ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells

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Zhuang S, Yan Y, Daubert RA, Han J, Schnellmann RG. ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells. Am J Physiol Renal Physiol 292: F440–F447, 2007. First published August 1, 2006; doi:10.1152/ajprenal.00170.2006.—Reactive oxygen species, including hydrogen peroxide (H2O2), are generated during ischemia-reperfusion and are critically involved in acute renal failure. The present studies examined the role of the extracellular signal-regulated kinase (ERK) pathway in H2O2-induced renal proximal tubular cells (RPTC) apoptosis. Exposure of RPTC to 1 mM H2O2 resulted in apoptosis and activation of ERK1/2 and Akt. Pretreatment with the specific MEK inhibitors, U0126 and PD98059, or adenosine virulent with a construct that encodes a negative mutant of MEK1, protected cells against H2O2-induced apoptosis. In contrast, expression of constitutively active MEK1 enhanced H2O2-induced apoptosis. H2O2-induced activation of caspase-3 and phosphorylation of histone H2B at serine 14, a postranslational modification required for nuclear condensation, which also were blocked by ERK1/2 inhibition. Furthermore, blockade of ERK1/2 resulted in an increase in Akt phosphorylation and blockade of Akt potentiated apoptosis and diminished the protective effect conferred by ERK inhibition in H2O2-treated cells. Although Z-DEVD-FMK, a caspase-3 inhibitor, was able to inhibit histone H2B phosphorylation and apoptosis, it did not affect ERK1/2 phosphorylation. We suggest that ERK elicits apoptosis in epithelial cells by activating caspase-3 and inhibiting Akt pathways and elicits nuclear condensation through caspase-3 and histone H2B phosphorylation during oxidant injury.

oxidative stress; renal proximal tubular cells; extracellular signaling-regulated kinase; phosphoinositide 3-kinase; histone

REACTIVE OXYGEN SPECIES (ROS) have been implicated in the pathogenesis of a variety of renal diseases, including ischemia-reperfusion and toxic acute renal failure, and cell death (7, 16, 28, 29, 33). ROS include superoxide anion, hydrogen peroxide (H2O2), and hydroxyl radical. Among them, H2O2 is a common mediator and is generated during hypoxia/reoxygenation and ischemia-reperfusion injury (18, 19, 28, 29). Therefore, understanding the signaling pathways of H2O2-induced cell death would provide important clues about the mechanisms of renal tubular cell injury and acute renal failure.

Caspases are a 12-member family of specific cysteine proteases. Among them, caspase-3 is a primary mediator in the effector phase of apoptosis induced by a variety of stimuli including H2O2 (8, 13, 22). Caspase-3 can be activated through intrinsic and extrinsic mechanisms. Intrinsic pathways are activated by some stress stimuli and lead to cytochrome c release from the mitochondria (13, 21). Released cytochrome c promotes the activation of caspase-9 through Apaf-1 and then activates caspase-3. Caspase-3 activation leads to cleavage and activation of caspase-activated DNase (CAD) and activated CAD is responsible for internucleosomal DNA fragmentation, a hallmark of apoptosis (40). Another hallmark of the terminal stages of apoptosis is chromatin condensation (10, 41). Although caspase-3 activation is also coupled to apoptotic nuclear condensation, the signaling molecules that transduce nuclear condensation remain poorly understood. Recently, histone phosphorylation, in particular, phosphorylation of histone H2B at serine 14, was associated with nuclear condensation in mammalian cells and yeast (1, 9). Caspase-3 induces phosphorylation of histone H2B in vitro and in vivo through cleavage of MST1 kinase, a ubiquitously expressed serine/threonine kinase (9).

Exposure of cells to oxidant stress also induces activation of multiple members of the mitogen-activated protein kinases (MAPK) including extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 kinase (35, 36), and the phosphoinositide 3-kinase (PI3K)/Akt pathway (42). Among these kinases, JNK and p38 pathways are frequently associated with induction of apoptosis, whereas the ERK and Akt pathways are thought to deliver survival signals that protect cells from apoptosis (42). However, increasing evidence reveals that ERK activation contributes to apoptosis in certain cell types and organs. For example, cisplatin induces ERK1/2 activation and inhibition of ERK-blocked apoptosis in renal epithelial cells (5, 23, 26). Furthermore, ERK inhibition by U0126 was renal protective in cisplatin-induced nephrotoxicity in mice (21).

Although ERK is activated in other forms of acute tubular cell injury such as renal ischemia-reperfusion injury in animals (31), the role of ERK in the pathogenesis remains to be established. With the use of in vitro models of ischemia-reperfusion injury, conflicting evidence exists concerning the role of ERK in apoptosis. Arany et al. (6) and Di Mari et al. (11) reported that ERK activation is required for cell survival in mouse renal proximal tubular cells (RPTC) exposed to H2O2. Other studies showed that ERK activation contributes to either apoptosis in OK cells following H2O2 treatment (25) or necrotic cell death in LLC-PK1 cells exposed to 2,3,5-tri-(glutathione-yl)hydroquinone, which induces toxicity via ROS generation and H2O2 (12, 32). Consequently, additional studies are needed to clarify the role of ERK in oxidant injury-induced apoptosis and the signaling thereof.

We previously made improvements to the culture of rabbit RPTC such that the degree of differentiation and similarity to
ERK contributes to apoptosis

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RPTC found in vivo is greater than other models of RPTC (27). Using this model and pharmacological and molecular approaches, we examined the role of ERK1/2 in H2O2-induced apoptosis and found that ERK is necessary for H2O2-induced apoptosis of RPTC. Furthermore, we provide evidence that ERK1/2-mediated apoptosis is through activation of caspase-3 and inactivation of PI3K/Akt pathway following oxidant injury and that nuclear condensation is the result of caspase-3-mediated histone H2B phosphorylation.

MATERIALS AND METHODS

Chemicals and reagents. Z-DEVD-FMK, Z-VAD-FMK, U0126, SB203580, SP600125, and PD98059 were obtained from Calbiochem (San Diego, CA). Rabbit anti-phospho-ERK1/2, anti-phospho-p38, anti-ERK1/2, anti-p38, anti-phospho-H2B-S14, anti-H2B, and anti-active caspase-3 were purchased from Cell Signaling (Boston, MA). All other chemicals were from Sigma (St. Louis, MO). All antibodies were used at 1:1,000 for immunoblot analysis.

Isolation and culture of renal proximal tubules and experimental protocols. Female New Zealand White rabbits were purchased from Myrtle’s Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in six-well or 35-mm tissue culture dishes under improved conditions as previously described (27). The culture medium was a 1:1 mixture of DMEM/Ham’s F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM l-glutamine, 1 mM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 mM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium. RPTC were used in control samples.

Replication-deficient adenovirus infection. Since RPTC in primary culture have a low efficiency of gene expression using common delivery methods, adenoviral expression vectors were used. The construction and characterization of recombinant adenoviruses containing the coding regions of the kinase active form of human MEK1 (Ad-MEK1ca) and kinase inactive form of human MEK1 (Ad-MEK1dn) driven by the cytomegalovirus immediate early promoter have been described previously (15). RPTC were infected with each virus at a multiplicity of infection (MOI) of 100 pfu for 2 h at 37°C in a humidified 5% CO2 incubator. Afterward, the cultures were placed in normal culture media for an additional 48 h and then exposed to oxidant injury for the time periods described in the figure legends. At an MOI of 100 pfu, ~100% of the cells showed expression of the viral gene insert as indicated by the X-gal assay of RPTCs introduced with recombinant adenoviruses expressing lacZ (data not shown).

Nuclear staining. After treatment cells were washed with PBS, fixed in methanol, and then stained with DAPI. Cells with condensed nuclei, and/or DNA fragmentation were considered to be apoptotic. Cells in five random fields (×40) were counted, and percentage of apoptotic cells was calculated.

MTT assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess cell viability (24, 30). MTT was added (final concentration of 0.5 mg/ml) to individual cultures. RPTC were incubated for an additional 1 h, and tetrazolium was released by dimethyl sulfoxide. Optical density was determined with a spectrophotometer (570 nm), and data were normalized to solvent-treated cultures.

Caspase-3-like activity assay. Caspase activity was measured by a colorimetric assay following the manufacturer’s instructions (BioVision, Palo Alto, CA). Cells were lysed in 0.1 M HEPES buffer (pH 7.4) containing 2 mM dithiothreitol, 0.1% 3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, and 1% sucrose. Cell lysates were incubated with a colorimetric substrate, Ac-DEVD-pNA, for 30 min at 30°C. The release of the chromophore p-nitroanilide was measured using a fluorescence spectrophotometer.

Immunoblot analysis. Cells were washed once with phosphate-buffered saline without Ca2+ and Mg2+ (PBS) and then suspended in the lysis buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.1 mg/ml bromphenol blue, 0.5% 2-mercaptoethanol). After sonication for 15 s, equal amounts of cellular protein lysates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Following treatment with 5% skim milk at 4°C overnight, the membranes were probed with various antibodies for 1 h followed by appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Bound antibodies were visualized by chemiluminescence detection on autoradiographic film.

Statistical analysis. Data are presented as means ± SD and were subjected to one-way ANOVA. Multiple means were compared using Tukey’s test. P < 0.05 was considered a statistically significant difference between mean values. Renal proximal tubules isolated from an individual rabbit represent a single experiment (n = 1) consisting of data obtained from three wells.

RESULTS

H2O2-induced apoptosis in RPTC. Primary cultures of RPTC were exposed to 1 mM H2O2, and cell viability was examined using the MTT assay. Cell viability was not changed during the initial 3 h after H2O2 exposure but decreased to 63 ± 4% at 4 h, 55 ± 8% at 5 h, and 52 ± 3% at 6 h (Fig. 1A). The 5-h time point was used for most of the subsequent studies. H2O2 exposure resulted in cell shrinkage, chromatin condensation, and increased caspase-3 activity, hallmarks of apoptosis (Fig. 1B and see Fig. 5A). With the use of Trypan blue staining as a marker of necrotic cell death, less than 5% of cells were necrotic during our experimental period (data not shown).

H2O2-induced activation of ERK, p38, and Akt in a time-dependent manner. Our previous studies showed that RPTC exposure to H2O2 resulted in ERK1/2, p38, and Akt activation, but not JNK (44, 45). Because these kinases are involved in the regulation of apoptosis and cell survival, we examined their activation profiles over longer time points. ERK1/2 was constitutively phosphorylated under basal conditions, and phosphorylation increased within 10 min following H2O2 exposure and persisted for 60 min (Fig. 2). The level of ERK1/2 phosphorylation began to decrease at 120 min and returned to basal levels at 180 min. p38 phosphorylation also increased within 10 min following H2O2 exposure and then decreased gradually over 240 min (Fig. 2). In contrast, H2O2-induced Akt phosphorylation increased at 60 min, remained elevated through 180 min, and returned to the basal levels at 240 min (Fig. 2). Total expression level of all these proteins did not change (Fig. 2). These data reveal that the time courses for activation of these kinases are different following oxidant injury in RPTC and may be associated with different functions.

Role of MAPK activation in H2O2-induced apoptosis. Since both pro- and anti-apoptotic roles for ERK have been reported in renal epithelial cells following oxidant injury (6, 30), we further evaluated the role of ERK activation in H2O2-induced cell death using U0126 and PD98059, two specific inhibitors of the ERK upstream kinase MEK1/2 (3, 14). Treatment of RPTC with H2O2 for 5 h resulted in apoptotic nuclear changes in 49 ± 7% of the cells (Fig. 3A). Inhibition of ERK using U0126 or PD98059 decreased RPTC apoptotic nuclear changes to
23 ± 8 and 28 ± 11%, respectively. The exposure of RPTC to U0126 or PD98059 alone for 5 h had no effect on apoptosis. Consistent with this observation, pretreatment of U0126 or PD98059 enhanced cell viability as measured by the MTT assay in H2O2-treated cells (see Fig. 8B). At the concentrations used, U0126 and PD98059 selectively inhibited the ERK1/2 phosphorylation as shown in our previous study (43).

We also examined the role of p38 and JNK pathways in H2O2-induced RPTC apoptosis. Preincubation of cells with SB203580, a p38 inhibitor, or SP600125, a JNK inhibitor, had no effect on apoptotic nuclear changes in H2O2-treated cells (Fig. 3B). These data reveal that ERK, but not p38 nor JNK, mediates apoptosis in RPTC following oxidant injury.

Effects of overexpression of constitutively active MEK1 and negative mutant of MEK1 on RPTC viability following H2O2 exposure. To confirm the role of ERK in H2O2-induced cell death, we evaluated the viability of RPTC overexpressing constitutive active MEK1 (Ad-MEK1ca) and negative mutant of MEK1 (Ad-MEK1dn) after H2O2 exposure. A further decrease in cell viability was observed in RPTC expressing Ad-MEK1ca and exposed to H2O2 (Fig. 4A). In contrast, overexpression of Ad-MEK1dn resulted in increased cell viability in RPTC exposed to H2O2 (Fig. 4C). The efficacy of both Ad-MEK1ca and Ad-MEK1dn was determined by immunoblot analysis. ERK1/2 phosphorylation levels increased and decreased in Ad-MEK1ca- and Ad-MEK1dn-
transfected cells, respectively (Fig. 4, B and D). These results confirm the participatory role of ERK1/2 activation in RPTC apoptosis.

Caspase-3-like activity mediates H$_2$O$_2$-induced apoptosis. Because many apoptotic stimuli induce apoptosis through activation of caspase-3 (8, 13, 22), ERK activation may induce cell death through a caspase-3-dependent pathway. To test this possibility, we examined whether H$_2$O$_2$ increased caspase-3 activity using DEVD-pNA as a substrate. RPTC exposure to H$_2$O$_2$ increased caspase-3 activity by 2.5-fold at 5 h (Fig. 5A). Caspase-3 activation was completely blocked by Z-VAD-FMK, a pan-caspase inhibitor, or Z-DEVD-FMK, a caspase-3, -6, and -7 inhibitor. Furthermore, Z-DEVD-FMK also blocked apoptotic nuclear changes (Fig. 5B). These data reveal that caspase-3 mediates apoptosis in H$_2$O$_2$-treated RPTC.

ERK acts upstream of caspase-3 during H$_2$O$_2$-induced apoptosis. Involvement of both ERK and caspase-3 in H$_2$O$_2$-induced apoptosis suggests the possibility that they may act in the same apoptotic pathway. Inhibition of ERK with U0126 or PD98059 blocked H$_2$O$_2$-induced caspase-3 activation (Fig. 6A). In contrast, neither Z-VAD-FMK nor Z-DEVD-FMK treatment altered the phosphorylation level of ERK1/2 induced by this oxidant (Fig. 6B). These data clearly illustrate that ERK acts upstream of caspase-3 in the signaling pathway which leads to apoptosis in H$_2$O$_2$-treated RPTC.

Effects of U0126 on phosphorylation of histone H2B induced by H$_2$O$_2$. The above studies revealed that nuclear condensation is dependent on caspase-3 activation. Because phosphorylation of histone H2B at serine 14 is associated with nuclear condensation during apoptosis (9), we determined whether H$_2$O$_2$ induces histone H2B phosphorylation in RPTC and, if so, whether this is mediated by ERK and/or caspase-3. Treatment with H$_2$O$_2$ induced histone H2B phosphorylation at S14 at 3 h and was persistent until 5 h (Fig. 7A). In the presence of U0126, the phosphorylation of H2B was inhibited largely (Fig. 7B). Similarly, incubation of cells with Z-DEVD-FMK blocked H2B phosphorylation (Fig. 7C). We suggest that the proapoptotic role of ERK in H$_2$O$_2$-treated RPTC apoptosis is through caspase-3 activation and, subsequently, the phosphorylation of histone H2B.

Blockade of PI3K/Akt enhances H$_2$O$_2$-induced cell death and attenuates the protective effect resulting from ERK inhibition. It has been reported that activation of PI3K/Akt is required for cell survival in cells exposed to a variety of stress stimuli, including H$_2$O$_2$ (4, 22). Given the effects of ERK1/2 inhibition on RPTC survival following H$_2$O$_2$ exposure, it is possible that ERK inhibition further activates PI3K/Akt-mediated survival signaling. To test this hypothesis, we examined the effect of ERK inhibition on Akt phosphorylation in RPTC exposed to H$_2$O$_2$. H$_2$O$_2$ treatment increased Akt phosphorylation, which was enhanced in the presence of U0126 (Fig. 8A). However, U0126 alone did not affect the basal level of Akt phosphorylation. In the presence of LY294002, a specific PI3K inhibitor, basal levels and H$_2$O$_2$-induced Akt phosphorylation were blocked. LY-
294002 had no effect on basal nor H2O2-induced ERK1/2 phosphorylation (Fig. 8A).

The effect of LY294002 inhibition of PI3K/Akt on cell viability following H2O2 injury in the presence or absence of U0126 was examined. As discussed above, inhibition of ERK1/2 with U0126 or PD98059 blocked H2O2-induced apoptosis in RPTC (Fig. 3A). If PI3K/Akt plays an important role in cell survival, then inhibition of PI3K/Akt should potentiate H2O2-induced decreases in RPTC viability; indeed this was observed (Fig. 8B). If ERK inhibition elicited cell survival through enhanced Akt phosphorylation, then inhibition of the PI3K/Akt pathway in the presence of ERK1/2 inhibition should partly decrease the cytoprotective effect of ERK1/2 inhibition alone; indeed this was observed (Fig. 8B). These inhibitors alone or in combination did not significantly affect cell survival during the course of the 5-h incubation (Fig. 8B). We suggest that although both PI3/Akt and ERK pathways are activated following oxidant injury in RPTC, they play opposite roles. Furthermore, induction of apoptosis by H2O2 injury is partially mediated by the inactivation of the PI3K-Akt pathway.

Fig. 4. Effect of overexpression of dominant negative MEK1 or constitutively active MEK1 on H2O2-induced loss of cell viability and ERK phosphorylation. RPTCs were transfected with adenovirus (multiplicity of infection = 100 pfu) encoding dominant negative MEK1 (Ad-MEK1dn), constitutive active MEK1 (Ad-MEK1ca), or encoding LacZ (Ad-Lac) for 24 h and then exposed to 1 mM H2O2 for 5 h (A and C) or 30 min (B and D). A and C: cell viability was assessed by MTT assay. Data are means ± SD of 3 independent experiments conducted in triplicate. Bars with different superscript letters are significantly different from each other (P < 0.05). B and D: cell lysates were analyzed by immunoblotting with antibodies to p-ERK1/2 or ERK. Representative blots are shown.

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Fig. 5. Effect of Z-VAD-FMK and Z-DEVD-FMK on caspase-3 activity and apoptosis. RPTC were pretreated with 100 μM Z-VAD-FMK (VAD), or 100 μM Z-DEVD-FMK (DEVD) for 1 h, and then exposed to 1 mM H2O2 for 5 h. Cell lysates were harvested for measuring caspase-3 activity (A) or apoptotic nuclei after DAPI staining. Apoptosis is expressed as the percentage of total cell number. Values are means ± SD from 3 independent experiments. Bars with different superscript letters are significantly different from each other (P < 0.05).
In this study, we investigated the role of the ERK1/2 pathway in apoptosis of RPTC in response to oxidant injury and showed that ERK1/2 is a critical mediator. This statement is based on the following observations. ERK1/2 are activated following oxidant injury, inhibition of the ERK signaling pathway either with PD98059 or U0126 blocked oxidant-induced loss of RPTC viability and apoptosis characterized by nuclear condensation, and overexpression of dominant negative mutant MEK1 blocked oxidant-induced loss of RPTC viability while expression of active MEK1 enhanced the loss of RPTC viability. Our results are in contrast with a report showing that ERK activation is associated with protective effect in mouse RPTC (6) and corroborate other studies indicating that ERK mediates cell death in renal epithelial cell lines (OK and LLC-PK1) (12, 25). The reason for the different results in the mouse RPTC is not clear but may be due to species or experimental protocol differences.

In addition to ERK1/2, p38 is also activated in RPTC following oxidant injury. However, activation of p38 kinase is not required for H$_2$O$_2$-induced apoptosis in RPTC as inhibition of p38 kinase by SB203580 did not affect the apoptotic nuclear condensation (Fig. 3B). Although JNK has also been reported to be associated with apoptosis in mouse RPTC following oxidant injury (6), the phosphorylation level of JNK did not change in H$_2$O$_2$-treated RPTC (45) and JNK inhibition, using SP600125, had no effect on apoptotic nuclear condensation (Fig. 3B). Of the MAPK pathways, we suggest that the ERK pathway uniquely mediates apoptosis in RPTC exposed to H$_2$O$_2$.

The mechanism by which ERK mediates apoptosis has not been well defined. Our studies revealed that H$_2$O$_2$-induced apoptosis is mainly mediated by caspase-3 and ERK inactivation blocked activation of caspase-3, suggesting that ERK induces apoptosis through activating the caspase-3-mediated apoptotic pathway. Because it was reported that ERK activation resulted in depolarization of mitochondrial potential and cytochrome c release (22), which are generally considered to be a prerequisite for activation of caspase-3, it is possible that ERK acts upstream of mitochondria in our model. Alternatively, ERK may act downstream of mitochondrial events and upstream of caspase-3. Consistent with this proposal, Nowak (26) reported that ERK inhibition blocked caspase-3 activation without affecting cytochrome c release from mitochondria. Further studies are needed to define the target(s) where ERK is coupled to the apoptotic pathway.

**Discussion**

In this study, we investigated the role of the ERK1/2 pathway in apoptosis of RPTC in response to oxidant injury and showed that ERK1/2 is a critical mediator. This statement is based on the following observations. ERK1/2 are activated following oxidant injury, inhibition of the ERK signaling pathway either with PD98059 or U0126 blocked oxidant-induced loss of RPTC viability and apoptosis characterized by nuclear condensation, and overexpression of dominant negative mutant MEK1 blocked oxidant-induced loss of RPTC viability while expression of active MEK1 enhanced the loss of RPTC viability. Our results are in contrast with a report showing that ERK activation is associated with protective effect in mouse RPTC (6) and corroborate other studies indicating that ERK mediates cell death in renal epithelial cell lines (OK and LLC-PK1) (12, 25). The reason for the different results in the mouse RPTC is not clear but may be due to species or experimental protocol differences.

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![Fig. 6. Effect of ERK inhibitors on caspase-3 activity and caspase inhibitors on ERK activation.](http://ajprenal.physiology.org/)

![Fig. 7. H$_2$O$_2$ induces H2B phosphorylation and the effects of caspase and ERK inhibition.](http://ajprenal.physiology.org/)
To gain further insight into the mechanisms by which ERK induces apoptosis, we evaluated the role of ERK in histone H2B phosphorylation. H2B phosphorylation at serine 14 is associated with condensed chromatin in apoptotic cells (9). Our data revealed that H2B phosphorylation at serine 14 was elevated in H2O2-treated RPTC, supporting a role for the ERK/Akt/H2B pathway in mediating condensed apoptotic chromatin.

ERK-mediated apoptosis also occurs through suppression of the PI3K-Akt pathway. PI3K/Akt elicits a survival signaling following stress, including oxidant injury (42). In line with this general view, exposure of RPTC to H2O2 induced Akt phosphorylation and pretreatment of cells with LY294002 reduced the viability of RPTC. However, unlike most observations in which Akt and ERK represent two separate survival pathways, inactivation of ERK resulted in elevated Akt phosphorylation and inhibition of the PI3K/Akt pathway partially reversed anti-apoptotic ERK inhibition. Thus, ERK induces apoptosis, in part, by suppressing the PI3K/Akt pathway. This pathway may be restricted to stress conditions because we observed that ERK inhibition only potentiated Akt phosphorylation in cells exposed to H2O2, not cells under basal conditions. Consistent with our observations, Sinha et al. (34) recently reported that inhibition of ERK1/2 not only prevented the decline in Akt activity but resulted in cell survival in kidney proximal tubular cells deprived of soluble survival factors. Interestingly, activation of ERK1/2 appears to be dependent on oxidative stress under this condition as addition of several different antioxidants and ROS scavengers prevented activation of ERK1/2 (34). The mediator(s) responsible for ERK-mediated suppression of Akt activation remains unclear but may be associated with activation of ribosomal S6 kinase 1 (rsk) and PDK1. In kidney epithelial cells subjected to growth factor withdrawal, rsk and PDK1 are found to coexist with ERK1/2 and Akt in a multimolecular complex (34). Additional studies are needed to determine whether these molecules are involved in apoptosis following oxidant injury.

It has been suggested that different durations of ERK phosphorylation are associated with different actions of ERK. For example, in situations where ERK enhances survival, the activation occurs rapidly and is more transient (2, 17) and in situations where ERK induces cell death the activation tends to be delayed and sustained (20, 38). However, this scenario was not observed in our study. As shown in Fig. 2, the phosphorylated ERK was elevated within 5 min following H2O2 exposure and only persisted for 2 h while loss of cell viability occurred after 3 h (Fig. 1A) when ERK phosphorylation level had returned to the basal level. These data suggest that prolonged activation of ERK may not be only factor in driving cells to undergo apoptosis. In support of this idea, acute and transient increases in ERK phosphorylation also occur immediately after reperfusion and mediate brain injury in a stroke model induced by transient occlusion of the middle cerebral artery (39). Therefore, once ERK is activated, the death signal may propagate later without needing additional ERK activation.

In summary, we suggest that ERK1/2 activation signals apoptosis in RPTC through at least two distinct pathways: activation of caspase-3 and inhibition of PI3K/Akt. Furthermore, ERK activation is coupled to phosphorylation of H2B at serine 14, a critical event for inducing nuclear condensation. Because ROS, including H2O2, is an important mediator in ischemia and toxicant-induced death of renal epithelial cell, it will be intriguing to further investigate the role of ERK1/2 in animal models of acute renal failure.
ERK CONTRIBUTES TO APOPTOSIS

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