p38 mitogen-activated protein kinase protects human retinal pigment epithelial cells exposed to oxidative stress

Cady E. Pocrnich,* MD; Hong Liu*; Mary Feng*; Tianqing Peng,† PhD; Qingping Feng,‡ PhD; Cindy M.L. Hutnik,*†§ MD, PhD

ABSTRACT • RÉSUMÉ

Objective: To investigate the role of p38 mitogen-activated protein kinase (MAPK) in human retinal pigment epithelial (RPE) cells exposed to acute oxidative stress.

Study Design: Experimental study.

Methods: Oxidative stress was induced by the chemical oxidant *tert*-butyl hydroperoxide (t-BOOH) in the human RPE cell line ARPE-19. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The phosphorylation of p38 MAPK was measured by Western blot analysis. Small interfering (si)RNA and plasmid DNA of a constitutive active mutant of a MAPK kinase (MKK6E) were transfected to knock down and activate p38 MAPK in ARPE-19 cells, respectively.

Results: t-BOOH induced ARPE-19 cell death in a time- and dose-dependent manner. t-BOOH increased phosphorylation of p38 MAPK. Both inhibition of p38 MAPK with a selective inhibitor SB203580 and knockdown of p38α MAPK with siRNA enhanced t-BOOH-induced ARPE-19 cell death (by 29% and 20%, respectively). Overexpression of MKK6E, a kinase upstream of p38 MAPK, increased phosphorylation of p38 MAPK and attenuated t-BOOH-induced ARPE-19 cell death by 16%. Preconditioning with a low dose of t-BOOH decreased the ARPE-19 cell death induced by a higher dose of t-BOOH by 13%. This protective effect was absent when ARPE-19 cells were pretreated with SB203580 for 1 hour before preconditioning.

Conclusions: Under the conditions tested, activation of p38 MAPK protects ARPE-19 cells against oxidant-induced death. Because RPE cells play a key role in retinal homeostasis, this finding may have important implications for retinal disease.

Objet : Investigation du rôle de la protéine-kinase activée par le mitogène p38 (MAPK) dans les cellules épithéliales pigmentaires de la rétine humaine (ÉPR), qui sont exposées à un stress oxydatif aigu.

Nature : Étude expérimentale.

Méthodes : Le stress oxydatif aigu a été induit par l'oxydant chimique hydroperoxyde de *tert*-butyle (t-BOOH) dans la lignée cellulaire ARPE-19 de la cellule ÉPR humaine. La viabilité cellulaire a été évaluée par l'essai de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). La phosphorylation de la MAPK p38 a été mesurée par l'analyse de buvardage Western. Une faible interférence d'ARN(si) et l'ADN plasmide d'un mutant constitutif actif de kinase MAPK (MKK6E) ont été transférés pour démolir puis activer la MAPK p38 dans les cellules ARPE-19, respectivement.

Résultats : Le t-BOOH a induit la mort des cellules ARPE-19 dans le délai et selon la dose utilisée. Le t-BOOH a augmenté la phosphorylation de la MAPK p38. L'inhibition de la MAPK p38 avec l'inhibiteur sélectif SB203580 et la démolition de la MAPK p38α dans les cellules ARPE-19 avec l'ARN(si) ont accentué la mort des lignées cellulaires ARPE-19 induite par le t-BOOH (de 29 % et 20 % respectivement). La surexpression de MKK6E, kinase en amont de la MAPK p38, a augmenté la phosphorylation de la MAPK p38 et atténué de 16 % la mort des cellules ARPE-19 induite par le t-BOOH. Le préconditionnement avec une faible dose de t-BOOH a réduit de 13 % la mort des cellules ARPE-19 induite par une plus forte dose de t-BOOH. Cet effet protecteur était absent lorsque les cellules ARPE-19 étaient traitées au préalable avec le SB203580 une heure avant le préconditionnement.

Conclusions : Selon les conditions soumises au test, l'activation de la MAPK p38 protège les cellules ARPE-19 contre une mort induite par un oxydant. Comme les cellules ÉPR jouent un rôle clé dans l'homéostase de la rétine, ces données peuvent avoir une implication importante dans les maladies de la rétine.

The retinal pigment epithelium (RPE) is a monolayer of postmitotic cells responsible for maintaining retinal homeostasis. The physiologic roles of the RPE include phagocytosis of membranes shed from photoreceptor outer segments and absorption of high-energy light.[1] These functions, as well as others, create oxidative load[2–6] and cause the retina to have a high rate of oxygen consumption.[1] Oxidative stress is now well recognized as a likely contributor to the development of age-related macular degeneration,[7–9] an important and prevalent cause of blindness characterized in its initial stages by dysfunction, deterioration, and loss of the RPE in the macular area.[1,7,8] The mechanisms by which

From *the Department of Ophthalmology, †the Department of Pathology, and ‡the Department of Physiology and Pharmacology, University of Western Ontario, London, Ont.; and §Clinical Investigation & Therapeutics, Lawson Health Research Institute, London, Ont.

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Correspondence to Cindy M.L. Hutnik, MD, Eye Clinic, Second Floor, St. Joseph's Health Care London, 268 Grosvenor St., London, ON N6A 4V2; cindyh@sjhc.london.on.ca

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oxidative stress mediates RPE cell death are not yet fully understood.

Oxidative stress is known to activate mitogen-activated protein kinases (MAPKs). The MAPK family consists of a large number of serine/threonine kinases that form a ubiquitous signal transduction system controlling fundamental cellular events such as proliferation, differentiation, survival, and apoptosis.[10–12] MAPKs are arranged in signaling cascades consisting of 3 levels: a MAPK kinase kinase (MKKK) activates a MAPK kinase (MKK), which finally leads to MAPK activation. MAPKs exert their downstream influences by phosphorylating effector proteins, especially transcription factors.[12,13] Distinct subfamilies of MAPK include the extracellular signal-regulated kinases (ERK), the c-Jun NH2-terminal kinases (JNK), and p38 kinases. These can be activated by oxidative stress, offering an opportunity for modulation of cell survival.[12]

However, the influence of MAPK activation on cell survival in the face of an oxidative challenge is not clear cut. The ERK cascade is the most comprehensively studied of the MAPK pathways and its activation is generally thought to mediate cell survival. However, although ERK has been shown to protect against oxidant-induced cell injury and death in most systems,[13–15] in others it appears to actually promote apoptosis.[16,17] Studies of oxidant-induced activation of JNK pathways have also provided conflicting results.[11,13,18,19] The role of p38 in the regulation of survival in oxidatively stressed cells has been equally controversial, with some studies supporting proapoptotic effects,[20–23] others supporting prosurvival effects,[24] and still others demonstrating neither.[13]

This report examines the role of p38 signaling in the ARPE-19 cell line following an acute exposure to the chemical oxidant *tert*-butyl hydroperoxide (t-BOOH). The phenomenon of preconditioning, in which exposure to a mild stressor confers protection against a more lethal challenge, is also explored. A better understanding of the intracellular mechanisms leading to RPE damage may provide insight into novel therapeutic targets for sight-threatening conditions.

Methods

Reagents

All cell culture reagents were purchased from Invitrogen (Burlington, Ont.), unless otherwise indicated. Antibodies were purchased from Cell Signaling Technology (Pickering, Ont.). MTT, t-BOOH, and SB203580 were purchased from Sigma-Aldrich (Oakville, Ont.).

ARPE-19 cell culture and treatment

The human RPE cell line ARPE-19 was purchased from American Type Culture Collection (Manassas, Va.). Cells were grown as previously described.[25] Before application of t-BOOH or SB203580, ARPE-19 cells were seeded at 3×10^4 cells/cm^2 onto 96-well plates or 60 mm dishes and grown in the culture media for 30 hours, followed by an overnight incubation in serum-free media to reach 100% confluence.

Transfection of ARPE-19 cells

siRNAs targeting p38α MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.) and a universal control siRNA was purchased from Qiagen (Germantown, Md.). The transfection of siRNA was carried out in ARPE-19 cells using TransMessenger Transfection Reagent (Qiagen), according to the manufacturer's instructions. Recombinant plasmid pMKK6E expressing an active mutant of MKK6 was kindly provided by Dr Jiahuai Han. The transfection of recombinant plasmid DNA was carried out in ARPE-19 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

MTT assay

The MTT reduction assay was used as an index of cell viability and was performed according to the manufacturer's instructions. Absorbances were normalized to the untreated control cultures, which represented 100% viability.

Protein extraction and Western blot analysis

Protein was isolated from confluent ARPE-19 cells as previously described.[25] Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed. Membranes were probed with antibodies against either p38 MAPK or phosphorylated-p38 (phospho-p38) MAPK, each at a dilution of 1:1000. Detection was performed using the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, Ill.). To ensure equal protein loading, nitrocellulose membranes were stripped in REblot (Chemicon International, Temecula, Calif.) and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody at a dilution of 1:10,000 (Cedarlane Laboratories, Burlington, Ont.).

Statistical analysis

All data are given as the mean ± SD of at least 3 experiments. Where applicable, differences between 2 groups were compared by the unpaired Student's *t* test. For multigroup comparisons, ANOVA, followed by Newman-Keuls test, was performed. A p value <0.05 was considered statistically significant.

Results

The MTT assay showed that RPE cell viability was decreased in both a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B) manner. To optimize our ability to examine any potentially protective effects of p38 MAPK activation, the dose and time combination (1 mM, 2.5 hours) producing approximately 60% to 70% viability was chosen for subsequent experiments, unless otherwise indicated.

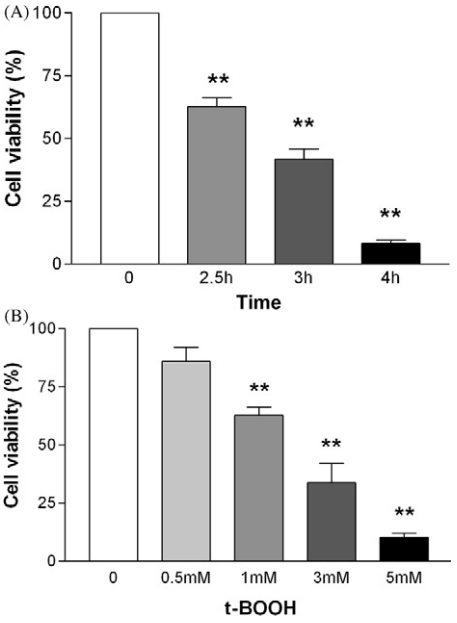

Fig. 1—Viability of retinal pigment epithelial (RPE) cells in response to treatment with *tert*-butyl hydroperoxide (t-BOOH). (A) Time course of RPE cell death after treatment with 1 mM t-BOOH. (B) Dose-dependent effect of a 2.5 h incubation with t-BOOH on RPE cell viability. **Significantly different from the control sample ($p < 0.001$; $n = 4$).

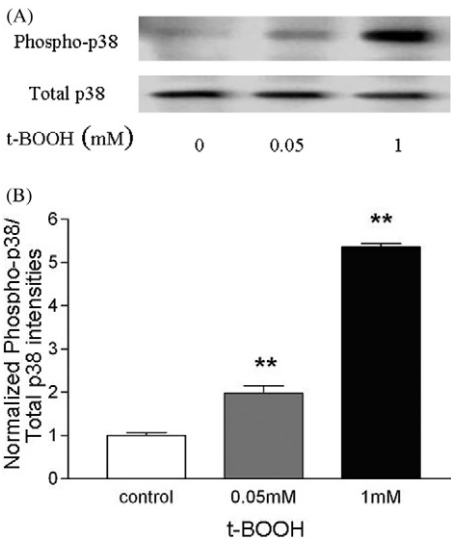

Fig. 2—The phosphorylation of p38 mitogen-activated protein kinase in response to treatment with *tert*-butyl hydroperoxide (t-BOOH). Retinal pigment epithelial cells were treated with t-BOOH (0.05 mM and 1 mM) or vehicle for 30 min. Cell lysate was analyzed by Western blot, and phosphorylated-p38 (phospho-p38) and total p38 were detected. (A) Western blot analysis. (B) Densitometric intensities of the phospho-p38 bands normalized to total p38, relative to control. **Significantly different from the control sample and from the sample treated with 0.05 mM t-BOOH ($p < 0.01$; $n = 3$).

t-BOOH induced phosphorylation of p38 MAPK within 30 minutes (Fig. 2). Because phosphorylation of p38 MAPK relates to the activity of functional p38 MAPK, these data suggest that t-BOOH activated p38 MAPK in ARPE-19 cells.

ARPE-19 cells were preincubated with a selective inhibitor of p38 MAPK known as SB203580 (10 μM) or vehicle for 1 hour, followed by t-BOOH for 2.5 hours. As shown in Fig. 3, pretreatment with SB203580 enhanced t-BOOH-induced RPE cell death by 29% compared with vehicle treatment. Substantiation of this finding was done using specific siRNA targeting p38α MAPK to knock down p38 MAPK signaling in ARPE-19 cells. Forty-eight hours after siRNA transfection, p38 MAPK protein was down-regulated (Fig. 4A). Consistent with the effect of SB203580, cells in which p38 MAPK had been knocked down displayed a 20% decrease in cell viability as compared with control cells (Fig. 4B), following a 2.5-hour incubation with 1 mM t-BOOH. Thus, our data suggest that inhibition of p38 MAPK increased ARPE-19 cell death induced by t-BOOH.

Overexpression of a constitutive active mutant of a MAPK kinase (MKK6E), a kinase upstream of p38 MAPK, was conducted. As expected, transfection with MKK6E for 6 hours led to an increase in phosphorylation of p38 MAPK in ARPE-19 cells (Fig. 5A). Consistently, overexpression of MKK6E significantly improved ARPE-19 cell viability after t-BOOH treatment by 16% in relation to pcDNA3 treatment (Fig. 5B). Thus, our data indicate that activation of p38 MAPK may protect ARPE-19 cells from t-BOOH-induced cell death.

To investigate whether p38 MAPK played a role in preconditioning-induced protection, ARPE-19 cells were first treated with a low concentration of t-BOOH (0.05 mM). This concentration activated p38 MAPK (Fig. 2) but did not induce appreciable cell death. Two

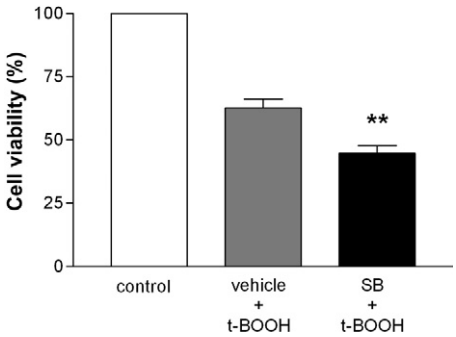

Fig. 3—The effect of a p38 mitogen-activated protein kinase selective inhibitor on *tert*-butyl hydroperoxide (t-BOOH)-induced cell death. Retinal pigment epithelial cells were preincubated with SB203580 (SB) (10 μM) or vehicle for 1 h, followed by treatment with t-BOOH (1 mM) for 2.5 h. Pretreatment with SB enhanced t-BOOH-induced cell death. **Significantly different from the sample preincubated with vehicle and treated with t-BOOH ($p < 0.001$; $n = 9$).

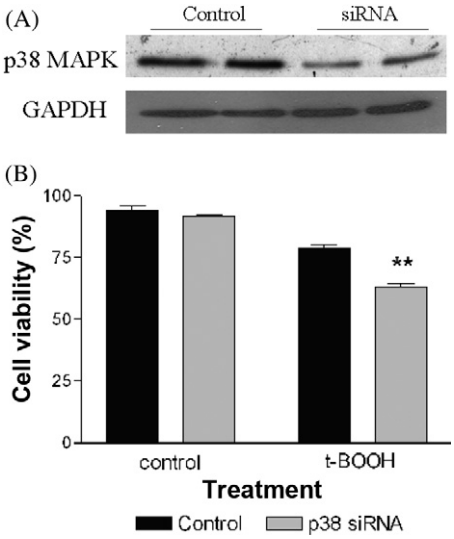

Fig. 4—The effect of a p38α mitogen-activated protein kinase (MAPK) specific small interfering (si)RNA on *tert*-butyl hydroperoxide (t-BOOH)-induced cell death. (A) Western blot analysis showing the down-regulation of p38 MAPK 48 h after siRNA transfection. (B) Treatment with p38 MAPK specific siRNA enhanced t-BOOH-induced cell death. **Significantly different from the sample treated with t-BOOH and control siRNA ($p < 0.001$; $n = 3$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

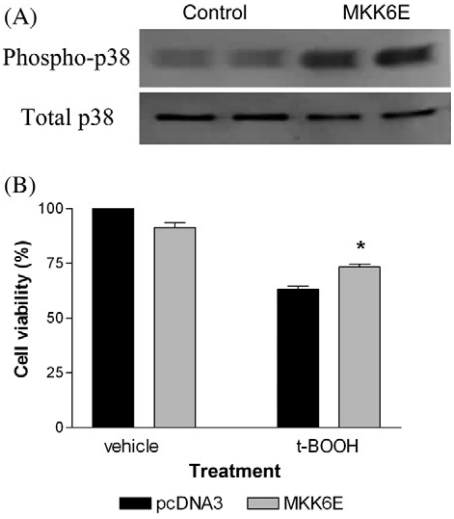

Fig. 5—The protective role of p38 mitogen-activated protein kinase (MAPK) activation by a constitutive active mutant of MAPK kinase (MKK6E) on oxidant stressed retinal pigment epithelial cells. (A) Western blot analysis showing the up-regulation of phosphorylated-p38 (phospho-p38) after transfection with a vector containing the MKK6E insert. (B) Cells were transfected with either empty pcDNA3 or MKK6E-pcDNA3. Cells were then treated with either vehicle or *tert*-butyl hydroperoxide (t-BOOH) and cell viability was assessed. MKK6E improved cell viability after t-BOOH treatment. *Significantly different from the other samples ($p < 0.05$; $n = 3$).

hours later, the cells were subjected to a higher concentration of t-BOOH (1 mM) for another 2.5 hours. Preconditioning with a low concentration of t-BOOH decreased cell death induced by a higher concentration of t-BOOH in ARPE-19 cells (Fig. 6). However, this protective effect was lost when ARPE-19 cells were pretreated with SB203580 for 1 hour prior to preconditioning. This suggests that the protective effect of preconditioning was mediated through p38 MAPK activation.

Conclusions

It has been proposed that oxidative stress may be a key contributor to RPE dysfunction, degeneration, and loss in retinal degenerative conditions. Consequently, the intracellular pathways by which oxidative stress mediates RPE cell death have been of considerable interest. One such pathway is the p38 MAPK signaling pathway, the stress activation of which has been widely reported in many cell systems. In this study, we demonstrated that t-BOOH activates p38 MAPK in ARPE-19 cells. Furthermore, activation of p38 protects against t-BOOH-induced cell death and mediates the protective effect of preconditioning by a low-dose t-BOOH in cultured ARPE-19 cells.

Other studies have examined the potential role of p38 MAPK in RPE cells under various conditions of stress using pharmacological inhibitors, and the results are controversial. Hecquet et al.[26] studied primary human RPE cells subjected to a serum-deprivation challenge which, predictably, induced RPE cell death. Serum deprivation was also found to activate p38 MAPK. Furthermore, inhibition of p38 activation reduced the degree of RPE cell death, whereas

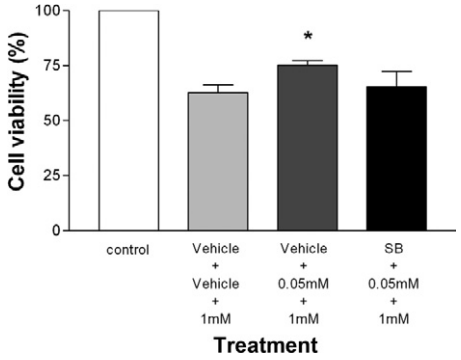

Fig. 6—The dependence of protective preconditioning on p38 mitogen-activated protein kinase activation. Retinal pigment epithelial cells were preincubated with SB203580 (SB) or vehicle for 1 h, followed by a 2 h treatment with either a low concentration of *tert*-butyl hydroperoxide (t-BOOH) (0.05 mM) or vehicle. The cells were then challenged by a high concentration of t-BOOH (1 mM). After 2.5 h, cell viability was assessed. SB203580 blocked the protective effect of preconditioning with t-BOOH. *Significantly different from all other samples ($p < 0.05$; $n = 9$).

enhancement of p38 activation increased the degree of RPE cell death under stress conditions. Ho et al.[27] published results consistent with those of Hecquet et al. [26] This time, ARPE-19 cells were challenged with hydrogen peroxide (H_2O_2). Once again, the stressor induced both cell death and p38 MAPK activation, and inhibition of p38 MAPK activation attenuated H_2O_2-induced ARPE-19 cell death. The results of both studies suggest that p38 MAPK can mediate oxidative stress-induced RPE cell death.[26,27] However, not all studies have provided results from which the same conclusion can be drawn. For instance, when Glotin et al.[28] challenged ARPE-19 cells with a low dose of t-BOOH, cell death was not accompanied by any marked change in the level of p38 MAPK activation. Furthermore, inhibition of p38 signaling had no significant effect on ARPE-19 cell viability in the face of the oxidative challenge. The results of Qin et al.[29] showed that although H_2O_2 caused transient activation of p38 MAPK signaling in ARPE-19 cells, p38 MAPK signaling again played no apparent role in mediating oxidative stress-induced cell death.

In this study, treatment of ARPE-19 cells with t-BOOH predictably induced cell death in a time- and dose-dependent manner, and also resulted in phosphorylation of p38 MAPK within 30 minutes at concentrations as low as 0.05 mM. This finding was not surprising, given that most previous papers have demonstrated stress activation of p38 MAPK,[26,27,29,30] although it is interesting to note that Glotin et al.[28] exposed ARPE-19 to 300 μM of t-BOOH and found no alteration in p38 MAPK phosphorylation at any time point ranging from 10 minutes to 6 hours. We next used 2 strategies to reduce p38 signaling: a pharmacologic (SB203580) approach and an siRNA approach. In both instances, t-BOOH-induced ARPE-19 cell death was increased. Consistently, enhancement of p38 MAPK signaling, which was accomplished by transfection with a constitutive active mutant of an upstream activator of p38 MAPK, produced a small but significant attenuation of t-BOOH-induced ARPE-19 cell death. Together, these results suggest that p38 MAPK activation is protective against oxidative stress-induced injury to ARPE-19 under the experimental conditions of this study.

In keeping with the protective findings described above, activation of p38 was also shown to be involved in the protective process of oxidant preconditioning. ARPE-19 cells exposed to a low dose of t-BOOH demonstrated phosphorylation of p38 MAPK without appreciable cell death in the time frame of the experiment. Preconditioning with this low dose of t-BOOH was relatively protective against higher concentrations of the oxidant. This small beneficial effect of preconditioning was lost with inhibition of p38 MAPK activation by SB203580, suggesting that the effect of preconditioning was mediated, at least in part, by p38 signaling. In other cell types, p38 MAPK signaling has also been implicated in the oxidative preconditioning process.[31] To our knowledge, this is the first demonstration that preconditioning through p38 MAPK activation can protect ARPE-19 cells against oxidant-induced death using both pharmacological and genetic/molecular approaches.

Collectively, our findings support a protective role for p38 MAPK activation in ARPE-19 cells exposed to an acute t-BOOH challenge. Although p38 signaling has been shown to protect against stress-induced apoptosis in some systems,[24] it is interesting to note that this is not the case for the RPE, where studies have shown p38 MAPK signaling either to mediate oxidative stress-induced death[26,27,30] or at least to have no effect on viability.[28,29] Clearly, the regulation of cell survival and death is dependent on a number of factors, giving emphasis to the versatility of signal transduction control. Factors accounting for these seemingly disparate outcomes may include differences in cell type, as well as the nature of the stress, its dosage, and the duration of the exposure.[11]

In summary, we have presented evidence that p38 MAPK activation decreases ARPE-19 cell death, whereas inhibition or knockdown of p38 MAPK activity leads to an increase in t-BOOH-induced ARPE-19 cell death. Ambiguities that continue to challenge our understanding of the p38 MAPK signaling pathway suggest that further investigation is warranted.

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