Sustained versus Transient ERK1/2 Signaling Underlies the Anti- and Proapoptotic Effects of Oxidative Stress in Human RPE Cells

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PURPOSE. Oxidative stress is thought to contribute to the pathogenesis of age-related macular degeneration (AMD), which involves retinal pigmented epithelial (RPE) cell death. However, signaling pathways involved in the oxidative-stress-induced RPE cell death are poorly understood. This study was conducted to investigate the involvement of the MAP kinase pathways during the induction of RPE cell death by oxidative stress.

METHODS. ARPE-19 cells were exposed to the oxidant tert-butyl hydroperoxide (t-BHP). Cell viability was assessed by cell counting and MTT-staining, and apoptosis was quantified by Western blot analysis and immunocytochemistry with specific anti-phospho protein antibodies. Specific pharmacologic inhibitors directed against the MAPKs were used to analyze the signaling involved in cell death of RPE cells exposed to t-BHP.

RESULTS. Exposure of RPE cells to t-BHP, associated with an increase in reactive oxygen species and intracellular glutathione depletion, induced time- and concentration-dependent apoptosis, which was associated with the accumulation of inactive ERK1/2 in cell nuclei and a transient and weak ERK1/2 activation. This activation was accompanied by a deactivation of P90RSK, the major target of ERK1/2 and consequently by the delayed activation of its transcription factor CREB. MEK1/2 inhibition completely suppressed the transient activation of ERK1/2 and completely blocked apoptosis, demonstrating the role of the MEK-ERK module in mediating oxidative-stress-induced RPE cell death. In contrast, neither JNKs nor p38 MAPKs were involved in mediating t-BHP-induced apoptotic signaling in RPE cells.

CONCLUSIONS. The results suggest that inhibiting the MEK-ERK module may allow the development of selective methods for treating oxidative-stress-induced RPE degeneration, such as AMD. (Invest Ophthalmol Vis Sci. 2006;47:4614-4623) DOI: 10.1167/iovs.06-0297

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cell death, suggesting that JNKs and the p38 MAPKs may participate in cell-death signaling in RPE cells.\(^{17}\)

We report the direct effects of oxidative stress on RPE cells caused by tert-butyl hydroperoxide (t-BHP). We examined the effect of t-BHP on the antioxidant pathways involved in the antiapoptotic defense against oxidative stress in RPE cells. We also analyzed the activation and phosphorylation kinetics of the JNK, p38 MAPK, and ERK1/2 signaling pathways and studied the effects of inhibiting these three kinases to determine the relationship between the activation of MAPKs and RPE cell apoptosis. We showed that the MEK-ERK module played a pivotal role in oxidative-stress–induced RPE cell apoptosis and suggest that the ERK1/2 signaling pathway is an important target for future therapeutic strategies against human RPE cell degeneration diseases such as AMD.

**Materials and Methods**

**RPE Cultures and Treatment of Cells**

ARPE-19 (generously provided by Leonard Hjelmeland, University of California, CA), were cultured in DMEM/F12 (Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 0.1 mg/mL streptomycin (complete culture medium).\(^{16}\) ARPE-19 is a nontransformed human RPE cell line that displays many differentiated properties typical of RPE in vivo.\(^{18,19}\) As serum depletion has been found to cause oxidative stress through the generation of superoxide ions and to activate members of the MAPK family in RPE cells,\(^{17}\) we applied t-BHP-mediated oxidative stress in 10% FCS-containing medium. t-BHP is a chemical oxidant and is a model compound of early intermediate of lipid peroxidation. Oxidative stress was performed as followed: confluent human RPE cells cultured in complete culture medium were washed with Hank’s balanced salt solution (HBSS) and fresh complete culture medium, to which the appropriate concentration of t-BHP was added (t-BHP-treated RPE cells). Control cell cultures consist of confluent human RPE cells cultured in complete culture medium, washed with HBSS and then stimulated with fresh complete culture medium in the absence of t-BHP (untreated control RPE cells). The effects of t-BHP on RPE cell viability and activation of the MAPK signaling pathways was followed over a 48-hour period of culture and a 6-hour period of culture, respectively. Time 0 of the kinetics corresponds to the moment of the replacement of the complete culture medium with fresh complete culture medium with (t-BHP-treated cells) or without (untreated control cells) t-BHP. Therefore, time 0 corresponds to unstimulated and untreated RPE cells and permits analysis of the initial basal levels of activated–phosphorylated MAPKs just before the oxidative stress.

In some experiments, we tested the effect of specific pharmacological inhibitors of signaling pathways. Inhibitors of MEK1/2 (U0126), JNK1-3 (SP600125), and p38 aβ MAPKs (SB202190; Calbiochem, La Jolla, CA) were added 2 hours before t-BHP treatment and then in combination with t-BHP. Stock solutions of each inhibitor were prepared in dimethyl sulfoxide (DMSO) and diluted in DMEM to give a final DMSO concentration not exceeding 0.1% in test solutions (a concentration having no effect on RPE cell death). Antioxidant defense signaling was stimulated with the following chemicals: N-acetylcycteine (NAC), glutathione monothiol ester (GME), trolox, and 3-aminotriazole (3-AT; Calbiochem); and the following antioxidant enzymes: catalase and SOD1 (Sigma-Aldrich, St. Louis, MO). Phosphatasases were inhibited with okadaic acid, calyculin-A, tautomycin, and orthovanadate (Calbiochem) by pretreating cells for 2 hours with the appropriate chemical before exposing the cells to t-BHP in combination with the chemical.

**Measurement of Intracellular Oxidation**

We measured extracellular reactive oxygen species (ROS) levels using a fluorescent method. We incubated t-BHP-treated and untreated RPE cells with 1 μM of dichlorodihydrofluorescein diacetate (H2DCFDA; Interchim, Montlucon, France) for 15 minutes at 37°C. The cells were then washed with phosphate-buffered saline (PBS), trypsin treated, collected in 500 μL of 1% PAF, and subjected to flow cytometry analysis, according to the manufacturer’s recommendations (Epics ALTRA; Beckman Coulter, Fullerton, CA).

**Assays for Cell Viability**

We determined the number of viable RPE cells by counting in a hemocytometer, trypan blue-excluding cells after adding 0.5% trypan blue, and by MTT staining (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), as previously described.\(^{20}\)

**Cell Cycle Progression and Cell Apoptosis Analysis**

We analyzed the cell cycle by determining the cell DNA content with the use of propidium iodide (PI) after 24 hours of culture. The cells were washed in PBS and fixed by incubation in ice-cold 70% ethanol for at least 2 hours at 4°C. They were then rehydrated in cold PBS, treated with 1 mg/mL RNase A (Roche, Indianapolis, IN), and stained with 50 mg/mL PI by incubation for at least 15 minutes at 4°C. The stained cells were analyzed by flow cytometry. An apoptotic process, with cleavage of the DNA in the oligonucleosomes, was indicated by a subG\(_1\) peak that was well separated from the G\(_0\)/G\(_1\) peak. We analyzed apoptotic cell death by two methods: terminal dUTP nick-end labeling (TUNEL; PCD kit; Roche) and double staining with Yopro-1 and PI, according to manufacturer’s recommendations. We observed and counted TUNEL-positive cell nuclei in three different fields within an ocular grid using a 25× objective on a microscope (Aristoplan, Leitz, Wetzlar, Germany). A minimum of 200 cells was counted per field. We calculated the percentages of apoptotic cell nuclei in comparison to the untreated control cells. The percentages of apoptotic (Yopro-1-positive) and necrotic (PI-positive) cells were determined by flow cytometry.

**Real-Time Polymerase Chain Reaction Analysis**

Total RNA was extracted from ARPE-19 cultures (TRizol; Invitrogen, Carlsbad, CA) by using a phenol-chloroform method. Two micrograms were reverse-transcribed (Sperscript II Reverse Transcriptase; Invitrogen). Amplification reaction assays contained 1× PCR master mix (SYBR Green; Invitrogen) and primers (Table 1) at optimal concentrations. PCR started at 95°C for 5 minutes and cycled 40 times at 95°C for 15 seconds and 65°C for 1 minute in a thermal cycler (model 7300 SDS; Applied Biosystem, Inc., [ABI] Foster City, CA). Forward and reverse primers were placed on two consecutive exons of the gene where possible. No-reverse-transcriptase controls were run in each assay to confirm lack of genomic DNA contamination. GAPDH was chosen as a suitable internal control because its expression was shown to be constant.
unaffected by t-BHP treatment in ARPE-19 cells. Validation experiments were performed to confirm equivalent PCR efficiencies for GAPDH and the target genes. Relative quantitation of gene expression was performed by the standard curve method (Prism 7700 Sequence Detection System; ABI User Bulletin number 2).

**Western Blot Analysis**

After inducing t-BHP–mediated oxidative stress, RPE cells were washed twice in PBS, lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 1 μg/mL leupeptin, and 1 μM pepstatin) and centrifuged at 4°C for 10 minutes at 10,000g. We used monoclonal antibodies directed against β-actin as an internal standard to check for protein loading. Cell lysates were mixed with 3× Laemmli buffer and heated for 5 minutes at 95°C. The lysates were then resolved by SDS-PAGE (12%-15% polyacrylamide gel) and transferred to polyvinylidene difluoride (PVDF) filters by electroblot. They were probed with polyclonal antibodies directed against ERK1/2, P90RSK, and cAMP-response element (CRE)-binding protein (CREB; 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), to determine the level of these kinases that were present during oxidative stress. Polyclonal antibodies directed against phospho-ERK1/2 (Thr182 and Tyr185), phospho-P90RSK (Ser381) and (Thr 60 and Ser364); phospho-JNK1/3 (Thr 183 and Tyr185); phospho-p38 MAPKs (Thr 180 and Tyr182); phospho-c-jun (Ser 73), and phospho-CREB (Ser133; dilution 1:1000, Cell Signaling Technology, Beverly, MA) were used to analyze the activation of intracellular signaling during t-BHP–induced RPE cell death. The primary antibodies were detected with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. We used chemiluminescent substrate to detect the secondary antibody, according to the manufacturer’s instructions, and the membrane was placed against autoradiograph film (Hyperfilm ECL; GE Healthcare, Piscataway, NJ). Quantification of the phosphorylation levels was performed (Image Station 2000MM and 1D3,6 software; Eastman Kodak, Rochester, NY).

**Statistical Analyses**

Statistical analyses were performed by computer (GraphPad Software Inc., San Diego, CA). Normality was tested with the Kolmogorov-Smirnov test. Differences between groups were compared by using the nonparametric Kruskal-Wallis test, and paired comparisons between groups were performed with the Mann-Whitney test. Data are expressed as the mean ± SD, and the differences was considered statistically significant at P < 0.05.

**RESULTS**

**Effect of t-BHP on RPE Cell Survival**

We first investigated the effect of a single treatment of t-BHP on cell survival in confluent, nonproliferating RPE cell cultures. Treatment with t-BHP (100–1000 μM) induced cell death in a dose-dependent manner in RPE cell cultures (Fig. 1A). At 100 μM, t-BHP had no lethal effect over 24 hours of culture (Fig. 1A). The t-BHP concentration necessary to induce 50% cell death (IC50) was 500 μM, whereas with 1000 μM t-BHP, approximately 30% of cells remained alive after 6 hours of treatment (Fig. 1A). By contrast, almost all RPE cells died after 12 hours of treatment with 500 μM t-BHP (Fig. 1A). Figure 1B shows the temporal evolution of cell death for a single treatment of 300 μM t-BHP. In these conditions, we clearly detected RPE cell death (31%) after only 6 hours of treatment.

We then investigated whether t-BHP-induced RPE cell death involved an oxidative-stress–signaling pathway. We observed an inversely correlated increase in intracellular production of ROS with cell survival during t-BHP treatment (Fig. 2A). Treatment of RPE cells with t-BHP (300 μM) reduced glutathione (GSH) levels by 63% after 6 hours of treatment (Fig. 2B).

The reduction in GSH levels remained the same at 500 μM t-BHP (Fig. 2B). Cell treatment with N-acetylcycteine (NAC, 1 mM), a GSH precursor, reversed the effect of t-BHP on GSH levels (Fig. 2B) and abolished t-BHP–induced cell death (Fig. 2C). Cell treatment with either glutathione monoethyl ester (GME, 3 mM), a GSH analogue, or trolox (25 μM), a derivative of vitamin E, completely blocked the lethal effect of t-BHP after 24 hours of culture (Fig. 2C). We then quantified the effects of t-BHP on the expression levels of these enzymes in RPE cells. T-BHP had no effect on the level of glutathione peroxidase expression, whereas it increased SOD2 expression by 96% after 4 hours of t-BHP treatment. Conversely, catalase expression decreased by 38% over the same time period (Fig. 2D). Therefore, we pretreated RPE cells with catalase. Neither catalase (100 U/mL) nor its specific inhibitor 3-aminothiozole (3-AT, 30 mM) altered t-BHP–induced cell death. Cell treatment with SOD1 (100 U/mL) also did not protect RPE cells from t-BHP–induced cell death (Fig. 2E).

**Characterization of the t-BHP-Induced RPE Cell Death**

We next wanted to determine the cell death process caused by t-BHP-induced oxidative stress in RPE cells. Approximately 96%
of control cells were detected corresponding to the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle, whereas 75.6% of the cells were detected in a well-separated peak corresponding to the sub-G<sub>1</sub> phase of the cell cycle after 48 hours of treatment with 300 μM t-BHP, and 97.5% of cells after 48 hours of treatment with 500 μM t-BHP (Fig. 3A). Cell treatment with 300 μM t-BHP also increased the number of TUNEL-positive cell nuclei by 8.8 times (27.3%) after only 6 hours of t-BHP treatment (Fig. 3B).

**FIGURE 2.** t-BHP induced production of ROS and cell death through oxidative stress in RPE cell cultures. Confluent cultures of quiescent RPE cells were cultured in the presence or absence of t-BHP (300 μM). (A) Production of extracellular reactive oxygen species was measured using H2DCFDA and flow cytometry. The effect of t-BHP on ROS production was analyzed together with cell survival, by MTT assay. The percentage of cell survival was calculated compared with untreated control cells (Ct). (B) The effect of different doses of t-BHP on the intracellular production of GSH was detected and quantified by a colorimetric method. Differences between groups were analyzed by the Kruskal-Wallis test (P = 0.0017) and the difference between pairs by Mann-Whitney test (P < 0.05). (C) The effects of pretreatment (1 hour before stimulation with t-BHP, 300 μM, with different antioxidant chemicals) on cell survival were analyzed after 24 hours of culture by using the MTT assay. The percentage of cell survival was calculated compared with untreated control cells. Differences between groups was tested by the Kruskal-Wallis test (P = 0.0001) followed by pair comparisons using the Mann-Whitney test (P < 0.05). Similar results were obtained in three independent experiments. Data are the mean ± SD.

**FIGURE 3.** Effect of t-BHP on cell cycle progression, apoptosis, and caspase activation in RPE cells. (A) Confluent cultures of quiescent RPE cells were treated with t-BHP (500 and 500 μM) and the sub-G<sub>1</sub> peak was analyzed by flow cytometry after PI staining after 48 hours of t-BHP treatment. Apoptosis was detected by both the (B) TUNEL method and (C) Yopro-1 and PI double-staining after 6 hours of culture. (B) The percentage of TUNEL-positive nuclei was calculated compared with untreated control cells. Pair differences were analyzed by the Mann-Whitney test (P < 0.05). (C) The percentage of apoptotic cells detected by Yopro-1/PI double-staining was determined by flow cytometry. (D) The activation of caspase-3 and -9 was detected by Western blot analysis Similar results were obtained in three independent experiments. *Control of activated caspase-3 in etoposide-treated HeLa cells.
Yopros-1 and PI double staining showed a 16.4-fold increase in the number of cells undergoing early apoptosis and a 3.9-fold increase in the number of cells undergoing late apoptosis after 6 hours of t-BHP treatment (Fig. 3C). Cell treatment with 300 μM of t-BHP induced caspase-9 activation, as revealed by the cleavage of pro-caspase-9 (47 kDa) into the active 17-kDa fragment after 10 minutes and over a 2-hour treatment period (Fig. 3D). By contrast, t-BHP-induced cell death did not induce cleavage of the inactive form of caspase-3 (32 kDa) and therefore did not activate caspase-3 (Fig. 3D).

**Roles of JNKS and p38 αβ MAPKs in the Apoptotic Signaling of t-BHP-Induced Oxidative Stress in RPE Cells**

We wanted to determine the intracellular pathways that participate in oxidative-stress-induced RPE cell death, to allow an anti-apoptotic strategy in RPE cells to be developed. Analysis of protein phosphorylation on Tyr and Thr showed that oxidative-stress-induced changes in the phosphorylation of these two residues over a 6-hour treatment period (Fig. 4A). Analysis of the protein phosphorylation of the MKKs of JNKS and p38 MAPKs—MKK4 and MKK3/6, respectively—showed that treatment of RPE cells with 300 μM t-BHP affected their activation. This finding showed an adaptive response of the cells to the oxidative stress rather than a rapid MKK-mediated activation of an apoptotic signaling in t-BHP-treated RPE cells (Fig. 4B). However, t-BHP did not markedly affect JNK and p38 MAPK activation compared with untreated control cells (Fig. 4C). JNK activation with a specific JNK inhibitor, SP600125, reduced cell survival in control untreated RPE cells by 60% (Fig. 4D) and did not protect the cells from t-BHP-induced apoptosis. JNK inhibition actually enhanced the lethal effects of t-BHP, as more than 98% of RPE cells died after treatment with SP600125 in the presence of t-BHP (Fig. 4D). We also treated cells with a specific p38 αβ MAPK inhibitor, SB202190, which did not affect cell survival of control RPE cells (Fig. 4D). However, it also did not rescue RPE cells from t-BHP-induced cell death, unlike the free radical scavenger NAC (Fig. 4D).

**Modulation of ERK1/2 Activation by Apoptotic Oxidative Stress in RPE Cells**

We investigated ERK1/2 activation during t-BHP-induced RPE cell death. ERK1/2 activation and phosphorylation levels were low before treatment with t-BHP (time 0; see Figs. 5A, 5C, 7B, 8A). Cell stimulation with 10% FCS in the absence of t-BHP (untreated control cells) induced a large increase in ERK1/2 phosphorylation levels after 10 minutes of culture. The ERK1/2 activation levels reached a plateau and remained at the same levels for 30 minutes (first phase of activation; Figs. 5A, 5C, 8A). Then the levels decreased slightly over the next 6 hours (second phase of activation). RPE cell treatment with 300 μM t-BHP in the presence of 10% FCS slightly affected the first- and second-phase of activation of ERK1/2, but substantially reduced the ERK1/2 phosphorylation levels during the second phase of activation (Fig. 5A). At 400 μM t-BHP, which induced approximately 75% RPE cell death after 12 hours of culture, reduced ERK1/2 activation levels in the first phase by 50% and completely abolished ERK1/2 phosphorylation in the second phase of activation, with the ERK1/2 phosphorylation levels returning to those of untreated control cells (Figs. 5A, 5B). We detected no activation of P90RSK, a major target of ERK1/2, in unstimulated and untreated RPE cells. Its activation kinetics matched those of ERK1/2 activation in untreated control cells (Fig. 5C). As for the deactivation of ERK1/2, the P90RSK activation kinetics was also substantially altered after t-BHP treatment. We observed a transient P90RSK phosphorylation induction phase after 10 minutes of t-BHP treatment, which then rapidly and completely disappeared (Fig. 5C). The activation kinetics of CREB, a directly activated substrate of ERK1/2-activated P90RSK, was also substantially altered after cell treatment with t-BHP (Fig. 5C). Phosphorylation/activation of CREB now occurred after 30 minutes of treatment. The levels of expression of total ERK1/2, P90RSK, and total CREB now occurred after 30 minutes of treatment. The ERK1/2-P90RSK-CREB signaling pathway was under the unique control of MEK1/2, in that its phosphorylation and activation kinetics matched those of ERK1/2 activation in untreated control cells.

**FIGURE 4.** Activation levels of the JNK and p38 MAPK signaling pathways were not altered by t-BHP and did not play a role in the protection of RPE cells against t-BHP-induced oxidative stress. Confluent cultures of quiescent RPE cells were cultured in the presence or absence of t-BHP (300 μM). Cells were lysed at the indicated time. Protein (30 μg) was reduced and subjected to SDS-PAGE and Western blot analysis with specific (A) anti-phospho-threonine (p-Thr) and anti-phospho-tyrosine (p-Tyr), (B) anti-phospho-MKK4 and anti-phospho-MKK3/6, and (C) anti-phospho-JNKs, anti-phospho-p38 kinases, and anti-phospho-c-jun antibodies. (B, C) Membranes were probed with an antibody directed against actin to control for equal loading. (D) Confluent quiescent RPE cells were pretreated with or without the JNK1-3 inhibitor, SP600125 (25 μM), the p38 αβ MAPK inhibitor, SB202190 (50 μM), the antioxidant, NAC (1 mM) for 2 hours and then cultured in the presence or absence of t-BHP (300 μM). The effects of JNK and p38 MAPK inhibition on cell viability were determined by the MTT colorimetric method. The percentage of cell survival was calculated compared with control untreated cells. The efficiency of the JNK and p38 MAPK inhibitors was controlled by the survival of serum-depleted RPE cells as previously described. Similar results were obtained in three independent experiments. The difference between groups was tested by the Kruskal-Wallis test (P < 0.05) followed by pair comparisons with the Mann-Whitney test (* P < 0.05).
Roles of MAP Kinase Phosphatases and Localization of ERK1/2 in Oxidative-Stress–Treated RPE cells

We then wanted to determine the precise molecular mechanism involved in converting sustained ERK activation to transient activation induced by oxidative stress. The active forms of ERK1/2 were not present in either the cytoplasm or the nucleus of unstimulated and untreated cells (Fig. 6A). Over a 6-hour period of culture, we detected very low levels of active ERK1/2 in the cytoplasm of untreated control RPE cells, whereas most of the nuclei were strongly stained (Figs. 6B, 6C). Immunohistochemical analysis of total ERK2 showed that ERK1/2 staining was confined to the cytoplasm of the majority of untreated and unstimulated RPE cells (Fig. 6G). However, a minority of cells present ERK1/2 staining to the nuclei. Over a 6-hour period of culture, we still detected ERK1/2 staining in the cytoplasm, but an intense ERK1/2 staining was detectable in the nuclei (Figs. 6H, 6I). Activation of ERK1/2 occurred for only a short period in the nuclei of apoptotic cells compared with untreated control cells. Phosphorylated–activated ERK1/2 was present in the nuclei of t-BHP–treated cells after only 10 minutes of serum stimulation (Figs. 6E, 6F). In contrast, total ERK1/2 was still detectable in the nuclei of most of the cells after 6 hours of t-BHP treatment, showing that unphosphorylated–inactive ERK1/2 remained in the nuclei and is the major form of ERK1/2 in RPE cells undergoing t-BHP–induced apoptosis (compare Figs. 6F and 6L).

Then we investigated whether the deactivation of ERK1/2 associated with RPE cell apoptosis involved MAP kinase phosphatases (MKPs). The expression levels of MKP3 decreased by 43%, whereas those of MKP1, PP1, and PP2A remained constant after t-BHP treatment (Fig. 7A). We next investigated whether t-BHP–mediated ERK1/2 deactivation may preferentially involve the hydrolysis of one of the two phosphorylated residues. We detected no differences between the pTyr and the pThr residues in the t-BHP–induced kinetics of ERK1/2 dephosphorylation during 6 hours of culture (Fig. 7B). Then, we studied the effect of MAPK/ERK kinase (MEK) inhibition by U0126 (10 μM) in t-BHP–treated RPE cells on the phosphorylation levels of ERK1/2, P90RSK, and CREB, and total ERK1/2, P90RSK, and CREB determined by Western blot analysis. Similar results were obtained in three independent experiments. (B) The and differences between groups were analyzed by Kruskal-Wallis test ($P = 0.0006$) followed by pair comparisons using Mann-Whitney test (*$P < 0.05$). Data are the mean ± SD.

Figures 5 and 6. Activation of ERK1/2 was substantially affected during oxidative-stress–induced apoptosis in t-BHP-treated RPE cell cultures. RPE cells were cultured in the presence or absence of t-BHP at the indicated concentrations and were lysed at the indicated time. Protein (30 μg) was reduced and subjected to SDS-PAGE and Western blot analysis with (A–C) specific anti-phospho-ERK1/2 and anti-ERK1/2 and (C) specific anti-phospho-P90RSK, anti-P90RSK, anti-phospho-CREB, and anti-CREB antibodies. The effect of MEK1/2 inhibition by U0126 (10 μM) in t-BHP-treated RPE cells on the phosphorylation levels of ERK1/2, P90RSK, and CREB, and total ERK1/2, P90RSK, and CREB was determined by Western blot analysis. Similar results were obtained in three independent experiments. (B) The and differences between groups were analyzed by Kruskal-Wallis test ($P = 0.0006$) followed by pair comparisons using Mann-Whitney test (*$P < 0.05$). Data are the mean ± SD.

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We next investigated the effect of blocking the transient activation of ERK1/2 to determine whether the transient activation of ERK1/2 mediates the oxidative-stress–induced apoptotic signaling in t-BHP-treated RPE cells. Treatment of untreated control cells with U0126, a highly specific-pharmacological–inhibitor of MEK1/2, did not alter cell survival over 24 hours of culture (Fig. 8A). By contrast, the complete inhibition of ERK1/2 activation by U0126 abolished cell death at 300 μM t-BHP (Fig. 8A). The complete blockade of apoptosis by MEK1/2 inhibition in cells treated with 500 μM of t-BHP, a dose that induced more than 90% cell death after 12 hours of culture, confirmed that transient ERK1/2 activation mediated the major signaling pathway that controls RPE cell death after exposure to oxidative stress (Fig. 8A). Cell treatment with U0124, a nonactive structural analogue of U0126, did not affect cell viability in untreated control cells and did not rescue cells from t-BHP–induced cell death (Fig. 8A). Cell treatment with U0126 decreased the number of TUNEL-positive cell nuclei by 6.9 times (25.2% of TUNEL-positive cell nuclei in t-BHP–exposed cells versus 3.7% of TUNEL-positive cell nuclei in t-BHP-exposed cells treated with U0126; Fig. 8B). In addition, NAC completely reversed the t-BHP-induced deactivation of ERK1/2, confirming that the regulation of ERK1/2 activation is due to oxidative stress (Fig. 8C).

**DISCUSSION**

**Role of ERK1/2 during Oxidative-Stress–Induced RPE Cell Death**

It has been suggested that ERK1/2 activation may be a general signaling defense against oxidative stress damage. Herein, we report a transient activation of ERK1/2 after a direct and acute oxidative stress induced by t-BHP. In unstimulated cells, ERK1/2 activation was sustained in 10% serum culture conditions, which shows that oxidative stress partially deactivates ERK1/2 in RPE cells. Our data seem to be opposite to that reported for the activation of ERK1/2 signaling after exposure to various types of oxidative stresses. However, these studies only looked at the initial basal levels of activated-phosphorylated ERK1/2 just before the oxidative stress. As the ERK1/2 activation kinetics were not studied in untreated control cells, the global effects of oxidants on ERK1/2 activation dynamics in these models are difficult to compare. We also showed that late deactivation of ERK1/2 was followed by the partial deactivation of P90rsk, which lies at the end of the ERK pathway and was associated with a delayed CREB activation. Our data suggest that the first phase of activation of ERK/P90rsk signaling is responsible for the apoptotic signal in RPE cells subjected to oxidative stress. As ERK1/2 activation alone may not predict subsequent cellular survival responses, we investigated the effects of inhibiting the transient ERK1/2 activation on cell survival in RPE cell cultures exposed to t-BHP. We showed that the complete inhibition of the first phase of ERK1/2 activation, after MEK1/2 inhibition, completely protected cells from t-BHP-induced oxidative stress. This observation is in apparent contrast with those in our previous study on the role of the FGFR1/FGF1/ERK1/2 signaling pathway in the protection of serum-starved, aged RPE cells. Several reasons may explain this difference. First, we showed a correlation only between the FGFR1/FGFR1 autocrine activation loop and the overactivation of ERK1/2 due to their own overexpression in bovine aged...
RPE cells. No experiments of ERK1/2 inhibition were undertaken to demonstrate the direct role of ERK1/2 in the protection of RPE cells against serum starvation. Second, overexpression of ERK1/2 was observed in aged bovine RPE cells after serum starvation, whereas the levels of ERK1/2 expression remained constant over the t-BHP treatment and were similar to those detected in control untreated human RPE cells. Third, aged bovine RPE cells after repeated culture passage (replicative senescence) are certainly different from normal human ARPE-19 cells. Fourth, the molecular mechanism induced by a

serum depletion-mediated metabolic stress is different from a direct chemical oxidant-induced oxidative stress performed in the presence of serum. Altogether, these data strongly suggest that the mechanisms by which we previously hypothesized that the FGF1/FGFR1-mediated overexpression and overactivation of ERK1/2 may confer cell resistance against cell death induced by serum depletion in bovine senescent RPE cells is not simply related to an oxidative stress and may involve several other processes.15 This conclusion is consistent with the study by Garg and Chang,24 who showed that inhibition of ERK1/2 signaling did not protect RPE cells from apoptosis, despite ERK1/2’s being activated by a combination of serum depletion and hydrogen peroxide. Other studies on established cell lines, primary neurons and animals subjected to a variety of oxidative stresses (hydrogen peroxide, peroxynitrite, zinc and iron, ischemia, and glutathione depletion) have used a similar experimental approach to ours (i.e., chemical inhibition of MEK1/2). These studies showed that specifically blocking ERK1/2 activation protects against oxidative-stress-induced cell death, irrespective of the strength and intensity of the ERK1/2 activation kinetics during oxidative stress.25–30

Mechanism Accounting for ERK1/2 Deactivation after Lethal Oxidative Stress in RPE Cells

Differences in outcome after the partial deactivation of ERK1/2 may depend both on the cell environment and the cell type expressing ERK1/2 and on the severity and kinetics of the oxidative injury. A microarray analysis in ARPE-19 cells comparing the respective effects of t-BHP and H2O2 showed that many genes were differentially regulated by these oxidants. This group included genes coding for signal transduction and apoptosis proteins, and transcription factors.51 Although the respective roles of t-BHP and H2O2 on the kinetics of ERK1/2 activation and ERK1/2-regulated transcription factor expression were not analyzed, the study confirmed the importance of the type of oxidant in the signal transduction-regulated transcription response in RPE cells. It has been shown recently that α-lipoic acid (LA) protects cultured human fetal RPE cells against t-BHP–induced cell death and apoptosis.32 It notable that the antioxidant effect of LA is mediated through ERK inactivation in different cell types.53

We focused on the subcellular localization of activated ERK1/2 under lethal oxidative stress, which may affect ERK1/2 activation. It has recently been shown that the subcellular localization that determines the protective effects of ERK1/2 depends on the types of apoptotic stimuli—cytoplasmic localization for serum depletion and nuclear localization for tyrosine kinase inhibition.34 Oxidative stress also appears to influence the subcellular trafficking and/or localization of activated ERK1/2 to downstream targets within distinct subcellular compartments underlies neurotoxic responses and therefore the survival response of stressed cells. We showed that oxidative stress influences the subcellular localization of ERK1/2 in RPE cells. Inactive ERK1/2 accumulates in the nucleus of apoptotic RPE cells. Recently, the blockade of active ERK1/2 nuclear translocation has been associated with neuroprotection against oxidative stress.56 This supports the key role of active ERK1/2 subcellular localization in ERK-mediated apoptotic signaling under oxidative stress. Changes in the subcellular localization of members of the ERK signaling pathway have also been observed in patients with different neurologic disorders. Nuclear accumulation of MEK1 has been observed during early stages of Alzheimer disease, whereas MEK1 never translocates to the nucleus in normal conditions.57 Alterations in the subcellular distribution of ERK1/2 and P90RSK have also been detected in the Parkinson disease–related Lewy body disease.38

FIGURE 8. The MEK-ERK module was the major pathway involved in the apoptotic signaling of t-BHP–mediated oxidative stress in RPE cells. (A, B) RPE cells were pretreated with or without the specific MEK1/2 inhibitor, U0126 (10 μM), or its inactive analogue, U0124 (10 μM), for 2 hours and then cultured, with or without t-BHP at the indicated concentrations. The effects of the inhibition of the MEK-ERK module on cell viability were determined by (A) the MTT colorimetric method and (B) the TUNEL method after 6 hours of culture. The percentage of cell survival and TUNEL-positive nuclei was calculated compared with untreated control cells (Ct). Difference between groups was tested by the Kruskal-Wallis test (A, P = 0.0006) followed by pair comparisons using the Mann-Whitney test (A, B, *P < 0.05). Similar results were obtained in three independent experiments. (C) RPE cells pretreated with or without NAC (1 mM) were cultured in the presence or absence of t-BHP at the indicated concentrations and were lysed at the indicated time. Protein (30 μg) was reduced and subjected to SDS-PAGE and Western blot with specific anti-phospho-ERK1/2 antibodies. Membranes were probed with antibodies directed against actin to control for equal loading.

Proapoptotic Role of ERK in RPE Cells
Moreover, it has been shown that CREB mediates a survival pathway that is altered during oxidative-stress-mediated neurodegenerative disorders, suggesting that the alteration of CREB activation contributes to oxidative-stress–induced neuronal dysfunction.59 This suggests that the mislocalization of the entire MEK/ERK/p90RSK/CREB signaling pathway may be involved in neurodegenerative diseases. Although the functional consequences of this alteration to subcellular localization are not known in these neurodegenerative diseases, we should analyze the subcellular distribution of the different members of this signaling pathway in the RPE cells of patients with AMD.

In conclusion, our study showed that oxidative stress provokes a major change in ERK1/2 activation kinetics. This change from a sustained to a transient activation similarly affects the activation of the downstream targets p90RSK and CREB. Transient activation is ultimately responsible for the GSH-depletion–associated apoptosis, as the selective blockade of MEK1/2, the direct upstream kinase of ERK1/2, completely protected cells from apoptosis. The lack of effect of MEK1/2 inhibition on RPE cells under normal conditions makes the components of the ERK1/2 signaling pathway potential targets for therapeutic treatments in retinal degeneration, such as AMD.

References


