NF- κ B/Cartilage Acidic Protein 1 Promotes Ultraviolet B Irradiation-Induced Apoptosis of Human Lens Epithelial Cells

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The apoptosis of human lens epithelial cells (HLECs) is a characteristic change that occurs during the development of cataracts. Ultraviolet B (UVB) is known to induce the generation of reactive oxygen species (ROS) and apoptosis in HLECs, and thus cause cataracts. Previously, we reported the functions of cartilage acidic protein 1 (CRTAC1) in UVB-treated HLECs. However, the underlying mechanism was not known. In this study, we found that CRTAC1 expression and nuclear factor-kappa B (NF- κ B) p65 nuclear translocation were elevated in capsule tissues of cataract patients in comparison with normal controls. The NF- κ B inhibitor, pyrrolidine dithiocarbamate (PDTC), alleviated UVB-induced apoptosis in HLECs; while activation of NF- κ B suppressed the effects of the ROS inhibitor, N-acetyl-L-cysteine (NAC), on UVB-treated HLECs. The expression and promoter activity of CRTAC1 was inhibited by PDTC and NAC. Moreover, the suppressed effects of CRTAC1 knockdown on UVB-induced ROS generation, cell apoptosis, nuclear translocation of NF- κ B p65, and p38 phosphorylation were attenuated by a p38 agonist. In contrast, the p38 inhibitor abolished the promotional effects of CRTAC1 overexpression on HLECs. Taken together, our results for the first time show that NF- κ B is a potential transcription factor for CRTAC1. The regulatory network involving NF- κ B, CRTAC1, and p38 may therefore play an important role in cataract formation.

Keywords: cataract, CRTAC1, NF- κ B, UVB, p38

Introduction

CATARACT IS THE MOST COMMON cause of blindness worldwide (>50%) (Pascolini and Mariotti, 2012). Our previous study with an adult Chinese population suggested that cataract is also the leading cause of monocular (61.2%) and binocular (66.8%) visual impairment in patients >60 years of age in China (Tang *et al.*, 2015). Numerous studies have identified risk factors for cataract, which mainly include age, trauma, environment, diseases, and genetics. The aging and apoptosis of human lens epithelial cells (HLECs), which are accelerated by oxidative stress-induced DNA damage, are a characteristic change that occurs during the development of cataracts (Li and Spector, 1996; Truscott, 2005). Ultraviolet (UV) light, especially ultraviolet B (UVB), is the most important environmental factor that causes cataracts (Li and Spector, 1996; Truscott, 2005; Ji *et al.*, 2015). UVB irradiation induces the formation of reactive oxygen species (ROS) and increases cell apoptosis in HLECs, thus causing the formation of cataracts (Li and Spector, 1996; Ji *et al.*, 2015, 2016).

Nuclear factor-kappa B (NF- κ B), a fast-acting transcription factor, ubiquitously resides in the cytoplasm as a p50/p65 heterodimer in an inactive form by binding with its inhibitor, I κ B (Maeda and Omata, 2008; Oeckinghaus and Ghosh, 2009; Tornatore *et al.*, 2012). Increasing evidence has shown that it can be activated by a wide variety of stimuli, including tumor necrosis factor- α (TNF- α), oxidants, and UV (Van Antwerp *et al.*, 1996; Dudek *et al.*, 2001; Muthusamy and Piva, 2010). Activated NF- κ B dissociates from I κ B and translocates into the nucleus, where it binds to the promoter of target genes and regulates the expression of these target genes (Maeda and Omata, 2008; Oeckinghaus and Ghosh, 2009; Tornatore *et al.*, 2012). The translocation of NF- κ B can inhibit or stimulate cell apoptosis in a variety of cell types (Van Antwerp *et al.*, 1996; Choi *et al.*, 2000; Dudek *et al.*, 2001; Yang *et al.*, 2008). In response to UVB, NF- κ B translocates into the nucleus and stimulates ROS production and cell apoptosis in HLECs cells (Lee *et al.*, 2005; Yao *et al.*, 2008; Srivastava and Ramana, 2009). Because damage to the epithelium initiates UV-induced cataracts, we hypothesized that NF- κ B may play a role in the pathogenesis of cataracts.

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The cartilage acidic protein 1 (*CRTAC1*) gene was first cloned from chondrocytes by Steck *et al.* (2001). *CRTAC1* was mapped to human chromosome 10q22, encoding a protein containing a Ca^{2+} -binding epidermal growth factor-like domain (Steck *et al.*, 2001, 2007). BMP4 stimulation leads to elevated expression of *CRTAC1* in human chondrocytes (Steck *et al.*, 2007). Previously, we reported that *CRTAC1* knockdown inhibited UVB-induced ROS production, cell apoptosis, TNF- α release, and p38 phosphorylation in HLECs (Ji *et al.*, 2016). However, the upstream regulators of *CRTAC1* in UVB-exposed HLECs still need to be determined.

In the present study, we showed that *CRTAC1* expression and NF- κ B p65 nuclear translocation were elevated in lens anterior capsular specimens from patients with cataracts. Using UVB-treated HLECs as a cell model, *in vitro* experiments suggested that *CRTAC1* was a candidate target gene of NF- κ B. In addition, *CRTAC1* overexpression promoted ROS generation and cell apoptosis via the activation of the p38 signaling pathway. Overall, our study suggested that a regulatory network involving NF- κ B, *CRTAC1*, and p38 may play an important role in cataract formation.

Materials and Methods

Human lens capsule specimens

The study protocol was reviewed and approved by the Ethics Committee of Eye & ENT Hospital of Fudan University (Shanghai, China; IRB number: 2013021). Anterior lens capsules were obtained from four cataract patients without other ocular diseases at the Department of Ophthalmology, the Eye & ENT Hospital of Fudan University (Shanghai, China), while control anterior lens capsules were obtained from the Shanghai Red Cross Eye Bank (Shanghai, China; Supplementary Table S1). Written informed consent was obtained from all participants before enrollment in the study.

Western blot assays

Cytoplasmic and nuclear fraction lysates were prepared from the lens capsule specimens and HLECs using a nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein quantification was conducted using a bicinchoninic acid assay kit (Sigma-Aldrich, St. Louis, MO). The lysates (25 μ g per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blot assays were conducted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a protein loading control. The sources of primary antibodies were as follows: *CRTAC1* (#ab102548, RRID:AB_10710442; Abcam, Cambridge, United Kingdom), p65 (#8242, RRID:AB_10859369; Cell Signaling Technology, Danvers, MA), histone H3 (#4499, RRID:AB_10544537; Cell Signaling Technology), phospho-p38 MAPK (#9211, RRID:AB_331641; Cell Signaling Technology), p38 MAPK (#9212, RRID:AB_330713; Cell Signaling Technology), and GAPDH (#5174, RRID:AB_10622025; Cell Signaling Technology).

Cell culture and UVB exposure

HLECs (Cell Bank of Academia Sinica, Shanghai, China) were maintained in Roswell Park Memorial Institute

(RPMI)-1640 medium (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS) in an incubator at 37°C in a 5% CO_2 incubator. HLECs at 80% confluence were washed twice with phosphate-buffered saline (PBS, pH 7.4), and exposed to 2 W/m^2 of UVB (wavelength: 297 nm; Photoelectric Instrument Factory, Beijing Normal University, Beijing, China) for 10 min as previously described (Ji *et al.*, 2016). After UVB irradiation, PBS was removed and fresh RPMI-1640 medium containing 10% FBS was added. Then, the cells were cultured for 24 h in the incubator and used for subsequent experiments.

Overexpression and inhibition of *CRTAC1*

To overexpress *CRTAC1*, its complete coding sequence was amplified and cloned into pLVX-puro (Clontech, Palo Alto, CA) with the following primers: *CRTAC1* forward, 5'-CGGAATTCATGGCTCCGAGCGCTGAC-3' and reverse, 5'-CGGGATCCTCAGCAGCTGGGCTCGC-3'. The empty vector served as a negative control. To prepare lentivirus, the constructs and packaging plasmids (psPAX2 and pMD2.G; Addgene, Watertown, MA) were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol, and 48–72 h later, lentiviruses were collected from the culture medium to infect HLECs.

To inhibit *CRTAC1* expression, *CRTAC1* siRNA (si*CRTAC1*, 5'-GCAGUGCCUCGGAUAUCUUUU-3') was purchased from Genepharma (Shanghai, China) and transfected into HLECs with Lipofectamine 2000 according to the manufacturer's protocol. Control siRNA (siNC; 5'-CAGUACUUUUGUGUAGUACAA-3') served as a negative control.

Experimental groups

To assess the function of NF- κ B in UVB-damaged HLECs, three groups were assigned: control, cells without UVB irradiation; UVB+Veh, cells treated with vehicle [dimethyl sulfoxide (DMSO)] for 1 h, and then exposed to UVB for 10 min; UVB+PDTC, cells were treated with 10 mM pyrrolidine dithiocarbamate (PDTC; Selleck Chemicals, Houston, TX) for 1 h, and then exposed to UVB for 10 min.

To characterize the relationship between NF- κ B and ROS, four groups, including control, UVB+Veh, UVB+N-acetyl-L-cysteine (NAC), and UVB+NAC+TNF- α , were assigned. Cells in the control group did not receive any treatment. Cells in the other three groups were exposed to UVB for 10 min. Before UVB exposure, cells in the UVB+Veh, UVB+NAC, and UVB+NAC+TNF- α were treated with DMSO, 10 mM NAC (Selleck Chemicals), and 10 mM NAC and 10 ng/mL TNF- α (Sigma-Aldrich), respectively.

To investigate the role of p38 and *CRTAC1* in UVB-treated HLECs, HLECs were transfected with siNC or si*CRTAC1* for 24 h, treated with 10 μ M anisomycin (Selleck Chemicals) or DMSO (Veh) for 1 h, and then exposed to UVB for 10 min.

To investigate the role of p38 activity in *CRTAC1* gene-induced apoptosis, HLECs were infected with lentivirus-expressing vector or *CRTAC1* overexpression (*CRTAC1* OE) for 24 h, and then treated with 10 μ M SB203580 (Selleck Chemicals).

Detection of apoptosis and ROS production

The HLECs were collected by trypsinization and washed twice with PBS. To detect cell apoptosis, the cells were incubated with annexin V-fluorescein isothiocyanate, and propidium iodide (Beyotime) at room temperature for 15 min. For analysis of ROS generation, the cells were probed with 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (Beyotime) for 20 min at 37°C. At the end of incubation, the samples were analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ).

Dual luciferase reporter assay

The CRTAC1 promoter sequence was cloned into a pGL3-basic plasmid (Promega, Madison, WI). HLECs were plated onto 12-well plates. After culturing overnight, the cells were transfected with CRTAC1 promoter plasmid and pRL-TK (Promega) with Lipofectamine 2000, and then treated as indicated. At 24 h after treatment, the cells were lysed and the dual luciferase reporter assay system (Promega) was used to determine luciferase activity according to the manufacturer's protocol.

Statistical analysis

The data from capsule tissues are expressed as the mean with 95% confidence intervals (CI). The results from three independent *in vitro* experiments are shown as the mean $\pm$ standard deviation (SD). Student's *t*-tests and one-way analysis of variance were performed to analyze the differences between two groups and among more than two groups, respectively, using SPSS statistical software for Windows, version 17.0. A value of $p < 0.05$ was considered statistically significant.

Results

CRTAC1 expression and NF- κ B p65 nuclear translocation were elevated in capsule tissues of cataract patients

We first evaluated protein expression of CRTAC1 and nuclear translocation of NF- κ B p65 in capsule tissues from cataract patients and normal controls by using western blotting (Fig. 1). Capsule tissues from cataract patients showed higher levels of CRTAC1 and nuclear translocation of NF- κ B p65 than those from normal controls. These findings suggested the involvement of CRTAC1 and NF- κ B in cataract formation.

NF- κ B inhibitor alleviated UVB irradiation-induced ROS production, cell apoptosis, and CRTAC1 expression

To assess the possible role of NF- κ B in cataract formation, HLECs were treated with an NF- κ B inhibitor, PDTC, followed by exposure to UVB. Figure 2A and B, show that PDTC significantly alleviated UVB treatment-induced ROS production and cell apoptosis ($6.47\% \pm 0.49\%$ vs. $15.3\% \pm 1.25\%$) when compared with vehicle. Furthermore, PDTC inhibited protein expression, mRNA expression, and promoter activity of CRTAC1 in UVB irradiation-exposed HLECs (Fig. 2C–E). Together, these results suggested that

NF- κ B was a potential transcription factor for CRTAC1 and correlated with UVB-induced HLECs apoptosis.

Activation of NF- κ B suppressed the effects of ROS inhibitor on UVB irradiation-damaged HLECs

It has been reported that the ROS inhibitor, NAC, significantly alleviates UVB irradiation-induced ROS production and cell apoptosis in HLECs (Rong *et al.*, 2019). The release of TNF- α , which induces nuclear translocation of NF- κ B p65 (Van Antwerp *et al.*, 1996), was reported to be elevated in UVB-treated HLECs (Ji *et al.*, 2016). In the present study, TNF- α exposure further promoted ROS production and apoptosis in UVB irradiation-induced cells (Supplementary Fig. S1). Moreover, HLECs were pretreated with TNF- α in the presence of NAC, and then exposed to UVB irradiation. NAC treatment resulted in attenuated ROS production (Fig. 3A), cell apoptosis (Fig. 3B), nuclear translocation of NF- κ B p65 (Fig. 3C), and CRTAC1 transcription (Fig. 3D–E) in response to UVB treatment; while the effects of NAC were clearly abolished by TNF- α . Taken together, the results showed that NF- κ B mediated ROS production and cell apoptosis of HLECs in response to UVB irradiation.

The p38 was a potential downstream factor of CRTAC1 in UVB-damaged HLECs

Our previous study reported that CRTAC1 knockdown suppressed the p38 signaling pathway in UVB-exposed HLECs (Ji *et al.*, 2016). To further investigate whether the p38 pathway was involved in the function of CRTAC1, HLECs transfected with siCRTAC1 were treated with the p38 agonist, anisomycin, while HLECs transfected with CRTAC1 OE were treated with a p38 inhibitor, SB203580. After UVB irradiation, CRTAC1 knockdown reduced ROS production (Fig. 4A), cell apoptosis (Fig. 4B), nuclear translocation of NF- κ B p65 (Fig. 4C), and p38 phosphorylation (Fig. 4D), which were completely reversed by anisomycin. In contrast, CRTAC1 overexpression increased ROS production, cell apoptosis, nuclear translocation of NF- κ B p65, and p38 phosphorylation, which were abolished by SB203580 (Fig. 5). Together, these results suggested that the effects of CRTAC1 on ROS and apoptosis were mediated by the p38 signaling pathway in UVB-exposed HLECs.

Discussion

CRTAC1 is a glycosylated extracellular matrix molecule, whose biological functions are not well-known. A few studies have reported its expression and functions in chondrocytes and cartilage tissues (Steck *et al.*, 2001, 2007). UVB exposure of HLECs is a widely used cell model to study the formation of cataracts (Li and Spector, 1996; Ji *et al.*, 2015, 2016). Previously, we reported that UVB-induced ROS generation and cell apoptosis were significantly attenuated in HLECs when CRTAC1 expression was knocked down (Ji *et al.*, 2016). In the present study, CRTAC1 overexpression promoted ROS generation and cell apoptosis of HLECs with or without UVB exposure (Supplementary Fig. S2). CRTAC1 protein expression and NF- κ B p65 nuclear translocation were elevated in lens

FIG. 1. CRTAC1 expression and NF- κ B p65 nuclear translocation were elevated in capsule tissues of cataract patients. (A) CRTAC1 expression, cytoplasmic p65, and nuclear p65 in capsule tissues from four cataract patients (C1–C4) and four normal controls (N1–N4) were assessed by western blotting. (B) The intensity of bands was quantified with GAPDH as a loading control. The results are expressed as the mean with 95% confidence intervals. ** $p < 0.01$; *** $p < 0.001$. CRTAC1, cartilage acidic protein 1; NF- κ B, nuclear factor-kappa B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

FIG. 2. The NF- κ B inhibitor alleviated UVB irradiation-induced cell apoptosis and CRTAC1 expression. HLECs were assigned to three groups: Control, UVB+Veh, and UVB+PDTC, and treated as described in the Materials and Methods section. ROS production (A) and cell apoptosis (B) were measured at 24 h post-UVB irradiation. Annexin V-negative/propidium-positive cells underwent apoptosis. CRTAC1 protein (C) and mRNA (D) expression were determined at 24 h after UVB irradiation. (E) The CRTAC1 promoter activity was assessed with dual luciferase reporter assays. *** $p < 0.001$ versus Control; ### $p < 0.001$ versus UVB+Veh. UVB, ultraviolet B; HLEC, human lens epithelial cell; Veh, vehicle; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species.

FIG. 3. Activation of NF- κ B suppressed the effects of ROS inhibitor on UVB irradiation-treated HLECs. HLECs, which were assigned to four groups and treated as described in the Materials and Methods section: Control, UVB+Veh, UVB+NAC, and UVB+NAC+TNF- α . At 24h after UVB irradiation, ROS production (A), cell apoptosis (B), CRTAC1 protein expression, cytoplasmic p65 and nuclear p65 (C), as well as mRNA expression (D) and promoter activity (E) of CRTAC1 were measured. *** p <0.001 versus Control; ## p <0.01; ### p <0.001 versus UVB+Veh; + p <0.05; ++ p <0.01; +++ p <0.001 versus UVB+NAC. TNF- α , tumor necrosis factor- α ; NAC, N-acetyl L-cysteine.

anterior capsules from patients with cataracts, suggesting a close association among CRTAC1, NF- κ B, and cataract formation.

UVB radiation, which is associated with cataract formation, induces ROS generation and nuclear translocation of NF- κ B (Taylor *et al.*, 1988; Yao *et al.*, 2009). NF- κ B, a transcription factor, has been suggested to stimulate ROS production and cell apoptosis in HLECs cells (Lee *et al.*, 2005; Yao *et al.*, 2008; Srivastava and Ramana, 2009). The NF- κ B signaling pathway has been reported to mediate

protective effects against H₂O₂-induced apoptosis and oxidative stress in HLECs (Bai *et al.*, 2018; Gu, 2018; Huang *et al.*, 2018). NF- κ B is also implicated in H₂O₂-induced injury in HLECs, which may be followed by cataractogenesis (Jin *et al.*, 2007). Consistent with these previous reports, in the present study, NF- κ B inhibitor PDTC significantly attenuated UVB-induced HLECs apoptosis. Inflammatory responses are frequently activated during UVB-induced cells apoptosis (Yoshizumi *et al.*, 2008). As an important cytokine, TNF- α can activate NF- κ B and initiate apoptosis

FIG. 4. p38 was a potential downstream factor of CRTAC1 in UVB-damaged HLECs. HLECs were transfected with CRTAC1 siRNA (siCRTAC1) or control siRNA (siNC) for 24 h, treated with 10 μ M anisomycin or DMSO (Veh) for 1 h, and then exposed to UVB irradiation for 10 min. After 24 h, ROS production (A) and cell apoptosis (B) were assessed by flow cytometry analyses. The levels of CRTAC1 (C), cytoplasmic p65, nuclear p65, p-p38, and p38 (D) were determined by western blotting. *** $p < 0.001$ versus siNC+Veh; ## $p < 0.01$; ### $p < 0.001$ versus UVB+siNC+Veh; ++ $p < 0.01$ versus UVB+siCRTAC1+Veh; +++ $p < 0.001$ versus UVB+siCRTAC1+Veh. DMSO, dimethyl sulfoxide.

FIG. 5. The p38 inhibitor attenuated CRTAC1 overexpression-induced ROS production and cell apoptosis. HLECs were transduced with CRTAC1 overexpressing virus (CRTAC1 OE) or control vector virus (Vector) for 24 h, and treated with 10 μ M SB203580 or DMSO (Veh). After 24 h, ROS production (**A**) and cell apoptosis (**B**) were assessed by flow cytometry. The levels of CRTAC1 (**C**), cytoplasmic p65, nuclear p65, p-p38, and p38 (**D**) were determined by western blotting. *** $p < 0.001$ versus Vector+Veh; ## $p < 0.01$ versus CRTAC1 OE+Veh; ### $p < 0.001$ versus CRTAC1 OE+Veh.

(Van Antwerp *et al.*, 1996). In the present study, TNF- α notably suppressed the inhibitory effects of the ROS inhibitor, NAC, on UVB-induced ROS production, and cell apoptosis. Together, these findings indicated that NF- κ B mediated ROS production and cell apoptosis in HLECs upon UVB irradiation (Fig. 6).

In addition, we found that PDTC significantly suppressed the expression and promoter activity of CRTAC1 in UVB-treated HLECs, suggesting that NF- κ B may serve as a transcription factor for CRTAC1. In contrast, TNF- α notably suppressed the inhibitory effects of NAC on NF- κ B p65 translocation and CRTAC1 transcription. These results suggested that NF- κ B, activated by ROS, may function in cataractogenesis by transcriptionally regulating CRTAC1 (Fig. 6). Moreover, our previous study suggested that CRTAC1 knockdown reduced TNF- α release in the culture medium of UVB-treated HLECs (Ji *et al.*, 2016). Thus, we hypothesize that a positive feedback loop may exist between

CRTAC1 and NF- κ B through TNF- α release (Fig. 6). Novel therapeutics for UVB-induced cataract formation may therefore be developed by targeting NF- κ B and CRTAC1.

The p38 mitogen activated protein kinase (MAPK) signaling pathway, activated by oxidative stress, cytokines, and growth factors, plays a crucial role in controlling growth, apoptosis, and differentiation (Cuadrado and Nebreda, 2010). Multiple factors have been shown to affect oxidative stress and cell apoptosis via the p38 MAPK signaling pathway in HLECs and cataract tissues (Zhou and Menko, 2004; Huang *et al.*, 2014; Bai *et al.*, 2015; Ji *et al.*, 2016; Gong *et al.*, 2018; Zhang and Huang, 2019). We have previously reported that CRTAC1 knockdown significantly attenuated UVB-induced apoptosis and decreased p38 phosphorylation in HLECs (Ji *et al.*, 2016). In the present study, the p38 agonist, anisomycin, reversed the effects of CRTAC1 knockdown on cell apoptosis, while the p38 inhibitor, SB203580, reversed the effects of CRTAC1 overexpression. The present data further indicated that inhibition of CRTAC1 decreased cell apoptosis by inactivating the p38 signaling pathway (Fig. 6). However, further experiments are required to determine how CRTAC1 activates the p38 signaling pathway.

In summary, our results suggested that NF- κ B/CRTAC1/p38 was involved in cataract formation. UVB exposure increased ROS production, which activated NF- κ B and promoted the transcription of CRTAC1. CRTAC1 further induced ROS production and cell apoptosis by activating the p38 signaling pathway. Our results were obtained *in vitro*, and whether such signal functions exist *in vivo* needs to be verified in animal models. However, the present study provides clues for further understanding the underlying molecular mechanisms that lead to cataractogenesis and facilitates the development of treatment strategies for cataracts.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1
Supplementary Figure S1
Supplementary Figure S2

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FIG. 6. A Schematic representation of the regulation of UVB-induced ROS production and apoptosis in HLECs by NF- κ B/CRTAC1/p38. Our previous study suggested that CRTAC1 played an important role in UVB-induced ROS generation, cell apoptosis, TNF- α release, and p38 phosphorylation in UVB-treated HLECs (Ji *et al.*, 2016). The present study indicated that NF- κ B mediated ROS production and cell apoptosis in UVB-treated HLECs by serving as a transcription factor for CRTAC1, and further showed that p38 was a potential downstream factor of CRTAC1 by using the p38 inhibitor, SB203580, and the p38 agonist, anisomycin.

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