ERK Activation Mediates Mitochondrial Dysfunction and Necrosis Induced by Hydrogen Peroxide in Renal Proximal Tubular Cells

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ABSTRACT

While tubular necrosis in acute renal failure is associated with excessive production of reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), the mechanism of ROS-induced cell necrosis remains poorly understood. In this study, we examined the role of the extracellular signaling-regulated kinase (ERK) pathway in H$_2$O$_2$-induced necrosis of renal proximal tubular cells (RPTC) in primary culture. Exposure of 60-70% confluent RPTC to 1 mM H$_2$O$_2$ for 3 hr resulted in 44% necrotic cell death, as measured by trypan blue uptake, and inactivation of MEK, the upstream activator of ERK, by either U0126 or PD98059 or over-expression of dominant negative mutant of MEK1, inhibited cell death. In contrast, over-expression of active MEK1 enhanced H$_2$O$_2$ induced cell death. H$_2$O$_2$ treatment led to the loss of mitochondrial membrane potential (MMP) in RPTC, which was decreased by U0126 and PD98059. Furthermore, inhibition of the MEK/ERK pathway decreased oxidant-mediated ERK1/2 activation and mitochondrial swelling in isolated renal cortex mitochondria. However, treatment with cyclosporin A (CsA), a mitochondrial permeability transition blocker, did not suppress RPTC necrotic cell death, loss of MMP and mitochondrial swelling. We suggest that ERK is a critical mediator of mitochondrial dysfunction and necrotic cell death of renal epithelial cells following oxidant injury. Oxidant-induced necrotic cell death was mediated by a CsA-insensitive loss of MMP that is regulated by the ERK pathway.
INTRODUCTION

Oxidants are generated in the kidney directly or indirectly following ischemia/reperfusion or exposure to toxicants. Consequently, oxidant injury has been implicated in the pathogenesis of acute nephropathies and nephrotoxic states. It is generally recognized that oxidant-induced cell death can occur through apoptosis and/or necrotic cell death. Apoptosis is a tightly orchestrated series of events that requires ATP and includes cell shrinkage, mitochondrial protein release, and caspase activation (Yu et al., 2001). In contrast, necrotic cell death is initiated by mitochondrial damage and ATP depletion and is associated with cellular swelling, and progressive increases in plasma membrane permeability (Liu et al., 2004). The extent of exposure to an insult determines the nature of cell death: milder insults induce apoptosis, whereas more severe insults usually cause necrotic cell death. Both forms of cell death are observed in injured kidneys in animal models and in patients with acute renal failure (ARF) (Devarajan, 2005).

Necrotic cell death is often considered to be a passive process involving severe loss of ATP, release of endoplasmic reticulum Ca\(^{2+}\), increases in cytosolic free Ca\(^{2+}\), influx of extracellular ions, and breakdown of the cytoskeletal-plasma membrane structure (Liu et al., 2004). Necrotic cell death is also regulated by the mitochondrial permeability transition (MPT) (Festjens et al., 2006).

The MPT is a process resulting in the permeabilization of the inner mitochondrial membrane that leads to loss of mitochondrial membrane potential (MMP), mitochondrial swelling, and rupture of the outer mitochondrial membrane (Brenner and Grimm, 2006). Whereas the molecular composition and regulation of MPT pores is being debated, it is thought that the MPT occurs after the opening of a channel, which is termed the
permeability transition (PT) pore. This classically defined MPT pore putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), CypD and possible other molecules (Baek et al., 2003). Cyclosporin A (CsA) binds to CypD and blocks MPT under many conditions; however, numerous reports show that MPT or the loss of MMP can occur in a CsA insensitive manner (Kushnareva et al., 2001; Kinsey et al., 2007a). This CsA insensitive, unregulated PT pore was first proposed by He and Lemasters to explain the finding that MPT can occur in a Ca\textsuperscript{2+}-independent fashion that is CsA insensitive (He and Lemasters, 2002). This unregulated PT pore or increased inner membrane permeability can be activated after exposure of mitochondria to oxidants such as tert-butyl hydroperoxide (He and Lemasters, 2002). The formation and regulation of this membrane permeability remains unclear.

In response to ROS and other stimuli, mitogen-activated protein kinase (MAPK) pathways are activated (Zhuang and Schnellmann, 2006). MAPK pathways are composed of extracellular signal-regulated kinases (ERK1/2), JNK and p38 pathways. Among them, activation of ERK1/2 is generally thought to confer a survival advantage to cells (Zhuang and Schnellmann, 2006). However, increasing evidence suggests that the activation of ERK1/2 also contributes to cell death in some cell types and organs under certain conditions (Zhuang and Schnellmann, 2006). For example, ERK activation occurs in animal models of ischemia-and trauma-induced brain injury, and cisplatin-induced renal injury, and inactivation of ERK reduces the extent of tissue damage (Zhuang and Schnellmann, 2006). ERK1/2 also is activated in neuronal and renal epithelial cells upon exposure to oxidative stress and toxicants or deprivation of growth factors, and inhibition of the ERK pathway blocks apoptosis (Zhuang and Schnellmann, 2006). ERK1/2 are
typically located in the cytosol and mitochondria (Yoon and Seger, 2006). While the role of cytosol ERK1/2 is well studied and involved in multiple cellular functions (Yoon and Seger, 2006), the role of mitochondrial ERK1/2 remains poorly understood. Recently, Nowak has shown that activation of the ERK pathway precedes mitochondrial dysfunction and is associated with the decrease in oxygen consumption in a model of sublethal injury produced by tert-butylhydroperoxide (Nowak et al., 2006). ERK1/2 are activated by ERK kinase1/2 (MEK1/2) by phosphorylation of threonine and tyrosine residues (Wolf, 2005).

Recently, our and other studies have demonstrated that ERK mediates apoptosis of renal epithelial cells following exposure to oxidants or nephrotoxicants (Nowak, 2002; Zhuang et al., 2007). Although necrotic cell death also occurs in response to these stimuli, the role of ERK in necrotic cell death and the mechanistic basis of ERK in this process remain poorly understood. In this study, we investigated the role of the ERK pathway in mitochondrial dysfunction and necrotic cell death using the naturally occurring oxidant, H₂O₂. Herein, our studies reveal that H₂O₂ elicits RPTC necrotic cell death and mitochondrial dysfunction through ERK dependent and independent pathways.

METHODS

Chemicals and reagents. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), SB203580 (4-(4-Fluorophenyl)-2-(4 methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) and PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] were obtained from Calbiochem (San Diego, CA). Antibodies to phospho-ERK1/2 or ERK1/2 were purchased from Cell
Signaling (Boston MA) and used at 1:1,000 for immunoblot analysis. All other chemicals were from Sigma (St. Louis, MO).

**Isolation and culture of renal proximal tubules and experimental protocols.**

Female New Zealand White rabbits (2-3 kg) 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 (Nowak and Schnellmann, 1996). The serum-free 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 µM 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 nM), and L-ascorbic acid-2-phosphate (50 µM) were added daily to fresh culture medium. RPTC were used in all experiments at approximately 60-70% confluency. When various pharmacological inhibitors were used, the same volume of dimethyl sulfoxide (DMSO) was added to the culture in control samples.

**Replication-deficient adenovirus infection.** The construction and characterization of recombinant adenoviruses containing the coding regions of the kinase active form of human MEK1 (Ad-caMEK1) and kinase inactive form of human MEK1 (Ad-dnMEK) driven by the cytomegalovirus immediate early promoter were provided by Dr. Jiahuai Han (The Scripps Research Institute)(Foschi et al., 1997). RPTC were infected with each virus at a multiplicity of infection (MOI) of 100 pfu for 2 hr at 37°C in a humidified, 5% CO₂ incubator. Afterward, the cultures were placed in culture media for an additional 48 hr and then exposed to oxidant injury for the time periods described in the figure legends.
At an MOI of 100 pfu, approximately 100% of the cells showed expression of the viral gene insert as indicated by the X-gal assay (data not shown).

**Assessment of necrotic cell death.** Necrotic cell death was assessed by the trypan blue exclusion assay and lactate dehydrogenase (LDH) release in the medium. At the end of protocol, 0.4% trypan blue was added into the culture dish. After ~5 min of equilibration, the cells in five fields (X 40) were counted using a microscope and the numbers of necrotic cells were expressed as the percentage of the total cell population. For LDH measurements, the cellular medium was collected, and the enzyme activity was determined spectrophotometrically using an assay kit and the instructions provided by the manufacturer (Sigma-Aldrich). Data were normalized to solvent-treated cultures.

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

**Measurement of annexin V and propidium iodide staining.** Annexin and PI staining were determined using flow cytometry as described previously with modifications (Cummings and Schnellmann, 2002). Briefly, media were removed, RPTC washed twice with phosphate-buffered saline (PBS), and incubated in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.4) containing annexin V-FITC (25 µg/ml) and PI (25 µg/ml) for 10 min. Cells were washed three times in binding buffer and then released from the monolayers using a rubber
policeman. Staining was quantified using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For each measurement 10,000 events were counted.

**Determination of MMP.** The MMP was assessed using JC-1, a lipophilic cation that can selectively enter into mitochondria. This mitochondrial dye, which normally exists in solution as a monomer emitting a green fluorescence, forms a dimer emitting red fluorescence in a reaction driven by the MMP. The change in fluorescence can be detected by flow cytometry with a decrease in the red fluorescence or the increase in green fluorescence intensity indicating mitochondrial depolarization. Cell staining was performed following the manufacturer's instructions. Briefly, 1 X 10⁶ cells were incubated with 10 mg/ml of JC-1 for 10 min at 37 °C, washed twice in cold PBS and staining determined using flow cytometry.

**Isolation of renal cortical mitochondria (RCM).** Following euthanasia, rabbit kidneys were removed by blunt dissection, cortical tissue was collected and placed in ice-cold mitochondrial isolation buffer containing (mM): sucrose 270, Tris-HCl 5, EGTA 1 (pH 7.4). RCM were isolated by differential centrifugation as previously described by Kinsey et al (Kinsey et al., 2007a).

**Mitochondrial swelling.** Isolated mitochondria were suspended at a concentration of 0.4 mg protein/ml in swelling buffer (150 mM KCl, 20 mM Tris-HCl, pH 7.4) in a 96-well plate and incubated with diluent, inhibitors or antioxidants at room temperature for 10 minutes. Ferrous sulfate heptahydrate (Sigma) to achieve a final Fe²⁺ concentration of 10 µM or diluent (swelling buffer) was then added to initiate oxidative stress as described previously (Hunter et al., 1963; Kinsey et al., 2007a). Mitochondrial swelling was measured using a SpectraMax 190 spectrophotometric plate reader (Molecular Devices,
Sunnyvale, CA) as the loss of optical density at 540 nm over time as previously described (Kinsey et al., 2007a).

**Measurement of cis-parinaric acid oxidation.** Lipid peroxidation in isolated mitochondria was measured using the fluorescent lipid, cis-parinaric acid as described previously (Kinsey et al., 2007a). Briefly, isolated mitochondria were suspended at a concentration of 1 mg protein/ml in swelling buffer and incubated on ice with cis-parinaric acid (6.4 µM) for 10 minutes. The mitochondria were centrifuged, the supernatant discarded, and the mitochondria resuspended (0.4 mg protein/ml) in deoxygenated swelling buffer. Mitochondria were added to a 96-well plate and incubated with diluent, inhibitors or antioxidants at room temperature for 10 minutes then treated with 10 µM Fe²⁺. Lipid peroxidation was measured as the loss of cis-parinaric acid fluorescence (excitation 320 nm, emission 405 nm) over time using a Fluoroskan Ascent fluorescent plate reader (Thermo Labsystems, Franklin, MA).

**Immunoblot analysis.** Cells were washed once with phosphate-buffered saline without Ca²⁺ and Mg²⁺, and then suspended in the lysis buffer (0.25 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.1 mg/ml bromphenol blue, 0.5% 2-mercaptoethanol). Mitochondria were harvested after various treatments and then suspended in the same lysis buffer. 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 hr 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 ± SE 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**

**H\(_2\)O\(_2\) induces necrotic cell death in RPTC.** Exposure of cells to H\(_2\)O\(_2\) can induce both apoptosis and necrosis, depending on the cell type, confluence status and H\(_2\)O\(_2\) concentration (Proskuryakov et al., 2003). To induce necrosis of RPTC, 60-70% confluent cells were treated with 1 mM H\(_2\)O\(_2\) for 0-3 hr and necrotic cell death was monitored using established markers, trypan blue and propidium iodide uptake, and LDH release. Exposure of cells to H\(_2\)O\(_2\) for 2 and 3 hr resulted in an increase trypan blue positive cells (Figure 1A and B). Only 7 ± 1% of cells were necrotic in the control group. As shown in Figure 1C, a 3 hr H\(_2\)O\(_2\) treatment resulted in a 2.3-fold increase in the level of LDH activity in the medium. Similar results were obtained by measuring PI staining using flow cytometry (Figure 1D).

Despite significant necrosis following oxidant injury, no increase in apoptosis, as assessed by annexin V binding using flow cytometry, was observed 3 hr after H\(_2\)O\(_2\) treatment (Figure 1D). These data reveal that subconfluent RPTC mainly undergo necrotic cell death in this model of oxidant stress. Since H\(_2\)O\(_2\)-induced necrotic cell death did not occur prior to 2 hr, 3 hr treatments with 1mM H\(_2\)O\(_2\) were used for the remaining studies.
Inhibition of the MEK/ERK pathway blocks \( \text{H}_2\text{O}_2 \)-induced RPTC necrotic cell death. To determine whether the MEK/ERK pathway mediates necrotic cell death, RPTC were pretreated with either U0126 or PD98059, two MEK1/2-specific inhibitors (Alessi et al., 1995; Favata et al., 1998) and then exposed to 1 mM \( \text{H}_2\text{O}_2 \) for 3 hr. \( \text{H}_2\text{O}_2 \)-induced release of LDH was blocked by U0126 and PD98059 (Figure 2A,C). Similarly, pretreatment with U0126 or PD98059 increased cell viability in \( \text{H}_2\text{O}_2 \) treated RPTC as reflected by the MTT assay (Figure 2B,D). In contrast, pretreatment of cells with SB203580, a specific inhibitor of p38 MAPK, at concentrations of up 20 \( \mu \text{M} \), or SP600125, an inhibitor of Jun N-terminal kinase (JNK) at doses of (20 \( \mu \text{M} \)) did not protect RPTC from death (data not shown). Thus, ERK is a critical mediator of \( \text{H}_2\text{O}_2 \)-induced RPTC necrotic cell death.

\( \text{H}_2\text{O}_2 \) induces phosphorylation of ERK1/2. To examine the activation of ERK1/2 in response to \( \text{H}_2\text{O}_2 \), the phosphorylation states of these proteins were determined by immunoblot analysis and anti-phospho-ERK1/2 antibodies. ERK1/2 phosphorylation occurred at 10 min, reached a maximal level at 30 min and persisted for at least 2 hr (Figure 3A). U0126 (10 \( \mu \text{M} \)) completely inhibited and PD98059 (100 \( \mu \text{M} \)) partially inhibited \( \text{H}_2\text{O}_2 \)-induced phosphorylation of ERK1/2 (Figure 3B,C). These results demonstrate that ERK1/2 are activated quickly and persistently after \( \text{H}_2\text{O}_2 \) exposure in this model.

Effect of expressing constitutively active MEK1 and negative mutant of MEK1 on RPTC necrotic cell death after \( \text{H}_2\text{O}_2 \) exposure. To confirm the role of ERK pathway in \( \text{H}_2\text{O}_2 \)-induced necrotic cell death, we evaluated LDH release and viability in RPTC over-expressing constitutively active MEK1 (Ad-caMEK1) or a dominant-
negative mutant of MEK1 (Ad-dnMEK1) after a 3 hr H2O2 exposure. Over-expression of Ad-dnMEK1 blocked LDH release in RPTC exposed to H2O2 to control levels while LDH release was potentiated in RPTC expressing Ad-caMEK1 and exposed to H2O2 (Figure 4A,B). Cell viability increased to control levels in RPTC over-expressing Ad-dnMEK1 and was less than H2O2 alone in RPTC over-expressing Ad-caMEK1 (Figure 4C,D). Ad-MEK1 or Ad-dnMEK1 did not significantly affect cell viability (Subramaniam et al., 2004; Zhuang et al., 2007). ERK1/2 phosphorylation levels increased and decreased in Ad-caMEK1 and Ad-dnMEK1 transfected RPTC, respectively, as demonstrated in our recent report (Zhuang et al., 2007). These results support the pharmacological inhibitor data that ERK1/2 are mediators of H2O2-induced RPTC.

H2O2 treatment results in the loss of MMP and is blocked by inhibition of the MEK1/ERK pathway. Previous studies have shown that H2O2 induced cell death is associated with the loss of MMP (Zhou et al., 2006) and recent studies suggested that ERK is constitutively expressed in mitochondria of RPTC (Nowak et al., 2006). To determine whether ERK plays a role in regulating H2O2 induced loss of MMP, we examined the effect of U0126 and PD98059 on the loss of MMP using flow cytometry following JC-1 staining. Mitochondrial depolarization was measured as an increase in green fluorescence intensity (Reers et al., 1995). Figure 5 illustrates that H2O2 treatment resulted in an increase in green fluorescence intensity, which was blocked by U0126 and decreased by PD98059. These results are consistent with the partial block of ERK1/2 phosphorylation by PD98059 (Figure 3C). We suggest that ERK mediates, in part, the loss of MMP and, in turn, necrotic cell death following H2O2 exposure.
Inhibition of the MEK/ERK pathway decreases ERK1/2 activation and mitochondrial swelling. To further investigate the role of ERK in oxidant-mediated mitochondrial damage, we examined the effect of U0126 and PD98059 on Fe$^{2+}$-induced ERK1/2 activation, mitochondrial swelling and lipid peroxidation in isolated RCM. The mitochondrial swelling induced by Fe$^{2+}$ occurs through production of ROS (Kinsey et al., 2007a). In response to Fe$^{2+}$, ERK1/2 were phosphorylated at 10 and 25 min. Pretreatment with U0126 (10 µM) completely blocked and PD98059 (100 µM) partially inhibited Fe$^{2+}$-induced phosphorylation of ERK1/2 (Figure 6). U0126 inhibited Fe$^{2+}$-induced mitochondrial swelling in a concentration-dependent manner with 1 µM U0126 partially blocking and 10 µM U0126 completely blocking mitochondrial swelling (Figure 7). PD98059 treatment led to a partial inhibition of mitochondrial swelling with equal effects at 50 and 100 µM. As a positive control, the antioxidant butylated hydroxyanisole (BHA) blocked Fe$^{2+}$-induced mitochondrial swelling (Figure 7). This is consistent with our statement that the mitochondrial swelling induced by Fe$^{2+}$ occurs through production of ROS (Kinsey et al., 2007a).

To determine the effect of U0126 and PD98059 on Fe$^{2+}$-induced mitochondrial lipid peroxidation, the rate of lipid peroxidation was determined using the fluorescent fatty acid, cis-parinaric acid. cis-Parinaric acid incorporates into membranes and loses fluorescence as it is oxidized. Treatment with Fe$^{2+}$ resulted in an increased rate of cis-parinaric acid oxidation (Figure 8). Fe$^{2+}$-induced cis-parinaric acid oxidation rate was blocked by pretreatment with 10 µM U0126 and the antioxidant BHA. However, treatment with 100 µM PD98059 or 1 µM U0126 did not show any inhibitory effect on Fe$^{2+}$-induced mitochondrial lipid peroxidation. These results provide evidence that
concentrations of U0126 and PD98059 that do not inhibit lipid peroxidation partially inhibit Fe$^{2+}$-induced mitochondrial swelling.

**H$_2$O$_2$-induced necrotic cell death and mitochondrial swelling are not sensitive to CsA.** Recent studies indicated that the mitochondrial permeability transition (MPT) plays an important role in necrotic cell death induced by Ca$^{2+}$ and H$_2$O$_2$ in fibroblasts and hepatocytes (Festjens et al., 2006). CsA binds cyclophilin D and blocks the MPT (Kallen et al., 1991). The primary goal of this experiment was to determine whether the oxidant-induced necrotic cell death and mitochondrial dysfunction is through the CsA-sensitive MPT. Treatment of RPTC with CsA at various concentrations (0.1 to 10 µM) did not block H$_2$O$_2$-induced LDH release nor loss of MMP at 3 hr (Figure 9A, B). These data suggest that the oxidant-induced RPTC necrosis is not mediated by the classical CsA-sensitive MPT.

Next, we examined the effect of CsA on Fe$^{2+}$-induced mitochondrial swelling. CsA (1 µM) did not block Fe$^{2+}$-induced mitochondrial swelling (Figure 9C) although our recent studies showed that 1 µM CsA was sufficient to block Ca$^{2+}$-induced MPT and mitochondrial swelling in these isolated mitochondria (Kinsey et al., 2007b). We suggest that oxidant stress does not induce classical MPT but induces an unregulated CsA-insensitive form of the MPT or a non-specific increase in inner membrane permeability.

**DISCUSSION**

Earlier Ca$^{2+}$ and calpain studies as well as recent studies suggest that necrotic cell death is mediated and regulated by enzymatic signals (Liu et al., 2004; Golstein and Kroemer, 2007). In this study, we demonstrate that inactivation of the MEK/ERK
pathway blocked oxidant-induced necrotic cell death of RPTC, implying that ERK is a critical signaling molecule that drives the necrotic response in renal epithelial cells.

As mitochondrial dysfunction has been identified as a key mechanism underlying both necrotic cell death and apoptosis (Kim et al., 2003), we examined the role of ERK in the regulation of mitochondrial dysfunction following oxidant exposure. In cultured RPTC, we showed that the MEK inhibitors, U0126 and PD98059, blocked and partially inhibited H₂O₂-induced decreases in MMP, respectively (Figure 5). In isolated RCM, we demonstrated that inhibition of the MEK/ERK pathway partially blocked the mitochondrial swelling (Figure 7). We suggest that ERK is functionally linked to mitochondrial dysfunction by promoting the loss of MMP. In support of this observation, Nowak et al., recently showed that ERK is constitutively expressed in the mitochondria of RPTC and activation of the ERK pathway precedes mitochondrial dysfunction (measured by decreases in basal, uncoupled and state 3 respirations and ATP production) in a model of sublethal injury produced by tert-butylhydroperoxide (Nowak et al., 2006). Furthermore, the decrease in oxygen consumption was partially reversed by PD98059 and U0126 (Nowak et al., 2006). Since mitochondrial dysfunction and loss of MMP lead to ATP depletion and initiate a necrotic response, ERK-mediated loss of MMP would represent a novel mechanism of renal tubular epithelial cell death.

The mechanism and mitochondrial target(s) of ERK that directly function to promote loss of MMP remain unclear. However, it does not appear that the decreased MMP is mediated through the CsA-sensitive MPT pore because CsA did not prevent the loss of MMP in RPTC nor mitochondrial swelling in isolated RCM following oxidant stress (Figure 9B and C). In line with our observations, the loss of MMP was also not prevented
by CsA in response to some other stimuli such as free fatty acids and thyroxine (Malkevitch et al., 1997; Sultan and Sokolove, 2001). Since CsA regulates the MPT pore through CypD, one of the primary components of the MPT pore (Festjens et al., 2006), our results also suggest that CypD is not involved in regulating the loss of MMP in renal epithelial cells exposed to H₂O₂. Similar to our observation, CypD is not required for necrotic cell death induced by staurosporine, TNFα and adenovirus-mediated Bax over-expression (Baines et al., 2005). In contrast, CypD has been reported to mediate necrosis of hepatocytes in response to this oxidant (Festjens et al., 2006). On this basis, we suggest that ERK mediates loss of MMP independent of CyD (CsA-resistant) in RPTC.

Currently, the molecular composition and regulation of the opening of the CsA-insensitive MPT pores or non-specific inner membrane permeability remains unknown. He and Lemasters have proposed that an “unregulated” PT pore is formed when oxidant and other stresses result in formation of large amphipathic protein aggregates whose size and number exceed the mitochondrial chaperone capacity, which normally regulates the PT pore (He and Lemasters, 2002).

In isolated mitochondria, we observed that 10 μM U0126 partially inhibited Fe²⁺-induced lipid peroxidation while 1 μM U0126 and 100 μM PD98059 had no effect (Figure 8). These inconsistent results may be attributed to the different capacities of these two inhibitors. U0126 inhibits active and inactive MEK1/2 while PD98059 inhibits inactive MEK1/2, and U0126 is more potent than PD98059 (Favata et al., 1998). In support of this notion, our data revealed that pretreatment with U0126 (10 μM) completely blocked and PD98059 (100 μM) partially inhibited Fe²⁺-induced phosphorylation of mitochondrial ERK1/2 (Figure 6).
Alternatively, U0126 may have more than one function. It has been reported that U0126 is able to inhibit formation of ROS induced by amyloid beta peptide in human neutrophil granulocytes (Andersen et al., 2003). Our observation that 10 µM U0126 blocks Fe\(^{2+}\)-induced lipid peroxidation in isolated mitochondria (Figure 8) is consistent with the study of Andersen et al., (Andersen et al., 2003) and provides evidence that U0126 may act as an antioxidant at higher concentrations. The observation that 1 µM U0126 and 50 and 100 µM PD98059, which had no effect on lipid peroxidation (Figure 8), partially blocked Fe\(^{2+}\)-induced mitochondrial swelling/injury supports the hypothesis that oxidant-induced loss of MMP occurs through ERK1/2 sensitive and insensitive pathways. These results are consistent with the cellular data in that PD98059 partially blocked the loss of MMP produced by H\(_2\)O\(_2\) and that 10 µM U0126 completely blocked the loss of MMP (Figure 3B and C).

ERK may contribute to MMP through a mechanism involving lipid peroxidation. It has been reported that ERK can directly activate cPLA\(_2\) by phosphorylation at serine 505 (Lin et al., 1993). cPLA\(_2\) activation results in hydrolysis of membrane phospholipids and release of free fatty acids including arachidonic acid (AA) which may alter MMP (Higuchi and Yoshimoto, 2002). Alternatively, our laboratory has shown that RPTC and RCM possess Ca\(^{2+}\)-independent PLA\(_2\)\(\gamma\) (iPLA\(_2\)\(\gamma\)). In contrast to cPLA\(_2\), iPLA\(_2\)\(\gamma\) protects against oxidant-induced lipid peroxidation, mitochondrial dysfunction and necrotic cell death (Cummings et al., 2002). PKC\(\varepsilon\) mediated phosphorylation appears to activate iPLA\(_2\)\(\gamma\) (25), but the effect of ERK activation on RCM iPLA\(_2\)\(\gamma\) activity has not been examined. It is possible that ERK mediated phosphorylation of iPLA\(_2\)\(\gamma\) decreases its activity or inhibits its repair functions, leading to increased lipid peroxidation. A MAPK
consensus motif has been recently identified in iPLA$_2$$\gamma$ (Tanaka et al., 2000) and additional studies are required to determine the effect of ERK activation on iPLA$_2$$\gamma$ activity in RPTC.

ERK activation has been implicated in tissue injury and inflammation in animal models with ARF and other renal diseases. Jo et al., showed that cisplatin-induced ARF is accompanied by activation of ERK1/2 and that inhibition of ERK1/2 with U0126 resulted in functional and histological protection in mice (Jo et al., 2005). Alderliesten et al., demonstrated that ERK1/2 are activated in a single-kidney rat model of I/R-induced acute kidney injury and inhibition of the ERK pathway with U0126 attenuated pathological damage to the kidney (Alderliesten et al., 2007). Furthermore, Gong et al., showed that adenovirus-mediated antisense ERK2 gene therapy attenuated chronic allograft nephropathy in a rat model of renal transplantation (Gong et al., 2006). In these studies ERK inhibition not only attenuated renal damage, but also decreased inflammatory responses, suggesting a role of ERK in mediating inflammatory pathway. While it is not clear whether ERK activation stimulates the release of inflammatory factors in renal epithelial cells, or other cell types in the kidney after oxidant injury, it is possible that ERK triggers the inflammatory response through induction of necrotic cell death. Necrotic cells can initiate pro-inflammatory signaling cascades by actively releasing inflammatory cytokines and by releasing their contents when they lyse (Proskuryakov et al., 2003).

In summary, our results demonstrate that ERK mediates mitochondrial dysfunction and necrotic cell death of renal epithelial cells following oxidant injury. Furthermore, ERK is functionally coupled to MMP through activation of a CsA-insensitive MPT pore
or a non-specific inner membrane permeability. This study, in conjunction with the proapoptotic role of ERK in RPTC, suggests that ERK is a critical mediator of renal epithelial cell death. Since ROS are generated in renal injury following ischemia/reperfusion or exposure to various insults, and both apoptosis and necrosis contribute to the pathogenesis of ARF, a better understanding of the mechanism by which ERK mediates necrotic cell death will help develop strategies to interrupt the cell death cascade, and thereby abrogate renal injury.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. **H₂O₂ induces necrotic cell death in RPTC.** A, RPTC were exposed to 1mM H₂O₂ for 3 hr (A,D) or for different times (B,C). Cells were stained with trypan blue and then photographed (A), or counted from five fields (X 40) (B). Media were harvested and LDH measured (C). Cells were stained with PI and annexin V and then analyzed by flow cytometry (D). Data are represented as the mean ± the S.E.M. of at least three separate experiments. Means with different superscript letters are significantly different from one another (P < 0.05).

Figure 2. **Effect of U0126 and PD98059 on LDH release and cell viability.** RPTC were incubated with 10 µM U0126 (A,B) or 100 µM PD98059 (C,D) for 1 hr and then exposed to H₂O₂ for 3 hr. LDH released in the medium (A,C) and cell viability (MTT assay) (B,D) were measured. The released LDH levels and cell viability are expressed as the percentage of control. Values are means ± S.E.M. from 3 independent experiments. Bars with different superscript letters are significantly different from each other (P < 0.05).

Figure 3. **Effect of U0126 and PD98059 on H₂O₂ induced phosphorylation of ERK1/2.** RPTC were exposed to 1 mM H₂O₂ for 0–3 hr (A) or pretreated with 10 µM U0126 (B), 100 µM PD98059 (C) for 1 hr and then exposed to H₂O₂ for 30 min (B,C). Cell lysates were analyzed by immunoblotting with antibodies to phospho-ERK1/2 (p-ERK1/2) and total ERK1/2. Representative immunoblots from 3 experiments are shown.
Figure 4. Effect of over-expression of dominant negative MEK1 or constitutively active MEK1 on H₂O₂-induced LDH release and loss of cell viability. RPTC were transfected with adenovirus (MOI = 100 pfu) encoding dominant negative MEK1 (Ad-dnMEK1), constitutively active MEK1 (Ad-caMEK1) or encoding LacZ (Ad-Laz) for 24 hr and then exposed to 1 mM H₂O₂ for 3 hr. LDH released in the medium (A,B) and cell viability (MTT assay) (C,D) were measured. LDH release and viability are expressed as percentage of controls. Data are mean +/- S.E.M. of three independent experiments conducted in triplicate. Bars with different superscript letters are significantly different from each other (P < 0.05).

Figure 5. Effect of U0126 and PD98059 on H₂O₂-induced reduction of mitochondrial membrane potential. RPTC were treated with 10 µM U0126 (A), 50–100 µM PD98059 (B) or diluent for 1 hr and then exposed to 1 mM H₂O₂ for 2 hr. JC-1 monomer staining was determined by flow cytometry and expressed as events relative to total events. Data are mean +/- S.E.M. of three independent experiments conducted in triplicate. Bars with different superscript letters are significantly different from each other (P < 0.05).

Figure 6. Effect of U0126 and PD98059 on Fe²⁺-induced phosphorylation of ERK1/2 in isolated mitochondria. A, RCM were exposed to Fe²⁺ for the indicated time (A) or pre-incubated with MEK inhibitors (10 µM U0126, 100 µM PD98059) 1 hr prior to exposure to 10 µM Fe²⁺ for 25 min (B). Mitochondrial lysates were analyzed by immunoblotting with antibodies to phospho-ERK1/2 (p-ERK1/2) and total ERK1/2.
Figure 7 Effect of U0126 and PD98059 pretreatment on Fe^{2+}-induced mitochondrial swelling. RCM were incubated with MEK inhibitors (U0126, PD98059) or the antioxidant butylated hydroxyanisole (BHA) 1 hr prior to exposure to Fe^{2+}. Mitochondrial swelling was measured as the decrease in optical density at 540 nm, (A). Swelling trace is representative of four separate RCM preparations. To quantify the effect of MEK inhibitors and BHA, the extent of swelling at 1500 seconds was compared between groups (B). Data are expressed as mean +/- S.E.M. of four separate RCM preparations; means with different superscript letters are significantly different from each other, p<0.05.

Figure 8. Effect of U0126 and PD98059 pretreatment on Fe^{2+}-induced mitochondrial lipid peroxidation. RCM were loaded with the fluorescent lipid cis-parinaric acid, pretreated with diluent, U0126, PD98059, or the antioxidant BHA, and then exposed to 10 µM Fe^{2+}. The loss of fluorescence (indicative of lipid peroxidation) was followed over time. To determine the rate of lipid peroxidation, the percent difference of the initial fluorescence in each treatment group from that of control was determined and the oxidation rate determined by linear regression analysis (A). All the fluorescence readings from 0 to 800 seconds for each group were used to determine the rates used to compare (B). Data are expressed as mean +/- S.E.M. of four separate RCM preparations, means with different superscript letters are significantly different from each other, p<0.05.
Figure 9. Effect of cyclosporin A (CsA) on H$_2$O$_2$-induced necrosis. (A), RPTC were treated with various concentrations of CsA for 1 hr and then exposed to 1 mM H$_2$O$_2$ for 3 hr, LDH release is expressed as the percentage of controls. (B), RPTC were exposed to 1 mM H$_2$O$_2$ for 2 hr in the presence or absence or 1 µM CsA and then incubated with 10 µM JC-1 for 30 min. JC-1 monomer staining was determined by flow cytometry and expressed as events relative to total events. (C), RCM were incubated with 1 µM CsA prior to exposure to Fe$^{2+}$. Mitochondrial swelling was measured as the decrease in optical density at 540 nm. Values are means ± S.E.M. from three independent experiments. Bars with different superscript letters are significantly different from each other, p<0.05.
Figure 1

A

Control

H₂O₂

Trypan blue (+) cells (%)

0 0.5 1 2 3 (h)

B

Relative LDH activity (%)

0 0.5 1 2 3 (h)

C

D

% Cells

Control H₂O₂

PI(+) Annexin V (+)

Cells

0 0 20 40 60 100 200 300

0 0 20 40 60

0 0 20 40 60

0 a a a b b

0 0 20 40 60

0 0 20 40 60

0 a a

Figure 2

A

Relative LDH activity (%)

0 50 100 150 200 250

-  +  -  +  H₂O₂

DMSO  U0126

B

% Viability

0 40 80 120

-  +  -  +  H₂O₂

DMSO  U0126
Figure 2

C

Relative LDH activity (%)

- - + -H₂O₂
DMSO PD98059

D

% Viability

- - + -H₂O₂
DMSO PD98059
Figure 3

A

\[ \text{H}_2\text{O}_2 \]

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>120 (h)</th>
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\[ \text{p-ERK1/2} \]

\[ \text{ERK1/2} \]

B

- - + + U0126
- + + - \text{H}_2\text{O}_2

\[ \text{p-ERK1/2} \]

\[ \text{ERK1/2} \]

C

- - + + PD98059
- + + - \text{H}_2\text{O}_2

\[ \text{p-ERK1/2} \]

\[ \text{ERK1/2} \]
Figure 4

A

Relative LDH Activity (%)

H₂O₂

Ad-Laz
Ad-dnMEK1

0 100 200 300

-  +  -  +

b a a

B

Relative LDH Activity (%)

H₂O₂

Ad-Laz
Ad-caMEK1

0 100 200 300 400

-  +  -  +

b c a

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Figure 4

C

% Viability

H₂O₂

Ad-Laz Ad-dnMEK1

D

% Viability

H₂O₂

Ad-Laz Ad-caMEK1
Figure 5

A

![Graph showing JC-1 monomer events for U0126DMSO and H₂O₂ treatments.]

B

![Graph showing JC-1 monomer events for DMSO, U0126, and PD98059 treatments with H₂O₂.]

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Figure 6

A

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B

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<th>U0126</th>
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<tr>
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<tr>
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C

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<th>PD98059</th>
<th>Fe²⁺</th>
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Figure 7

A

B

Swelling

(% Initial OD)

Control
$\text{Fe}^{2+}$ 10 µM
$\text{Fe}^{2+}$ + BHA 25 µM
$\text{Fe}^{2+}$ + U0126 1 µM
$\text{Fe}^{2+}$ + U0126 10 µM
$\text{Fe}^{2+}$ + PD98059 50 µM
$\text{Fe}^{2+}$ + PD98059 100 µM

Swelling

(\% OD at 1500 sec.)

- + + + + + Fe$^{2+}$
- - 25 - - - - BHA (? M)
- - - 1 10 - - U0126 (? M)
- - - - - 50 100 PD98059 (? M)
Figure 8

A

B

cis-Parinaric acid oxidation rate

% Initial Fluorescence

0 200 400 600 800

% of iron alone

0 40 80 120

0 10 100

Control

Fe²⁺ 10 µM

Fe²⁺ + BHA 25 µM

Fe²⁺ + U0126 1 µM

Fe²⁺ + U0126 10 µM

Fe²⁺ + PD98059 100 µM

Seconds

Figure 8

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Figure 9

A

Relative LDH Activity (%)

0 50 100 150 200 250

H2O2

CsA (µM)

0 0 0.1 1 10 10

B

JC-1 monomer events (% total)

0 10 20 30 40 50

H2O2

DMSO CsA

C

Swelling (ΔOD550 at 1500 sec.)

0.00 0.10 0.20 0.30

Con CsA Fe2+ Fe2+ + CsA

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