Apoptosis (2006) 11:1899–1908
DOI 10.1007/s10495-006-9403-6

Activation of mitogen-activated protein kinases is essential for hydrogen peroxide -induced apoptosis in retinal pigment epithelial cells

T.-C. Ho · Y.-C. Yang · H.-C. Cheng · A.-C. Wu · S.-L. Chen · H.-K. Chen · Y.-P. Tsao

Published online: 21 August 2006

Abstract Retinal pigment epithelial (RPE) cells are constantly exposed to oxidative injury while clearing byproducts of photoreceptor turnover, a circumstance thought to be responsible for degenerative retinal diseases. The mechanisms of hydrogen peroxide (H_2O_2)-induced apoptosis in RPE cells are not fully understood. We studied signal transduction mechanisms of H_2O_2-induced apoptosis in the human RPE cell line ARPE-19. Activation of two stress kinases (JNK and p38) occurs during H_2O_2 stimulation, and H_2O_2-mediated cell death was significantly reduced by their specific inhibition. Exposure to a lethal dose of H_2O_2 elicited Bax translocation to the mitochondria and release of apoptosis-inducing factor (AIF) from the mitochondria, both of which were abolished by either JNK- or p38-specific inhibitors. Both H_2O_2-induced cell death and JNK/p38 phosphorylation were partially inhibited by *C. difficile* toxin B, inhibitor of Rho, Rac, and cdc42. Use of pull-down assays revealed that the small GTPase activated by H_2O_2 is Rac1. This study is the first to demonstrate that H_2O_2 induces a Rac1/JNK1/p38 signaling cascade, and that JNK and p38 activation is important for H_2O_2-induced apoptosis as well as AIF/Bax translocation of RPE cells.

Keywords JNK · p38 · Rac1 · RPE · AIF · Hydrogen peroxide

Introduction

Human retinal pigment epithelial (RPE) cells form the outer blood-retina barrier between the photoreceptors and the choriocapillaris. The physiological function of RPE cells, removing photoreceptor turnover byproducts, leaves them constantly exposed to a number of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) [1]. Oxidative injury is thought to play an essential role in the degeneration, dysfunction, or loss of RPE cells, and may be responsible for several retinal degenerative diseases, including age-related macular degeneration (AMD) [2, 3].

Although several previous studies have demonstrated that RPE cell death, when induced by different oxidants *in vitro* and *in vivo*, proceeds through the apoptosis pathway, few have investigated upstream signaling events mediating this stress response [4–11]. Mitogen-activated protein kinases (MAPKs), including stress-activated c-Jun NH2-terminal kinase (JNK), p38 kinase and extracellular signal-regulated kinase (ERK), have been found to respond to a variety of extracellular stimuli and to determine cell fate under stress [12, 13]. It is generally accepted that ERK activation is essential for cell survival, whereas activation of JNK and p38 is thought to play an important role in cell death signaling

Y.-C. Yang and T.-C. Ho contributed equally to the work described herein.

T.-C. Ho · H.-C. Cheng · A.-C. Wu · H.-K. Chen · Y.-P. Tsao
Department of Ophthalmology, The Mackay Memorial Hospital, Taipei, Taiwan

Y.-C. Yang
Mackay Medicine, Nursing and Management College, Taipei, Taiwan

Y.-C. Yang
School of Medicine, Taipei Medical University, Taipei, Taiwan

S.-L. Chen · Y.-P. Tsao (✉)
Department of Microbiology and Immunology, The National Defense Medical Center, Taipei, Taiwan
e-mail: yptsao@yahoo.com

S.-L. Chen
Department of Microbiology, The School of Medicine, National Taiwan University, Taipei, Taiwan

[14, 15]. While high-level and persistent activation of JNK1 and p38 has been observed in RPE cell death induced by serum depletion, involvement of these kinases in the oxidative stress response has not been established [16].

A recent study of apoptosis identified apoptosis-inducing factor (AIF) as a molecule capable of mediating a unique mechanism that is different from the caspase 3-mediated pathway [17–19]. AIF is localized in the mitochondrial intermembrane space [17]. Release of AIF from the mitochondria followed by its translocation to the nucleus induces chromatin condensation and large-scale DNA fragmentation in a caspase-independent manner [19, 20]. The Bcl-2 protein family member Bax has been reported to translocate from the cytoplasm to the mitochondria in response to certain death signals [21]. Bax translocation causes permeabilization of the mitochondrial membrane and the release of apoptogenic factors like AIF [22]. AIF translocation, as opposed to caspase 3 activation, was recently shown to be the major pathway of oxidant-induced RPE cell death; however, the upstream signaling for this event remains unclear [7].

Constitutively activated mutant forms of Rac and Cdc42, of the Rho-family GTPases, have been reported to induce JNK and p38 kinase activations [23–25]. Rho GTPases belong to the Ras superfamily of monomeric G proteins. The three most studied Rho GTPases, RhoA, Rac1, and Cdc42Hs, participate in a wide variety of cellular responses that include mediating cell survival or death signals. For example, Cdc42-mediated JNK activation is required for nerve growth factor withdrawal-induced neuronal death [26]. Osmolarity-induced cellular shrinkage of NIH3T3 cells activates Rac, followed by activation of p38 MAPK, resulting in apoptosis [27]. However, the involvement of Rho-family GTPases in oxidative stress signaling has not been established. Moreover, the relationship in RPE cells between Rho-family GTPases and stress kinases, such as JNK and p38, remains unclear.

The RPE constitutes the blood-retinal barrier and plays an essential role in the maintenance of photoreceptor function. However, this physiological role renders it vulnerable to oxidative stress. Finding strategies for protecting RPE cells from oxidative stress-induced cytotoxicity is therefore an important objective. In the present study we have examined H_2O_2-induced death signaling in cultured RPE cells. Our results suggest that the early signaling cascade includes the small GTPase Rac1, and that JNK and p38 MAPK are involved.

Materials and methods

Reagents

H_2O_2, 4′,6-diamidino-2-phenylindole (DAPI), and other pharmacological and general biochemical reagents were purchased from Sigma (St. Louis, MO, USA). SB203580, SP600125 and *Clostridium difficile* toxin B were purchased from Calbiochem (San Diego, CA, USA).

Cell culture

The human RPE cell line ARPE-19, obtained from American Type Culture Collections (Manassas, VA, USA), was cultured in Dulbecco's modified essential medium (DMEM)-F12 supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin/streptomycin and 2.5 mM glutamine in a 37°C incubator with 5% CO_2.

Cell viability

ARPE-19 cells were seeded in 10% FBS-DMEM-F12 medium in 96-well microculture plates (12,000 cells/well) (Nunclon, Roskilde, Denmark) for 24 hr. The culture medium was then replaced by 0.5% FBS-DMEM-F12 medium for 16 hr. At the time, the cell density before H_2O_2 treatment was around 22,000 cells/well of 96-well microculture plates. Cells were then fed with serum-free medium containing either 10 μM SP600125, 20 μM SB203580 or 5 ng/ml *Clostridium difficile* toxin B for 1 hour followed by 1 mM H_2O_2 treatment for 2 hr. Cells were then washed with PBS and fed with 10% FBS-DMEM-F12 medium for additional 16 hr to remove residual H_2O_2. Cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and confirmed by the trypan blue exclusion assay [6, 28].

Cell Lysis, Fractionation, and SDS-PAGE

Following H_2O_2 treatment, ARPE-19 cells were scraped into lysis buffer (150 μL/35 mm well) containing 20 mm HEPES (pH 7.4), 1% SDS, 150 mm NaCl, 1 mm EGTA, 5 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 100 μM sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. The lysate was incubated on ice for 15 min. Cell debris was removed by centrifugation at 15,000 rpm for 15 min at 4°C. Total cell lysate was separated into cytoplasmic and nuclear fractions using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL, USA). Mitochondria were extracted using the Mitochondria Isolation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Each cellular fraction was then resolved on a 12% SDS-PAGE and then electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA).

Western blot analysis

Samples were probed with Anti-Active p38 polyclonal antibody (Promega, Madison, WI, USA), Anti-Active JNK

polyclonal antibody (Promega, Madison, WI, USA), anti-phospho-HSP27 (Ser78) antibody (Upstate Biotechnology, Lake Placid, NY, USA), phosphor-c-Jun (ser73) antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-AIF polyclonal primary antibody (Santa Cruz Biotechnology Inc., CA, USA) or anti-human monoclonal Bax antibody (BD Biosciences Clontech, Palo Alto, CA), according to the manufacturer's instructions, and then washed three times in TBST. Antibodies against JNK (Santa Cruz Biotechnology Inc., CA, USA), p38/SAPK2, HSP27, c-Jun (60A8) (Upstate Biotechnology, Lake Placid, NY, USA) or actin (Sigma-Aldrich Corp, St. Louis, MO, USA) were used to verify equal loading of protein. Next the blots were incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse (Amersham Biosciences, Piscataway, NJ, USA) secondary antibody diluted in PBS with 0.1% Tween (PBS-T) for 1 h, and then washed three times in TBST prior to visualization using an enhanced chemiluminescent technique. X-ray films were scanned on the Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using Labworks 4.0 software.

Detection of AIF translocation into the nucleus

Immunocytochemistry was performed as described elsewhere, using a goat anti-AIF polyclonal primary antibody (Santa Cruz Biotechnology Inc., CA, USA) diluted 1:200, and an FITC-conjugated rabbit anti-goat secondary antibody (Sigma, St. Louis, MO, USA) diluted 1:60 [6]. The cells were mounted with DAPI containing anti-fade mounting medium (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA) and viewed with an epi-fluorescence microscope (Olympus Optical Co., LTD, Melville, NY, USA).

Pull-down assay for Cdc42, Rac1, and Rho activation

ARPE-19 cells were deprived of serum for 16 hr and then treated with 1 mM H_2O_2 ranging from 10 min to 2 hr and detached from the culture plate by scraping. Cells were lysed in Mg^{2+} lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA) containing 10 μg/ml leupeptin and 10 μg/ml aprotinin. The lysates were briefly centrifuged to remove cell debris. A small aliquot of the resulting supernatant was immediately removed for detection of total Rac1, Cdc42, or RhoA protein by immunoblotting. To precipitate activated Cdc42 or Rac1, supernatants were incubated with 10 μg/sample of GST-PBD beads (Upstate Biotechnology, Lake Placid, NY, USA). GST-rhotekin beads (Upstate Biotechnology, Lake Placid, NY, USA) were used for activated Rho precipitation. The supernatants were then mixed by inversion for 1 hr at 4°C and pelleted beads were washed three times with Mg^{2+} lysis buffer. Aliquots of the original cell lysates and the PBD- and rhotekin-precipitates were subjected to 15% SDS–PAGE, transferred to PVDF membranes, and immunoblotted for Cdc42, Rac1, and Rho.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The Mann-Whitney U test was used to determine statistically significant differences: P values < 0.05 were considered significant.

Results

H_2O_2 induces JNK and p38 MAPK activation

We used Western blot analysis to determine whether JNK and p38 phosphorylation can be induced by H_2O_2 exposure. Previous studies in our laboratory indicated that 1 mM H_2O_2 is lethal to ARPE-19 cells, but 0.2 mM H_2O_2 does not affect cell viability [29]. ARPE-19 cells were treated with 0.2, 1, or 2 mM H_2O_2 for intervals ranging between 20 to 180 min. Whole cell extracts were prepared and analyzed using antibodies against the active phosphorylated forms of JNK and p38. As shown in Fig. 1(A), JNK1 (MW 46 kDa) and p38 are activated 20 min after H_2O_2 treatment. The peak phosphorylation of JNK1 occurred 40 min after treatment; activation was maintained for up to 60–90 min, depending on H_2O_2 dose. Phosphorylation of p38 peaked 60 min after H_2O_2 treatment, was sustained for up to 90 min, and remained detectable for 2–3 hr. There were no obvious changes in total p38 or JNK (data not show) (Fig. 1(A)). Therefore, H_2O_2 activated JNK1 and p38 phosphorylation.

Phosphorylation of JNK1 and p38 occurred at the non-lethal dose (0.2 mM) of H_2O_2; however, a 2- to 3-fold higher level was observed following treatment with a lethal dose of 2 mM (Fig. 1(B) and (C)). This implied that critical levels of JNK and p38 phosphorylation must be achieved to trigger the death signal.

Effects of JNK and p38 on H_2O_2-induced cell death

We next determined whether activation of JNK1 or p38 was essential for H_2O_2-induced cell death. As shown in Fig. 2(A), the viability of cells treated with 1 mM H_2O_2 was markedly decreased. However, following pretreatment with a JNK inhibitor (10 μM SP600125) or a p38 inhibitor (20 μM SB203580) for 2 hr, the 1 mM H_2O_2-induced cell death was reversed by 91 ± 11% and 78 ± 2%, respectively, when compared to control ($P < 0.05$). In control experiments, 2 hr exposure of cells to 10 μM SP600125, 20 μM SB203580, or to the solvent DMSO were not cytotoxic to ARPE-19 cells (data not shown).

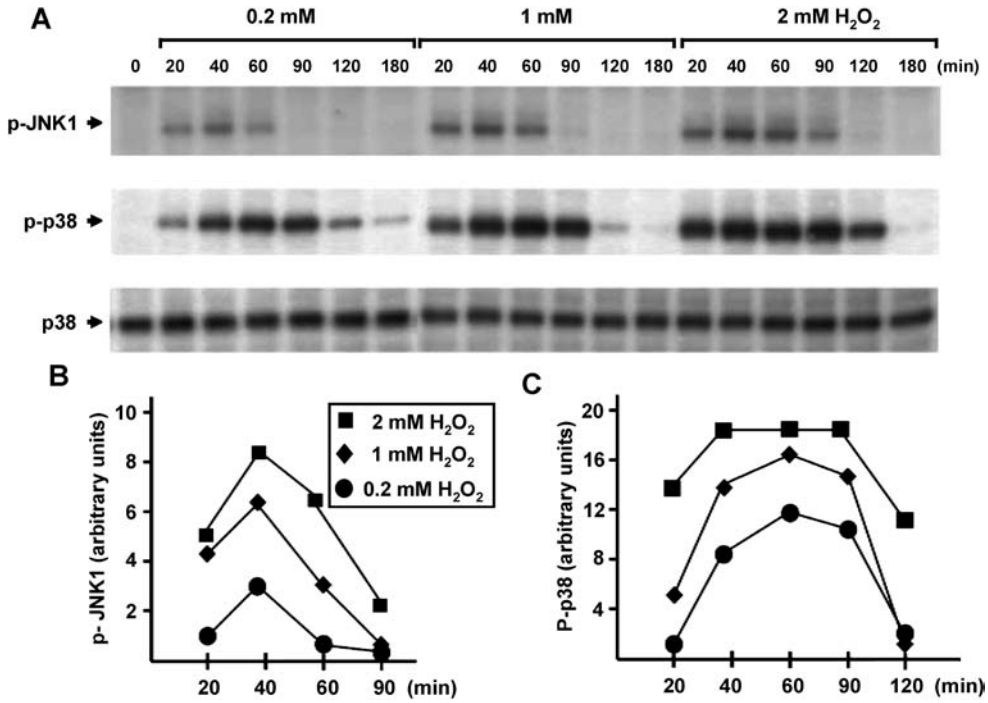

Fig. 1 Concentration-dependent profile and time course of changes in H_2O_2-stimulated MAPK phosphorylation. **(A)** Serum-starved ARPE-19 cells were exposed to 0.2, 1 or 2 mM H_2O_2 for times indicated. Cell lysates were resolved on 12% SDS-PAGE gels followed by Western blotting with specific antibodies against the active phosphorylated forms of JNK and p38 (p-JNK and p-p38), then stripped and reprobed with anti-p38 antibodies as a loading control (lower panel). An additional study yielded equivalent results. Intensities of p-JNK1 **(B)** and p-p38 MAPK **(C)** were determined by densitometry

Since the activation of JNK and p38 seems to play an essential role in H_2O_2-mediated cell death, we further explored the specificity of the inhibitors of p38 and JNK. SP600125 selectively inhibited JNK-mediated phosphorylation of c-Jun, and SB203580 inhibited p38-mediated phosphorylation of HSP27 in H_2O_2-activated ARPE-19 cells. As shown in Fig. 2(B), Western blot analysis revealed that the phosphorylated c-Jun level was not affected by the SB203580 (10–20 μM) and phosphorylated HSP27 level was not affected by SP600125 (5–10 μM).

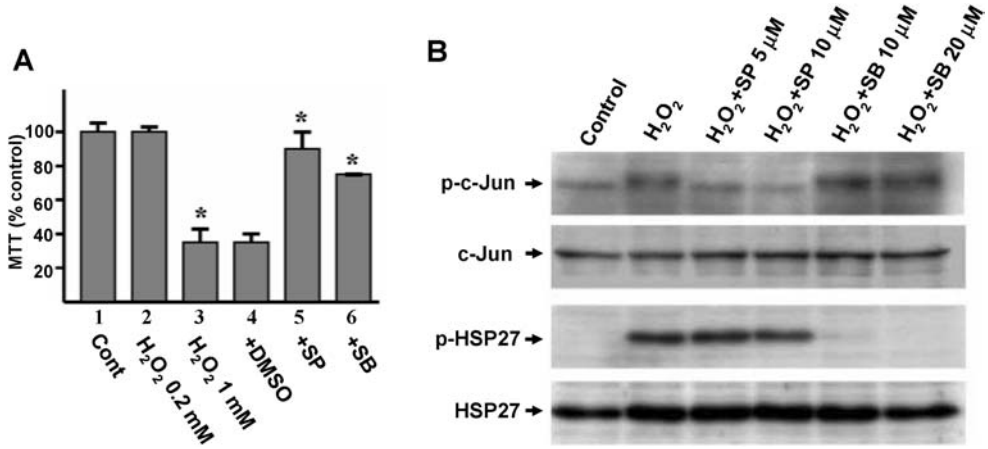

Fig. 2 Activation of JNK and p38 plays an essential role in H_2O_2-mediated cell death. **(A)** MTT viability assay following a 16 h recovery period of untreated ARPE-19 cells (column 1), cells treated with 0.2 mM and 1 mM H_2O_2 for 2 h (columns 2 and 3), and cells pretreated with the chemicals indicated, before stimulation with 1 mM H_2O_2 for 2 hr (columns 4–6). Values expressed as % control. Data presented are means $\pm$ SD (n $=$ 4). $^*P < 0.05$ vs. untreated cells. **(B)** Specificity of the p38 and JNK inhibitors. Western blot analysis of serum-starved ARPE-19 cells (control) and the cells pretreated for 1 hr with the SP600125 or SB203580 at the indicated concentrations then incubated with 1 mM H_2O_2 for an additional 40 min using specific antibodies against phosphorylated forms of c-Jun (substrate of activated JNK) or HSP27 (substrate of activated p38). Loading equality was confirmed with antibodies against unphosphorylated c-Jun and HSP27. The experiment was repeated two times with similar results. Cont, untreated control; SB, SB203580 (a p38 MAPK inhibitor); SP, SP600125 (a JNK inhibitor)

H_2O_2-induced nuclear morphology changes can be abolished by inhibitors of p38 and JNK

The effect of kinase inhibitors on nuclear morphology in H_2O_2-treated ARPE-19 cells was assessed by DAPI staining. The normal nuclear morphology of untreated cells is shown in Fig. 3(A) (a). In contrast, H_2O_2 exposure resulted in small nuclei with partially condensed chromatin (Fig. 3(A), (d)); $82 \pm 12\%$ of cells showed this abnormal nuclear morphology (Fig. 3(B)). We also noted an absence of apoptotic bodies and chromatin fragmentation in these H_2O_2-treated cells, which is consistent with previous reports of atypical apoptosis morphology in RPE [7]. Pretreatment with SB203580 and SP600125 prior to H_2O_2 exposure reduced the number of cells with abnormal nuclear morphology to 32% and 28%, respectively (Fig. 3(B)). These findings indicated that inhibitors of p38 and JNK1 significantly reduce H_2O_2-induced nuclear morphological changes.

H_2O_2-induced AIF translocation was blocked by inhibitors of p38 and JNK

In untreated cells, AIF was localized predominantly outside the nucleus with a dot-like pattern (Fig. 3(A), b). H_2O_2 exposure led to robust AIF staining in both cytosol and nucleus (arrowheads, Fig. 3(A), e), indicating that H_2O_2 triggered translocation of AIF from mitochondria to the nucleus. However, only $31 \pm 7\%$ of SB98059-treated cells and $22 \pm 8\%$ of SP600125-treated cells showed nuclear localization of AIF (Fig. 3(B)). The decrease is indicative of a regulatory role for JNK1 and p38 in AIF nuclear translocation, further establishing the significance of kinase signaling in H_2O_2-induced cell death.

To further demonstrate that H_2O_2-induced JNK and p38 activation is required for AIF translocation, the inhibitors effect on AIF translocation was examined by Western blotting. Results revealed that either SP600125 (5 μM and 10 μM) or SB203580 (10 μM and 20 μM) prevented AIF translocation to the cytosol and nuclei fraction (Fig. 4(A)). H_2O_2-induced AIF translocation was not affected by the DMSO solvent. The Bcl-2 family member Bax has been shown to cause release of AIF and subsequent caspase-independent cell death [22]. As shown in Fig. 4(B) (blot 2), Western blot analysis revealed that H_2O_2 induced mitochondrial localization of Bax. The Bax targeting to mitochondria was also attenuated in cells pretreated with either SP600125 (10 μM) or SB203580 (20 μM). The Western blot analysis also showed a significant increase in Bax protein, which was observed up to 4 hr after H_2O_2 treatment as compared to basal levels (Fig. 4(B), blot 1). Taken together, the selective inhibition of p38 and JNK prevented the H_2O_2-induced release of AIF from the mitochondria (Figs. 3(A) and 4(A)) and significantly attenuated the H_2O_2-induced increase in mitochondrial localization of Bax (Fig. 3(B)).

Effect of *C. difficile* Toxin B on H_2O_2-induced cell death and on JNK and p38 activation

The above findings led us to hypothesize that inhibition of upstream effectors of JNK1 or p38 would block H_2O_2-mediated cell death. For inhibition, *C. difficile* toxin B was selected for its very high degree of substrate specificity for Rho, Rac, and Cdc42, but not other small G proteins [30]. ARPE-19 cells were pretreated for 4 hr with 5 ng/ml toxin B prior to treatment with 1 mM H_2O_2 and the MTT viability assay was performed. The viability of toxin B-pretreated cells was increased by $78 \pm 7\%$ as compared to cells treated with H_2O_2 alone ($P < 0.05$), suggesting that small GTPases are involved in H_2O_2-induced death signaling (Fig. 5(A)). Pretreatment with toxin B alone was not toxic to ARPE-19 cells (data not shown).

We used Western blot analysis of toxin B-pretreated cells to establish that JNK1 and p38 kinases are activated by Rho family small G proteins during H_2O_2 exposure. Exposure to 1 mM H_2O_2 for 40 and 60 min induced a marked activation of JNK1 and p38 (Fig. 5(B), p-JNK1 and p-p38 panels), whereas pretreatment of cells with toxin B resulted in only modest activation of both kinases following H_2O_2 exposure (Fig. 5(B), p-JNK and p-p38 panels). Total JNK and p38 protein levels were not changed. The levels of JNK1 and p38 phosphorylation in cells treated with toxin B plus1 mM H_2O_2 remained higher than those for cells treated with 0.2 mM H_2O_2. These findings are consistent the hypothesis that JNK and p38 phosphorylation mediates H_2O_2 cytotoxicity. Reductions in JNK1 and p38 phosphorylation by toxin B led to partial protection.

We were also interested in discovering which specific member of the Rho-family GTPases is responsible for p38 and JNK activation. Without H_2O_2 exposure, GST-PBD beads precipitated only small amounts of Cdc42 and Rac1 (Fig. 5(C)). Exposure of cells to 1 mM H_2O_2 resulted in a prominent increase in the amounts of active Rac1, but no increase of Cdc42 (Fig. 5(C)). Activation of Rac1 was detected after 10 min and reached its maximum 20 min after stimulation, earlier than JNK and p38 activation (compare Fig. 5(D) and Fig. 1). This suggested that Rac1 regulated the phosphorylation of JNK1 and p38 during H_2O_2 treatment.

Discussion

In this study we investigated signaling mechanisms occurring during H_2O_2-induced RPE cell death. We demonstrated that H_2O_2 exposure markedly increased JNK and p38 activity in human ARPE-19 cells. H_2O_2-mediated cell death was

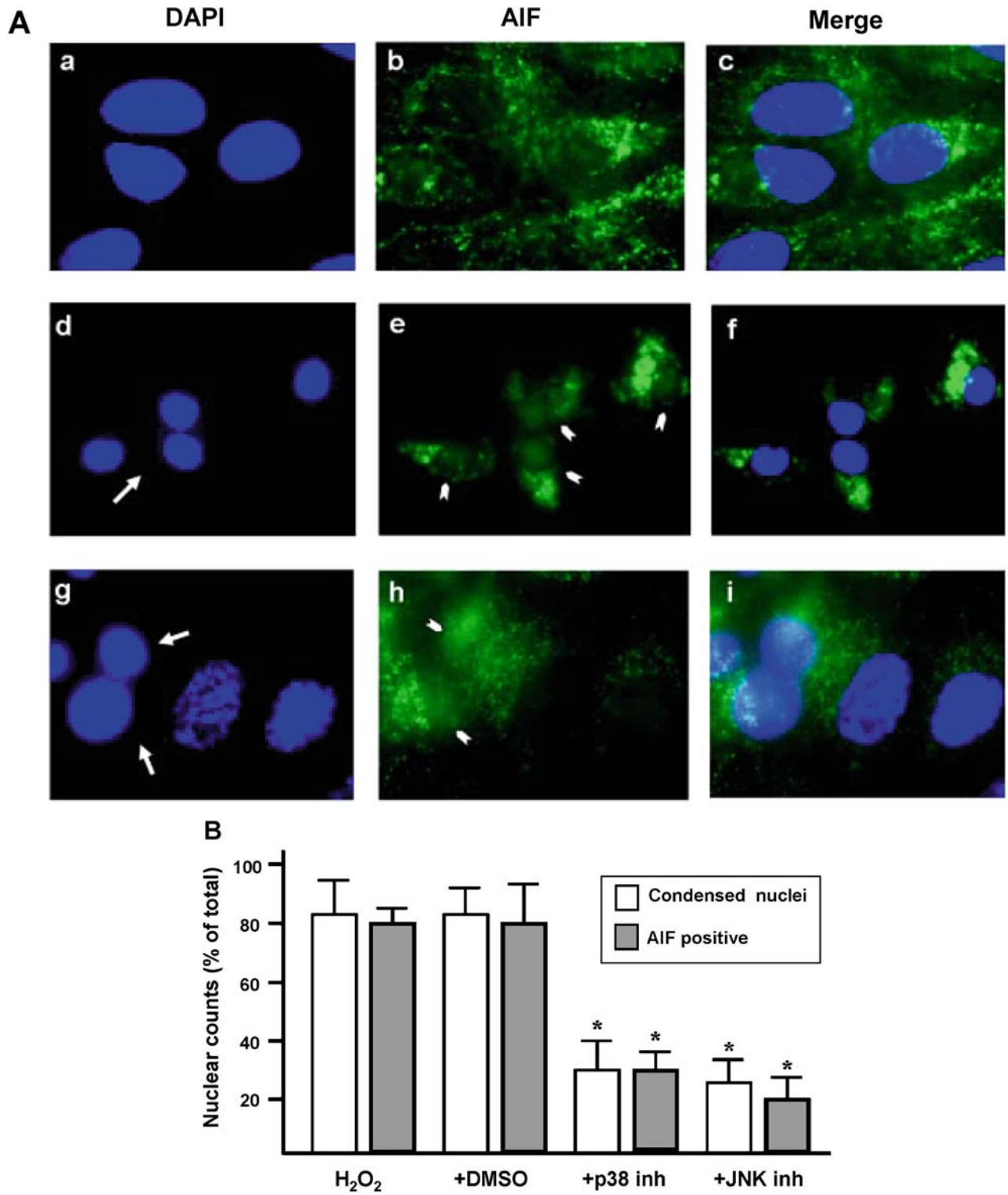

Fig. 3 JNK and p38 inhibitors block H_2O_2-induced chromatin condensation and AIF translocation. **(A)** Untreated ARPE-19 cells (a–c), cells treated with 1 mM H_2O_2 for 2 h (d–f) or pretreated with SB203580 (20 μM, 2 h) then either exposed to 1 mM H_2O_2 for 2 h (g-i) were fixed and double-stained with AIF (green) and DAPI (blue) after a 16 h recovery period. Arrows in panels (d) and (g) point to partially condensed nuclei. Arrowheads in panels (e) and (h) indicate AIF located in nuclei. Representative photomicrographs from duplicate experiments are shown. **(B)** Percentages of ARPE-19 cells, treated with 1 mM H_2O_2 or pretreated with SB203580 (20 μM, 2 h) or SP600125 (10 μM) or DMSO solvent (2 μM) then either exposed to 1 mM H_2O_2 for 2 h as described above, displaying nuclear AIF and DAPI staining. Values were obtained by averaging five fields per slide in which approximately 150 cells per slide were counted. Results expressed are means $\pm$ SD from three independent experiments. (*, $P < 0.05$ versus untreated samples)
(continued on next page)

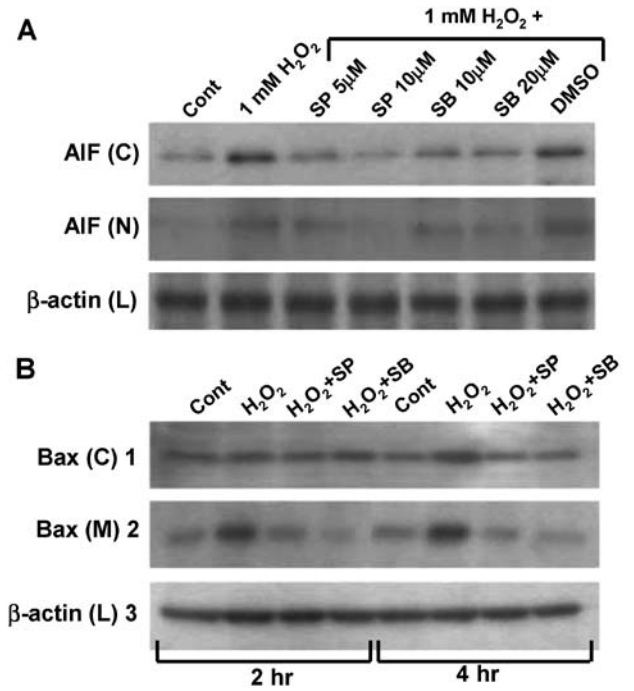

Fig. 4 Effect of inhibition of p38 and JNK on cellular localization of AIF and Bax in ARPE-19 cells undergoing H_2O_2-mediated cell death. Aliquots containing equal amounts of protein from cytosolic (C), nucleus (N) and mitochondrial (M) fractions or whole cell lysates (L) were subjected to SDS-PAGE and Western blot analysis. **(A)** H_2O_2-induced translocation of AIF from the mitochondrial to cytosolic and nucleus fractions is inhibited by either p38 or JNK inhibitors. ARPE-19 cells were treated with 1 mM H_2O_2 for 4 hr or pretreated with SP600125 (SP; JNK inhibitor) or SB203580 (SB; p38 inhibitor) at the indicated concentrations for 1 hr before treatment with 1 mM H_2O_2 for 4 hr, or were left untreated as controls. **(B)** H_2O_2-induced translocation of Bax from the cytosol to the mitochondria is inhibited by either p38 or JNK inhibitors. ARPE-19 cells with or without inhibitor (SP600125; 10 μM, SB203580; 20 μM) pretreatment for 1 hr were exposed to 1 mM H_2O_2 for the times indicated. β-actin was probed to indicate similar loading of protein extract for each treatment

significantly reduced by treatment with inhibitors of JNK and p38 MAPK. Moreover, a lethal dose of H_2O_2 resulted in both Bax and AIF translocation, which was abolished by JNK and p38 MAPK inhibitors. We also found that H_2O_2 substantially increased the amount of activated Rac1. H_2O_2-induced JNK1 and p38 MAPK phosphorylation was partially inhibited by *C. difficile* toxin B, indicating JNK1 and p38 MAPK activation is mediated, at last in part, by activity of a small GTPase. Collectively, these findings suggest a hierarchical model for H_2O_2-related death of ARPE-19 cells, in which H_2O_2 induces signaling through the Rho family of small G proteins to activate JNK and p38, leading to apoptosis. Inhibition of either JNK or p38 kinase prevented apoptosis as well as the translocation of Bax and AIF. These results indicate that activation of both JNK and p38 signaling are the signaling molecules involved in H_2O_2-induced cell death. This may be valuable information for the development of agents to protect RPE cells from oxidative damage.

Inhibition of either JNK or p38 produced marked inhibition of H_2O_2-induced cell death. Inhibition of serum deprivation-mediated death of ARPE-19 cells by JNK and p38 inhibition has been reported previously, but possible mechanisms were not explored [16]. In the present study, we found that inhibition of only one of these two signaling pathways is sufficient to inhibit cell death to a large extent (Fig. 2(A)). This would suggest a mutually dependent interplay of JNK and p38 activation in signaling H_2O_2-mediated cell death. This notion is, however, based on the specificity of kinase inhibitors used in the study. To ensure the inhibitor specificity, the phosphorylation of c-Jun and HSP27 was examined. As shown in Fig. 2(B), phosphorylation of c-Jun and HSP27 was abolished specifically only when its corresponding upstream kinase was inhibited. Nevertheless, lack of cross reactivity between the p38 inhibitor and the JNK inhibitor does not exclude potential overlapping inhibitory effects on other kinases. How JNK and p38 affect each other and whether other kinases are also involved in this interplay remains to be determined.

Our observation that AIF translocation can be abolished by specific inhibitors provides further evidence that activated JNK and p38 are indeed the signaling molecules involved in this phenomenon (Figs. 3 and 4(A)). These findings are consistent with previous observations in retinoid derivative-treated human respiratory epithelial cells and ceramide–treated neuronal cells [31, 32].

Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals induce a conformational change of Bax, leading to its mitochondrial translocation [33, 34]. Bax translocation plays a critical role in the mitochondria-induced release of cytochrome c and induces release of AIF from mitochondria [22, 35]. Here we report that in ARPE-19 cells H_2O_2-induced JNK and p38 activation mediates Bax translocation (Fig. 4(B)). The relationship between JNK, p38 and Bax, however, remains unclear. A recent report demonstrated that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax dependent mitochondrial apoptotic pathway [36]. Nevertheless, the involvement of the BH3-only members of the Bcl-2 intracellular protein family in H_2O_2-induced Bax translocation, and the link with JNK and p38 signaling remains to be determined.

The Rho-family GTPases (Rho, Rac1, and cdc42) participate in a wide variety of cellular responses, including cell death. These GTP binding proteins have been shown to mediate apoptosis through activating downstream stress-activated kinases such as p38 and JNK [23–25]. In this study, *C. difficile* toxin B decreased H_2O_2-induced JNK1 and p38 phosphorylation (Fig. 5(B)), indicating that Rho GTPases mediate activation of these kinases in RPE cells. This finding, considered together with the effects of the GTPase inhibitor,

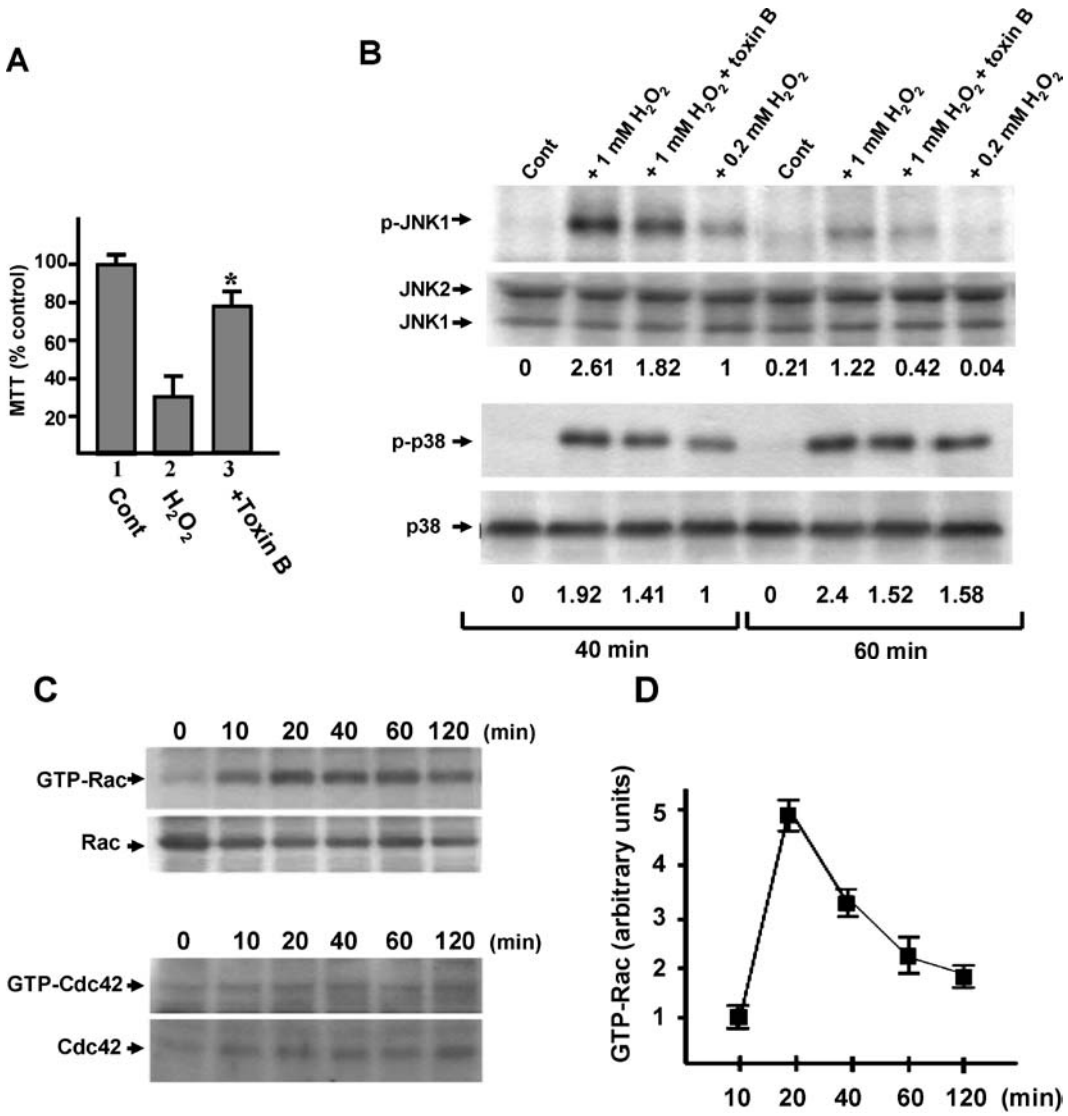

Fig. 5 *C. difficile* toxin B inhibits H_2O_2-induced ARPE-19 cell death and MAPK activation. **(A)** MTT viability assay. ARPE-19 cells were treated with 1 mM H_2O_2 for 2 hr, pretreated with 5 ng/ml *C. difficile* toxin B for 4 hr before treatment with 1 mM H_2O_2 for 2 hr, or left untreated as controls: expressed as % control. Variations shown represent SD from three independent experiments (n = 4 dishes). $^*P < 0.05$ compared with H_2O_2-treated cells. **(B)** Western blot analysis. ARPE-19 cells were treated with 1 mM or 0.2 mM H_2O_2 for 40 or 60 min as indicated, or treated with 5 ng/ml *C. difficile* toxin B for an additional 4 hr before treatment with 1 mM H_2O_2. Samples were subjected to SDS-PAGE and Western blot analysis. Phosphorylated JNK and p38 kinases were detected with anti-active-form antibodies. The total levels of JNK and p38 kinases were analyzed with anti-JNK and anti-p38 kinase antibodies and used as equal loading control. Values represent the fold difference of the quantified p-JNK and p-p38 signals relative to total levels of JNK and p38 loading control, compared with controls treated with 0.2 mM H_2O_2 for 40 min. The experiment was repeated two times with similar results. **(C)** Pull down assay. Cells were treated for times indicated with 1 mM H_2O_2, lysed, and active Rac1 and Cdc42 were precipitated using GST-PBD beads. Captured proteins were separated by SDS-PAGE and analyzed by Western blotting. The results shown are from three independent experiments. Whole cell lysates were also analyzed by Western blot analysis to estimate the levels of Rac1 and Cdc42. **(D)** Values for GTP-Rac were normalized to total Rac. The values represent the means ± SD of the optical density ratio from three independent experiments

supports the notion that JNK-p38 signaling is essential for H_2O_2-induced cell death. The specific small GTPase responsible for p38 and JNK activation was suggested to be Rac1 (Fig. 5(C)). The level of activated RhoA was found to be unchanged by H_2O_2 treatment (data not shown). This is the first report linking Rac1 signaling to H_2O_2-induced RPE cell death. In addition, comparison of the activation time course of Rac1, JNK and p38 during H_2O_2 exposure lead to the suggestion that Rac1 is an upstream mediator of JNK1/p38 signaling. First, the appearance of GTP-Rac occurred 10 min prior to JNK1/p38 activation (compare Fig. 5(D) with Fig. 1(B) and (C)). Second, after cells were treated with 1 mM H_2O_2 for 2 hr, the increased levels of GTP-Rac returned to basal levels, and the levels of JNK1/p38 phosphoryla-

tion showed a pattern consistent with the changes in Rac1 (compare Fig. 5(C) with Fig. 1(A)).

Interestingly, the degree of activation of JNK1 and p38 MAPK seems to be critical for H_2O_2-induced cell death. Our results revealed that a lethal dose H_2O_2 induces a much higher degree of JNK1 and p38 activation than a non-lethal dose H_2O_2 (Fig. 1(B) and (C)). In addition, H_2O_2-induced cell death can be significantly reversed by toxin B at a concentration that only modestly inhibited JNK1 and p38 activation (Fig. 5(B)). It is possible that adequate levels of activation of p38 and JNK are required to promote Bax/AIF translocation and result in H_2O_2-induced RPE injury.

The ARPE-19 cell line is derived from a primary RPE tumor, and is widely used as an *in vitro* model for investigation of the molecular pathways and related biochemical and cellular functions of human RPE. Since the physiological function of RPE cells is responsible for clearance of oxidants derived from photoreceptor turnover, ARPE-19 cells have been used to explore oxidant-mediated insults and related protective factors in a number of recent studies. Hepatocyte growth factor (HGF), pigment epithelium-derived factor, keratinocyte growth factor, sigma receptor ligands and neuroactive steroids have been shown to prevent oxidant-induced apoptosis in ARPE-19 cells [7, 29, 37–39]. In these studies, pretreatment with HGF abrogated oxidant-mediated AIF translocation; however, the underlying mechanism has not been elucidated [7]. The present study provides a hierarchical model for an H_2O_2-induced signaling cascade related to AIF-mediated apoptosis in ARPE-19 cells that may prove useful for future studies of protective strategies to prevent RPE cell death or injury.

Acknowledgments The authors thank Willian J. Freed Ph. D. (NIDA IRP, Nation Institutes of Health, DHNS, U.S.A.) for editing the manuscript. The authors thank Ju-Yun Wu and I-Pin Choung for excellent technical support. This study was supported by grants from National Science Council, Taiwan (NSC 94 - 2314 - B - 195 - 002, 94 - 3112 - B - 195 - 001) and Mackay Memorial Hospital (MMH-E-95006, MMH-9501).

References

1. Miceli MV, Liles MR, Newsome DA (1994) Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. Exp Cell Res 214:242–249
2. Winkler BS, Boulton ME, Gottsch JD, Sternberg P (1999) Oxidative damage and age-related macular degeneration. Mol Vis 5:32–43
3. Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP (2000) Oxidative damage and protection of the RPE. Prog Retin Eye Res 19:205–221
4. Volobouева LA, Liu J, Suh JH, Ames BN, Miller SS (2005) (R)-alpha-lipoic acid protects retinal pigment epithelial cells from oxidative damage. Invest Ophthalmol Vis Sci 46:4302–4310
5. Kasahara E, Lin LR, Ho YS, Reddy VN (2005) SOD2 protects against oxidation-induced apoptosis in mouse retinal pigment epithelium: implications for age-related macular degeneration. Invest Ophthalmol Vis Sci 46:3426–3434
6. Strunnikova N, Zhang C, Teichberg D, et al (2004) Survival of retinal pigment epithelium after exposure to prolonged oxidative injury: a detailed gene expression and cellular analysis. Invest Ophthalmol Vis Sci 45:3767–3777
7. Zhang C, Baffi J, Cousins SW, Csaky KG (2003) Oxidant-induced cell death in retinal pigment epithelium cells mediated through the release of apoptosis-inducing factor. J Cell Sci 116:1915–1923
8. Jin GF, Hurst JS, Godley BF (2001) Hydrogen peroxide stimulates apoptosis in cultured human retinal pigment epithelial cells. Curr Eye Res 22:165–173
9. Sparrow JR, Cai B (2001) Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by Bcl-2. Invest Ophthalmol Vis Sci 42:1356–1362
10. Howes KA, Liu Y, Dunaief JL, et al (2004) Receptor for advanced glycation end products and age-related macular degeneration. Invest Ophthalmol Vis Sci 45:3713–3720
11. Del Priore LV, Kuo YH, Tezel TH (2002) Age-related changes in human RPE cell density and apoptosis proportion in situ. Invest Ophthalmol Vis Sci 43:3312–3318
12. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Sci 270:1326–1331
13. Chang L, Karin M. (2001) Mammalian MAP kinase signalling cascades. Nature 410:37–40
14. Ono K, Han J (2000) The p38 signal transduction pathway: activation and function. Cell Signal 12:1–13
15. Davis RJ (2000). Signal transduction by the JNK group of MAP kinases. Cell 103:239–252
16. Hecquet C, Lefevre G, Valtink M, Engelmann K, Mascarelli F (2003) Activation and role of MAP kinase-dependent pathways in retinal pigment epithelium cells: JNK1, P38 kinase, and cell death. Invest Ophthalmol Vis Sci 44:1320–1329
17. Susin SA, Lorenzo HK, Zamzami N, et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446
18. Lorenzo HK, Susin SA, Penninger J, Kroemer G (1999) Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ 6:516–524
19. Susin SA, Daugas E, Ravagnan L, et al (2000) Two distinct pathways leading to nuclear apoptosis. J Exp Med 192L:571–580
20. Daugas E, Susin SA, Zamzami N, et al (2000) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J 14:729–739
21. Goping IS, Gross A, Lavoie JN, et al (1998) Regulated targeting of BAX to mitochondria. J Cell Bio 143:207–215.
22. Cregan SP, Fortin A, MacLaurin JG, et al (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol 158:507–517
23. Gallo KA, Johnson GL (2002) Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol 3:663–672
24. Minden A, Lin A, Claret FX, Abo A, Karin M (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 81:1147–1157
25. Zhang S, Han J, Sells MA, et al (1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 270:23934–23936
26. Bazenet CE, Mota MA, Rubin LL (1998) The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. Proc Natl Acad Sci USA 95:3984–3989

27. Friis MB, Friborg CR, Schneider L, et al (2005) Cell shrinkage as a signal to apoptosis in NIH 3T3 fibroblasts. J Physiol 567:427–443
28. Garg TK, Chang JY (2003) Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: Prevention of cell death by AG126 and 15-deoxy-delta 12, 14-PGJ2. BMC Ophthalmol 3:5
29. Tsao YP, Ho TC, Chen SL, Cheng HC (2006) Pigment epithelium-derived factor inhibits oxidative stress-induced cell death by activation of extracellular signal-regulated kinases in cultured retinal pigment epithelial cells. Life Sci (in print).
30. Prepens U, Just I, von Eichel-Streiber C, Aktories K (1996) Inhibition of Fc epsilon-RI-mediated activation of rat basophilic leukemia cells by Clostridium difficile toxin B (monoglucosyltransferase) J Biol Chem 271:7324–7329
31. Boisvieux-Ulrich E, Sourdeval M, Marano F (2005) CD437, a synthetic retinoid, induces apoptosis in human respiratory epithelial cells via caspase-independent mitochondrial and caspase-8-dependent pathways both up-regulated by JNK signaling pathway. Exp Cell Res 307:76–90
32. Stoica BA, Movsesyan VA, Knoblach SM, Faden AI (2005) Ceramide induces neuronal apoptosis through mitogen-activated protein kinases and causes release of multiple mitochondrial proteins. Mol Cell Neurosci 29:355–371
33. Gross A, Jockel J, Wei MC, Korsmeyer SJ (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 17:3878–3885
34. Bellosillo B, Villamor N, Lopez-Guillermo A, et al (2002) Spontaneous and drug-induced apoptosis is mediated by conformational changes of Bax and Bak in B-cell chronic lymphocytic leukemia. Blood 100:1810–1816
35. Gross A, Jockel J, Wei MC, Korsmeyer SJ (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 17:3878–3885
36. Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci USA 100:2432–2437
37. Geiger RC, Waters CM, Kamp DW, Glucksberg MR (2005) KGF prevents oxygen-mediated damage in ARPE-19 cells. Invest Ophthalmol Vis Sci 46:3435–3442
38. Bucolo C, Drago F, Lin LR, Reddy VN (2006) Sigma receptor ligands protect human retinal cells against oxidative stress. Neuroreport 17:287–291
39. Bucolo C, Drago F, Lin LR, Reddy VN (2005) Neuroactive steroids protect retinal pigment epithelium against oxidative stress. Neuroreport 16:1203–1207