation [13].

Inflammatory response often occurs during UVB irradiation induced-apoptosis in HLECs. When studying on ultraviolet B radiation-induced cell death, Caricchio et al. determined the critical role of ultraviolet dose in inflammation [15]. Moreover, TNF- α is an important mediator of immunity and inflammation, and it was reported to be involved in UVB-induced apoptosis of keratinocytes [16]. Wozniacka et al. reported that three months of chloroquine treatment appeared to block UVB-induced up-regulation of IL-1 β , IL-6 and TNF- α expression in non-diseased skin of systemic lupus erythematosus patients [17].

It is well established that apoptosis is mainly regulated by Bcl-2 family proteins, in which Bcl-2 protein has negative effect in cellular apoptotic pathway while Bax protein (Bcl-2-homologous protein) can reverse the suppression effect of Bcl-2 in apoptosis [18, 19]. Bax expression is increased but Bcl-2 expression is decreased during UVB-induced apoptosis in cataract, suggesting both proteins play pivotal roles in cataract formation [2]. Experimental and clinical evidences have indicated that mitogen activated protein kinase (MAPK) signal pathway plays a key role in regulating cell apoptosis [20]. There are six distinct groups of MAPK involved in mammalian apoptosis, including p38 MAPK and JNK which are activated by extracellular stimuli such as ultraviolet irradiation, genotoxic agents, and oxidative stress. MAPK signal pathway could also be activated by growth factors as well as inflammatory and cytokine stimulation. Especially, p38 MAPK plays an important role in controlling apoptosis, cell cycle arrest, growth inhibition and differentiation, while JNK is initially activated in response to a variety of stress signals and plays a critical role in death receptor-initiated extrinsic as well as mitochondrial intrinsic apoptotic pathways [20, 21].

The homeostasis of cellular calcium (Ca^{2+}) is essential for maintaining lens clarity in the lens epithelial cells. Both abnormal Ca^{2+} levels and inappropriate Ca^{2+} signaling are related to many clinical disorders such as stroke, obesity, cardiac dysfunction, aging and Alzheimer's disease [22-25]. It is worth noting that Ca^{2+} signaling is a key element of apoptotic signaling pathways. Brnjic et al. showed that Ca^{2+} signaling is important in mediating sustained JNK activation during apoptosis, and cisplatin-induced apoptosis is dependent on generation of ROS and calcium signaling [26]. Cartilage acidic protein 1 (CRTAC1) is a matrix component

with high affinity for integrins. It is profusely expressed in cartilage, a mostly avascular tissue [27]. The corneal expression of CRTAC1 indicated that its function may be related to the avascular environs. CRTAC1 is reported to function as a calcium-binding protein and involved in calcium-signaling pathways, but its role in UVB irradiation induced-apoptosis in cataract has not been fully elucidated [28, 29].

Previously we have investigated HLECs under different intensities of UVB irradiation with different exposure time, and discovered the effects of UVB irradiation involved in regulation of apoptosis related genes, mitochondrial dysfunction and caspase program. In this study, we further investigated UVB irradiation induced-apoptosis in cataract by inhibiting CRTAC1 expression in HLECs. The levels of ROS, Ca^{2+} , $\Delta\Psi\text{m}$, and two apoptosis associate proteins (Bcl-2 and Bax) in HLECs were measured. Oxidative stress, inflammatory response, and apoptosis signaling pathways were also evaluated in order to understand the mechanisms of CRTAC1 gene involvement in UVB irradiation induced-apoptosis in the HLECs.

Materials and Methods

Cell culture

Human lens epithelial cell line (HLEC) was obtained from the Cell Bank of Academia Sinica (Shanghai, China), cultured using RPMI-1640 medium (Hyclone, China) supplemented with 10 % fetal bovine serum (FBS), 1 % glutamine and 1 % penicillin/streptomycin, and grown at 37 °C in an incubator (Thermo, USA) with 5 % CO_2 . Cells were seeded when growing into the log phase, and further grown to 80 % confluence for experiments.

UVB treatment

The UVB treatment was executed by using a UV-B radiometer (Photoelectric Instrument Factory, Beijing Normal University, China) with a total output of 4 W/m² at 297 nm wavelength. Before treatment, HLECs were washed twice with phosphate buffered saline (PBS, pH 7.4) to remove residual serum and non-attached cells. Washed HLECs were re-suspended in fresh medium and cultured in an incubator (Thermo, USA) at 37 °C with 5 % CO_2 .

Inhibition of CRTAC1 expression by siRNA transfection

CRTAC1 siRNA target sequence (NM_001206528.2) was cloned into pLVX-AcGFP-C1. The siRNA construct was transfected into HLECs using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's protocol. Inhibition efficiency of CRTAC1 was determined by real-time PCR and western blot analysis. Model group and empty vector group were used as negative controls (NC).

Flow cytometry analysis of apoptosis, ROS production, calcium concentration and mitochondrial membrane potential ($\Delta\Psi\text{m}$) level

Before analysis, the HLECs were trypsinized, centrifuged and re-suspended twice in PBS. Re-suspended HLECs (1×10^5 cell/mL) were treated under UVB irradiation at 2 W/m² for 60 min. For apoptosis detection, the HLECs were collected and incubated in 5 μL annexin buffer and 5 μL propidium iodide (PI) for 10 min; for ROS detection, the HLECs were incubated with culture medium containing 10 μM DCFH-DA for 30 min; and the HLECs were incubated with Fluo-3 (10 μM , Sigma) for 30 min for determination of intracellular calcium concentration. Additionally, the level of $\Delta\Psi\text{m}$ in HLECs was measured using cationic dye JC-1 assay kit as our previous study [2].

Determination of SOD, MDA, LDH, and T-AOC levels

To determine the oxidative stress status in UVB irradiation induced-apoptosis in CRTAC1-inhibited HLECs, the levels of SOD, MDA, LDH and T-AOC were measured using commercial test kits according to the protocols of the manufacturers. These commercial test kits were MDA kit (Njjcbio A003), SOD kit (Njjcbio A001), LDH kit (Njjcbio A020-1), and T-AOC kit (Njjcbio A015), all purchased from the Jiancheng Bioengineering Institute (Nanjing, China).

ELISA analysis of TNF- α , IL-6 and IL-10

Inflammatory responses in HLECs were evaluated by analyzing the concentrations of three pro-inflammatory cytokines: TNF- α , IL-6 and IL-10. ELISA kits (Biosource International, Camarillo, California, USA) were used according to the manufacturer's instructions.

Real-time PCR analysis of CRTAC1 gene expression

The CRTAC1 gene expression was analyzed by real-time PCR. Total RNA was isolated using Trizol (Invitrogen, USA). The cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, USA). Real-time quantitative PCR was performed using an ABI 7300 (Applied Biosystem, USA) thermal cycler. The reaction volume was 25 μ L containing 12.5 μ L SYBR Green Mix, 0.5 μ L each of forward and reverse primers, 9.5 μ L RNase-free water and 2 μ L cDNA. The PCR program was as following: DNA denaturing at 95 °C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. The PCR primers for amplification of CRTAC1 gene were: 5'- CTGCGACAATGAGAATGG -3' (forward) and 5'- CATCACGGTTGAAGTCAG -3' (reverse); the PCR primers for internal control gene, GAPDH, were 5'-CACCCACTCCTCCACCTTTG-3' (forward) and 5'-CCACCACCCTGTTGCTGTAG-3' (reverse). The mRNA expression of CRTAC1 gene was compared with GAPDH expression and the result was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Total proteins of HLECs were extracted using RIPA lysis buffer as previously reported [3]. Bio-Rad Protein Assay Kit was used to quantify the extracted proteins, and 20 μ g proteins of each sample were subjected to 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting and assay were carried out using the following antibodies: CRTAC1 antibody (abcam, Ab102548), Bax antibody (Santa, Sc-493), Bcl-2 antibody (Santa, Sc-492), p38 antibody (CST, #9212), p-p38 antibody (CST, #9211), JNK1/2 antibody (CST, #9252), p-JNK1/2 antibody (CST, #9255), CasR antibody (abcam, Ab137409), CaMKII antibody (abcam, Ab52476), and GAPDH antibody (CST, #5174). GAPDH protein was used as internal control. The band intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Each sample was analyzed three times and the data was presented as mean value $\pm$ SD. Statistical analyses including one-way analysis of variance (ANOVA) were performed using SPSS 17.0 software. Statistical significance was marked * ($p < 0.05$) or ** ($p < 0.01$).

Results

Inhibition of CRTAC1 expression in HLECs

In this study, we intended to investigate the role of CRTAC1 gene in UVB-induced apoptosis in HLECs. Three groups of HLECs were employed including model group, empty vector group, and CRTAC1 interference group. CRTAC1 expression in each group was investigated at both transcriptome and proteome levels. As shown in Fig. 1A, the expression of CRTAC1 gene in interference group was only 0.05, much lower than those in model group and empty vector group (the expressions of CRTAC1 gene in model group and empty vector group were nearly the same). Western blot analysis (Fig. 1B) showed that CRTAC1 protein in interference group was also lower than those in the other two groups (CRTAC1 protein levels in model group and empty vector group were also quite similar). These results confirmed the decreased expression of CRTAC1 gene in the interference group, while the expression of CRTAC1 gene in empty vector group had no obvious difference from that in model group.

Inhibition of CRTAC1 expression reduced UVB irradiation induced-apoptosis in HLECs

Previous studies have shown that UVB irradiation could efficiently induce HLECs apoptosis in a time and dose dependent manner [2]. The annexin V-FITC/propidium iodide (PI) staining results revealed that the percentage of primary apoptotic cells in CRTAC1 interfe-

rence group was fewer than those in model group and empty vector group (Fig. 2). The percentages of late apoptotic cells and necrotic cells in interference group were similar to those in the other two groups. The results indicated that inhibition of CRTAC1 expression could reduce UVB irradiation induced-apoptosis in HLECs.

Inhibition of CRTAC1 reduced ROS production and intracellular Ca^{2+} concentration, but induced a higher level of $\Delta\Psi\text{m}$

Oxidative stress has been reported to be associated with UVB irradiation induced-apoptosis in the development of cataract. To evaluate the status of oxidative stress in HLECs after exposure to UVB irradiation, a dye named DCFH-DA was used for the detection of ROS. Measured DCFH-DA fluorescence intensity in the CRTAC1 interference group was significantly lower when compared to those in model group and empty vector group ($p < 0.01$), indicating ROS level in HLECs was decreased after inhibition of CRTAC1 expression (Fig. 3A).

Previous reports showed that the elevation of intracellular free Ca^{2+} concentration was involved in apoptosis development [26]. Here, we confirmed the role of Ca^{2+} concentration in the model group by 1, 2-bis (2-amino-phenoxy) ethane- N,N,N',N' -tetraacetic acid-tetraacetoxymethyl ester (BAPTA-AM) treatment as the previous study

[30]. As shown in Fig. 4 and Fig. 5, chelating intracellular Ca^{2+} with BAPTA-AM reduced Ca^{2+} concentration and inhibited the apoptosis in HLECs. On the other hand, the effect of inhibition of CRTAC1 on the intracellular Ca^{2+} concentration was also determined. In CRTAC1 interference group, the intracellular Ca^{2+} concentration was 87.19 nM, while in model group and empty vector group, the intracellular Ca^{2+} concentrations were 184.87 nM and 180.67 nM, respectively (Fig. 3B). The results demonstrated that the intracellular Ca^{2+} concentration in HLECs was significantly decreased after inhibition of CRTAC1 expression.

The level of $\Delta\Psi\text{m}$ was also measured to further investigate the effects of inhibition of CRTAC1 on the status of oxidative stress of mitochondrial in HLECs. Obviously, the level of $\Delta\Psi\text{m}$ in HLECs of CRTAC1 interference group was significantly higher than those in model group and empty vector group ($p < 0.01$) (Fig. 3C), indicating the inhibition of CRTAC1 expression may lower oxidative stress and mitochondrial dysfunction in HLECs.

Fig. 1. The confirmation of CRTAC1 expression in interference group. (A) Real-time PCR analysis of CRTAC1 gene expression; (B) Western blot analysis of CRTAC1 proteins. ** represents p value among those three groups less than 0.01.

Fig. 2. Flow cytometry analysis of apoptosis exposure to UVB irradiation in HLECs.

Measurement of SOD, MDA, LDH and T-AOC levels

SOD, MDA, LDH, and T-AOC levels associated with oxidative stress in HLECs were determined using commercial assay kits (Table 1). Both SOD and T-AOC levels in CRTAC1 interference group were significantly increased when compared to those in model group and empty vector group ($p < 0.01$). On the contrary, the levels of MDA and LDH in HLECs were significantly decreased in CRTAC1 interference group when compared to those in model group and empty vector group ($p < 0.01$). These results again suggested that the inhibition of CRTAC1 expression lowered oxidative stress in HLECs.

Measurement of TNF- α , IL-6 and IL-10 concentrations

It is well known that inflammatory responses often occur after exposure to UVB irradiation, so we investigated the inflammatory responses in the three HLECs groups after exposure to UVB irradiation. The levels of TNF- α , IL-6 and IL-10 were measured by ELISA. The concentrations of TNF- α and IL-6 in CRTAC1 interference group were significantly decreased when compared to those in model group and empty vector group ($p < 0.01$, Fig. 6A and Fig 6B). However, the concentration of IL-10 in CRTAC1 interference group was 282.29 ng/L, significantly higher than those in model group and empty vector group ($p < 0.01$, Fig. 6C). The results suggested that inhibition of CRTAC1 expression alleviated inflammatory response in HLECs exposed to UVB irradiation.

Western blot analysis of Ca²⁺ associated-proteins, apoptosis-regulated proteins, and proteins involved in p38 MAPK and JNK pathways

Ca²⁺ associated-proteins, apoptosis-regulated proteins, and proteins involved in p38 MAPK and JNK pathways were analyzed by western blot (Fig. 7 and Table 2). The calcium-sensing receptor (CaSR) level is closely related to intracellular Ca²⁺ concentration in HLECs,

Fig. 3. Flow cytometry analysis of ROS production (A), calcium concentration (B) and mitochondrial membrane potential ($\Delta\Psi_m$) level (C) exposure to UVB irradiation in HLECs. ** represents p value among those three groups less than 0.01.

Fig. 4. Flow cytometry analysis of calcium concentration in model group (A) and treatment group (B). HLECs were treated by 10 μ mol/L BAPTA-AM in treatment group.

Fig. 5. Flow cytometry analysis of apoptosis cells in model group (A) and treatment group (B). HLECs were treated by 10 μ mol/L BAPTA-AM in treatment group.

Table 1. Determination of levels of SOD, MDA, LDH, and T-AOC exposure to irradiation in HLECs. ** represents *p* value among those three groups less than 0.01

Group	Model group	Empty vector group	CRTAC1 interference group
SOD activity (U/mL)	149.56 $\pm$ 1.69	150.96 $\pm$ 0.47	170.9 $\pm$ 0.97**
MDA concentration (nmol/mL)	5.56 $\pm$ 0.44	5.86 $\pm$ 0.61	1.61 $\pm$ 0.11**
LDH activity (U/L)	953.27 $\pm$ 98.46	1015.58 $\pm$ 94.08	436.14 $\pm$ 30.04**
T-AOC level	17.02 $\pm$ 1.18	15.62 $\pm$ 2.35	26.31 $\pm$ 0.74**

Table 2. The exact values of the intensity of western blot analysis including CaSR, Bax, Bcl-2, p-p38, CaMKII, p38, p-JNK1/2, and JNK1/2 proteins. GAPDH protein was used as an internal control

Protein Name	Model group	Empty vector group	CRTAC1 interference group
CaSR	11824.974	12474.56	5661.104
Bax	17970.673	18739.288	4037.731
Bcl-2	6276.004	7927.075	16802.024
p-P38	16437.066	15709.924	1957.456
CaMK II	8791.317	8983.903	1804.426
P38	28309.752	28676.581	26914.581
p-JNK1/2	11769.338	11908.288	3052.669
JNK1/2	25483.539	24057.075	23082.196
GAPDH	30068.054	30904.681	31790.024

and the western blot result revealed that CaSR protein level in CRTAC1 interference group was decreased when compared to CaSR levels in model group and empty vector group. Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is another Ca²⁺-associated protein which

Fig. 6. ELISA analysis of TNF- α , IL-6 and IL-10 concentrations exposure to UVB irradiation in HLECs. ** represents *p* value among those three groups less than 0.01.

Fig. 7. Western blot analysis of CaSR, Bcl-2, Bax, p-p38, CaMKII, p38, p-JNK1/2, and JNK1/2 proteins exposure to UVB irradiation in HLECs. GAPDH protein was used as an internal control.

Fig. 8. Western blot analysis of Bcl-2 and Bax proteins in model group and treatment group (treated by JNK inhibitor). GAPDH protein was used as an internal control.

is known for its roles in regulating calcium homeostasis, and when investigated by western blot, its level in CRTAC1 interference group was lower than those in the other two groups.

The protein levels of two apoptosis-related proteins were also determined: Bcl-2

is a suppressor of apoptosis, and Bax is a contributor to apoptosis. The expression of Bax protein in CRTAC1 interference group was lower than in model group and empty vector group. On the contrary, the expression of Bcl-2 protein was higher than in the other two groups.

Four other proteins, p-p38, p38, p-JNK1/2, and JNK1/2, are components of p38 MAPK and JNK pathways, and they have been reported to function in the control of apoptosis. JNK pathway is initially activated in response to a variety of stress signals, and p38 MAPK pathway is strongly activated by environmental and genotoxic stresses. Especially, the JNK signaling pathway could affect members of the Bcl-2 family by inactivating Bcl-2 proteins but activating Bax proteins, which was also demonstrated here by the JNK inhibitor in western blot analysis (Fig. 8) as the previous study [31]. In this study, the expression of p-p38 protein was decreased in CRTAC1 interference group, when compared to its levels in model group and empty vector group, but the expression of p38 protein in CRTAC1 interference group was very similar to those in the other two groups. For the JNK pathway, the expression of p-JNK1/2 protein in CRTAC1 interference group was lower than in model group and empty vector group, while the expressions of JNK1/2 protein was similar in all three groups. These results indicated that CRTAC1 gene might be involved in UVB irradiation induced-apoptosis in HLECs by regulating the expression of apoptotic proteins (Bcl-2 and Bax) or proteins associated with calcium signaling, p38 MAPK and JNK pathways.

Discussion

Cataract is one of the leading eye diseases that cause vision loss, and although it's possible to treat cataract by surgery to remove the diseased lens and replaced with a clear one, it is still difficult to have efficient control of cataract in the near future [32-34]. One major obstacle in treating cataract is that the underlying mechanism of cataract development is still not clearly understood. Previous reports have indicated that UVB irradiation can inflict damages to HLECs, induce apoptosis, and eventually cause cataract. In the present study, we further investigated UVB irradiation induced-apoptosis and cataract in HLECs by focusing on the function of CRTAC1 gene. Three HLEC groups, the model group (regular HLECs), CRTAC1 interference group (inhibition of CRTAC1 expression through siRNA interference), and empty vector control group (HLECs transfected with interference construct vector only) were used in the study. The percentage of primary apoptotic cells, ROS production, Ca^{2+} concentration, and $\Delta\Psi_m$ level, were determined in all three groups of HLECs. Moreover, the levels of SOD, MDA, LDH and T-AOC associated with oxidative stress, concentrations of TNF- α , IL-6 and IL-10 that are involved in inflammatory responses, and proteins participating in regulation of apoptosis and apoptotic signaling pathways (including Ca^{2+} signaling, p38 MAPK and JNK pathways) were also determined. These results are useful in understanding the role of CRTAC1 gene in UVB irradiation induced-apoptosis and cataract in HLECs.

Apoptosis is an early event in the development of UVB-induced cataract. During apoptosis, mitochondria in lens cells rapidly generate reactive oxygen species (ROS), and high levels of ROS could put lens cells at risk of oxidative stress [35]. We have previously discovered that UVB irradiation could efficiently cause apoptosis in HLECs in both time-dependent and dose-dependent manner. ROS production in UVB irradiated HLECs was increased, and levels of T-AOC, MDA and SOD (which are associated with oxidative stress) changed accordingly, suggesting mitochondrial dysfunction in HLECs after UVB irradiation. In CRTAC1 interference group HLECs, real time PCR and western blot analysis both confirmed the inhibition of CRTAC1 gene expression. When CRTAC1 interference group cells were exposed to UVB irradiation, ROS production, MDA concentration, and LDH activity were decreased; $\Delta\Psi_m$ level, SOD activity and T-AOC level were increased. These results indicate less apoptosis, lower oxidative stress, and less mitochondrial dysfunction in HLECs after inhibition of CRTAC1.

UVB-induced apoptosis in HLECs is a multifactorial event including inflammatory responses that generate various inflammatory cytokines and chemokines [36]. It has been demonstrated that an important pro-inflammatory cytokines, TNF- α , could initiate apoptotic

cell death in UVB-irradiated HLECs apoptosis. Schwarz et al. reported that UVB light induces the release of TNF- α by keratinocytes in sunburn cells (apoptotic keratinocytes) [37]. Two other pro-inflammatory cytokines, IL-6 and IL-10, were also reported to be involved in UVB-induced apoptosis. IL-6 expression is significantly lower in UVB-irradiated skin cells while large amounts of IL-10 are produced in the meantime [17, 38]. In our study we found that inhibition of CRTAC1 gene expression could reduce the amounts of TNF- α and IL-6, but increase IL-10 in interference group. The results indicate that inhibition of CRTAC1 gene could possibly play a role in reducing UVB-induced apoptosis via alleviating inflammatory responses.

Many proteins are involved in the regulation of apoptosis, and Bcl-2 protein family is the most important contributor [39]. Among this family, Bcl-2 protein is a suppressor of apoptosis while Bax protein is a contributor to apoptosis. We measured the expression of these two apoptosis-related proteins, and western blot analysis revealed that in CRTAC1 interference group, Bax protein level was decreased and Bcl-2 was increased. The results suggest that inhibition of CRTAC1 gene expression may reduce UVB-induced apoptosis by regulating Bax and Bcl-2 protein levels.

The apoptotic signaling pathways are essential for oxidative stress-induced apoptosis in HLECs, especially pathways involving mitogen-activated protein kinases (MAPK) pathways such as p38 and Jun amino-terminal kinases (JNK) pathways [20]. So we further evaluated the expression of four proteins involved in p38 and JNK pathways, and western blot results showed that the expressions of p-p38 and p-JNK1/2 proteins were decreased in CRTAC1 interference group, indicating less activated p38 and JNK pathways. The findings indicate that inhibition of CRTAC1 may reduce apoptosis by inactivating p38 and JNK signaling pathways. Moreover, the lower levels of Ca²⁺, CaSR, and CaMKII protein in CRTAC1 interference group suggest that inhibition of CRTAC1 may also result in less activated calcium-signaling pathways. Altogether, the inactivation of calcium-signaling pathways could further lead to inactivation of MAPK signal pathways including p38 and JNK signaling pathways, which could reduce oxidative stress and apoptosis in UVB irradiated HLECs, and finally delay the development of cataract.

In summary, here for the first time we identified the possible roles of CRTAC1 gene *in vitro* experiments, and revealed the underlying mechanism of CRTAC1 gene involvement in UVB irradiation induced-apoptosis in the HLECs. The information from our study could serve as a baseline for future studies (such as *in vivo* experiments) especially those aimed at discovering potential new prevention and treatment strategies for cataract.

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Disclosure Statement

The authors declare no competing financial interest.

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