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Signal Transduction Pathways Involved in Oxidative Stress-Induced Intestinal Epithelial Cell Apoptosis

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ABSTRACT

Necrotizing enterocolitis (NEC) is a devastating inflammatory condition of the gut that occurs in premature infants. Ischemia-reperfusion gut injury with production of reactive oxygen species (ROS) is thought to contribute to NEC; the exact cellular mechanisms involved are largely unknown. The purpose of this study was to determine the intracellular signaling transduction pathways involved in oxidative stress–induced intestinal epithelial cell apoptosis. H_2O_2 treatment resulted in rat intestinal epithelial cell apoptosis in a dose- and time-dependent manner; the caspase inhibitor, zVAD-fmk, blocked this response. Western blotting was performed to determine phosphorylation of kinases and ELISA was used to assess DNA fragmentation, as a measure of apoptosis. A rapid increase in phosphorylation of extracellular signal-related kinase (ERK)1/2, c-Jun N-terminal kinase (JNK)1/2, and Akt was noted. Inhibition of ERK and JNK decreased H_2O_2-induced apoptosis. Additionally, inhibition of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) attenuated and enhanced H_2O_2-mediated apoptosis and mitochondrial membrane potential decrease, respectively. Furthermore, activation of PKC reduced the Akt phosphorylation, whereas inhibition of PKC attenuated H_2O_2-mediated activation of caspase-3 and enhanced the H_2O_2-induced Akt phosphorylation. This study shows that activation of multiple signaling transduction pathways occurs during oxidative stress–induced intestinal epithelial cell injury. In contrast to ERK, JNK, and PKC, PI3-K/Akt may play an important role as a protective cellular signaling pathway during this process. **(*Pediatr Res* 58: 1192–1198, 2005)**

Abbreviations

ERK1/2, extracellular signal-regulated kinases 1 and 2
JNK1/2, c-Jun N-terminal kinases 1 and 2
MAPK, mitogen-activated protein kinase
NEC, necrotizing enterocolitis
PARP, poly (ADP-ribose) polymerase
PI3-K, phosphatidylinositol 3-kinase
PKC, protein kinase C
PMA, phorbol-12-myristate-13-acetate
RIE-1, rat intestinal epithelial
ROS, reactive oxygen species

NEC, characterized by inflammation, ischemia, and necrosis of the intestine, is a devastating condition in premature infants (1). Recent advances in the care of premature infants born with respiratory insufficiency have resulted in increased survival for extremely small premature infants and, as a result, the incidence of NEC has steadily risen. There are numerous presumed risk factors for NEC, which include perinatal stress, hypoxia, mesenteric ischemia-reperfusion, and hyperosmolar enteral feedings (2). In particular, a potential link between ROS produced by ischemia-reperfusion injury to the gut and the development of NEC has been suggested by several studies (3–5). However, the exact cellular signaling involved in oxidative stress–mediated intestinal epithelial cell apoptosis, which may occur during NEC, has not been clearly defined.

Cells undergo oxidative stress when levels of ROS exceed the counter-regulatory antioxidant capacity of the cell (6). Cellular responses to oxidative stress can vary from growth arrest to cell death depending upon the stress stimuli, duration of exposure, cell type, and surrounding cell environment (7). ROS-mediated cell damage is implicated in the pathogenesis of a variety of diseases (7). In the gastrointestinal tract, ROS-induced injury endothelial dysfunction is considered to be an important cellular mechanism in indomethacin-induced gastric mucosal injury (8) as well as in the colonic inflammation associated with ulcerative colitis (9). The exact cellular signaling involved in ROS-mediated intestinal cell injury in NEC is not well defined.

Oxidative stress is known to induce apoptosis in a variety of cell types by activating intracellular cell death signaling cascades (6). On the other hand, oxidative stress can also trigger the activation of certain signaling pathways that protect against cell death (10,11). For example, treatment with hydrogen

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peroxide (H_2O_2) to generate ROS activates members of the MAPK family (ERK1/2, JNK1/2, and p38 MAPKs) and PI3-K. It is generally accepted that ERK1/2 and PI3-K activation promotes cell survival by activating antiapoptotic signaling pathways, whereas activation of JNK and p38 MAPK is associated with cell death (12–14). However, recent studies have suggested an apoptotic function for mitogen-activated protein kinase kinase (MEK)/ERK in H_2O_2-mediated signals (15,16). In addition, PI3-K activation, resulting in phosphorylation of its downstream effector Akt (phospho-Akt), appears to play an essential role in cell survival by preventing the oxidative stress–induced cell death (11).

Considering the pivotal role of ROS in intestinal inflammation, we determined the intracellular signaling transduction pathways involved in the H_2O_2-induced RIE-1 cell injury in the present study. We show that H_2O_2 induces apoptosis in RIE-1 cells with increased phosphorylation of ERK1/2, JNK1/2, and Akt. Inhibition of MEK/ERK1/2, JNK, and protein kinase C (PKC) attenuated, whereas inhibition of PI3-K enhanced H_2O_2-mediated apoptosis. Moreover, we found that inhibition of PKC results in the enhanced Akt phosphorylation and attenuates H_2O_2-induced RIE-1 cell death.

MATERIALS AND METHODS

Reagents. Wortmannin, PMA, and H_2O_2 were purchased from Sigma Chemical Co. (St. Louis, MO). GF109203x, U0126, and SP600125 were from Calbiochem (San Diego, CA). Rabbit polyclonal anti-ERK1, rabbit polyclonal anti-phospho-ERK1/2, rabbit polyclonal anti-caspase-3, and rabbit anti-PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-actin antibody was purchased from Sigma Chemical Co. Rabbit anti-JNK antibody, rabbit anti-phospho-JNK antibody, rabbit anti-phospho-Akt (Ser473) antibody, and rabbit anti-Akt antibody were purchased from Cell Signaling (Beverly, MA). Tissue culture media and reagents were obtained from Invitrogen (Carlsbad, CA). Polyvinylidene diflouride PVDF membranes were from Millipore Corp. (Bedford, MA). The enhanced chemiluminescence system was purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture. RIE-1 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and cultured at 37°C under an atmosphere containing 5% CO_2. All experiments were performed on cells within 6 wk of culture from liquid nitrogen stocks and free of *Mycoplasma* contamination. To eliminate the potential ROS scavenger action of DMSO (17) in vehicle, we used the same concentrations of DMSO (0.1%) for both inhibitors and vehicle.

DNA fragmentation assay. Cells were plated in 96-well plates 24 h before treatment. DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA^{Plus} kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions.

Protein extraction and Western blot analysis. Cells were lysed with TNN buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM NP40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/mL each of aprotinin, leupeptin, and pepstatin A] at 4°C for 30 min. Lysates were clarified by centrifugation (10,000 $\times$ *g* for 30 min at 4°C), and protein concentrations were determined using the method described by Bradford (18). Total protein (100 μg) was resolved on a 10% polyacrylamide gel and transferred to polyvinylidene diflouride membranes. Filters were incubated overnight at 4°C in a blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20), followed by a 1-h incubation with primary antibodies at 4°C overnight. Filters were washed three times in a blocking solution and incubated with horseradish peroxidase-conjugated second antibodies for 1 h at room temperature. After three additional washes, the immune complexes were visualized by enhanced chemiluminescence system.

JC-1 mitochondrial membrane potential detection. The mitochondrial membrane potential was analyzed using MitoProbe JC-1 Assay kit (Molecular Probes, Eugene, OR). The collapse in the electrochemical gradient across the mitochondrial membrane was measured using a fluorescent cationic dye 5,5′,6,6,-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolo-carbocyanin iodide, known as JC-1. This dye exhibits potential dependent accumulation in mitochondrial matrix (19). Cells (1×10^6) were incubated with 2 μM JC-1 for 15 min at room temperature in darkness. Cells were washed twice with PBS at 4°C, resuspended in 0.5 mL PBS, and analyzed on a FACSCalibur flow cytometer.

Statistical analysis. Results are expressed as the mean $\pm$ SEM. The data in the figures were analyzed using the Kruskal-Wallis test and assessed at the 0.05 level of significance.

RESULTS

H_2O_2 induces apoptosis in RIE-1 cells. H_2O_2 treatment resulted in RIE-1 cell death in a dose-dependent manner with a significant induction of cell death at concentrations of 100–500 μM (Fig. 1*A*). The mechanism of apoptosis is mediated by sequential activation of caspases (20). To confirm whether H_2O_2 is inducing apoptosis in RIE-1 cells through caspase activation, we next pretreated cells with the pan-caspase inhibitor, zVAD-fmk (20), before H_2O_2 treatment. Pretreatment with zVAD-fmk effectively inhibited apoptosis induced by H_2O_2 in a dose-dependent manner, suggesting a role for caspase activation in H_2O_2-mediated induction of apoptosis (Fig. 1*B*).

To further confirm the activation of caspase pathway in the apoptotic effect of H_2O_2 on RIE-1 cells, we next determined protein expression of cleavage products of caspase-3 and PARP after treatment with H_2O_2. Treatment with H_2O_2 resulted in activation of caspase-3, as demonstrated by cleavage of pro-caspase-3 noted by increased expression of the M_r 17,000 cleavage product (*i.e.* active caspase-3). In addition, cleavage of PARP (M_r 85,000 cleavage product) was also noted (Fig. 1*C*).

H_2O_2 activates ERK1/2, JNK, and Akt. We found that RIE-1 cells undergo apoptosis when exposed to H_2O_2 in a dose- and time-dependent manner. Oxidative stress is known to activate multiple signal transduction pathways in many experimental systems (6,7). To identify the signaling mechanisms activated by H_2O_2 in RIE-1 cells, we assessed the phosphorylated protein expression of ERK, JNK, and Akt. Treatment with H_2O_2 resulted in marked increases in phosphorylation of ERK1/2 and JNK1/2, without affecting the total protein expression levels (Fig. 2). The activation of ERK1/2 and JNK1/2 occurred at 25 min after the addition of H_2O_2 (500 μM), peaked at 30–60 min, and returned to basal level by 2 h. Interestingly, H_2O_2 treatment resulted in sustained activation of Akt throughout the time course.

Effects of protein kinase inhibition on H_2O_2-mediated apoptosis. To further delineate the signaling pathways involved in H_2O_2-mediated apoptosis, RIE-1 cells were pretreated for 30 min with inhibitors to JNK (SP600125), ERK1/2 upstream kinase MEK1 (U0126), or combination of both for 30 min before treatment with H_2O_2 (500 μM). Cell death was assayed at 3 h after addition of H_2O_2. As expected, H_2O_2 induced RIE-1 cell death; this induction was partially attenuated by treatment with either SP600125 or U0126 (Fig. 3*A*). However, combination treatment did not show synergistic inhibition of cell death. We also confirmed the inhibitory effect of U0126 on the MEK/ERK1/2 pathway by determining phosphorylated protein level of ERK1/2 by Western blot. As noted previously, phospho-ERK1/2 was increased in H_2O_2-treated RIE-1 cells,

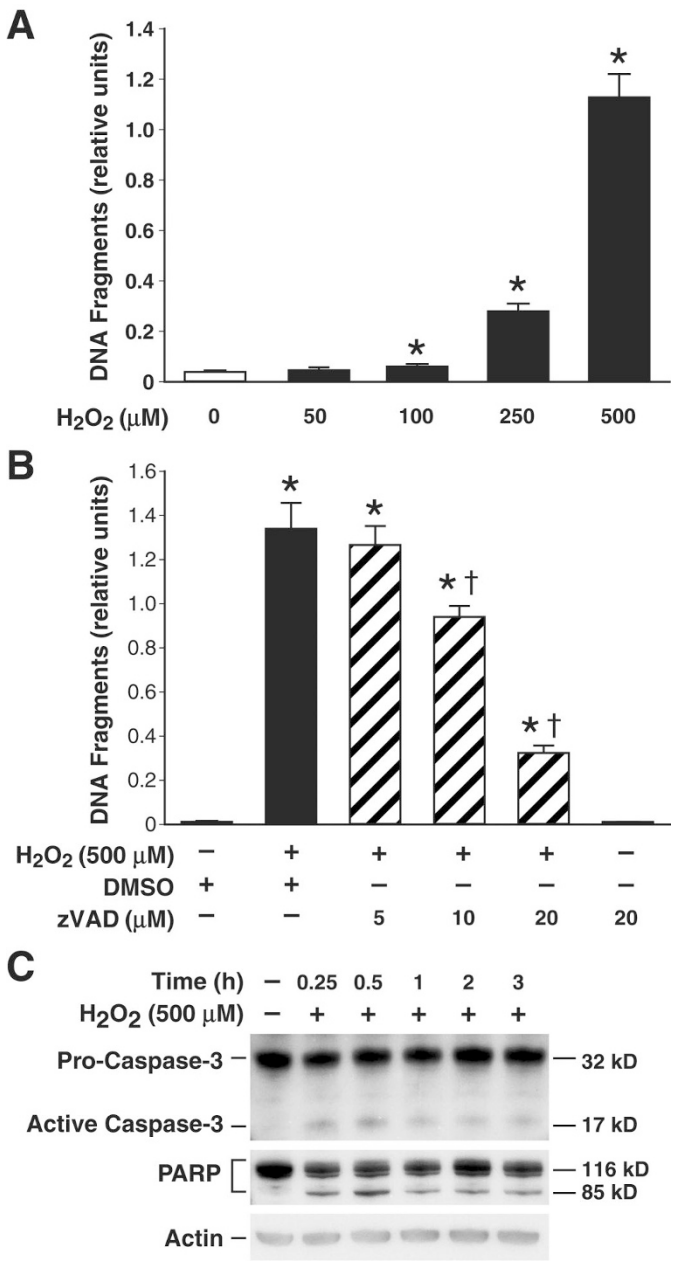

Figure 1. H_2O_2 induces RIE-1 cell apoptosis. (*A*) RIE-1 cells were plated for 24 h before treatment with H_2O_2 for 3 h or (*B*) pretreated with zVAD-fmk for 30 min before treatment with H_2O_2. Apoptosis was estimated by a DNA fragmentation ELISA (data represent triplicate determinations ± SEM; * $p < 0.05$ *vs* control; † $p < 0.05$ *vs* H_2O_2 alone). Experiments were repeated twice. (*C*) RIE-1 cells were treated with H_2O_2 for various time points. A representative Western blot from three separate experiments is shown here.

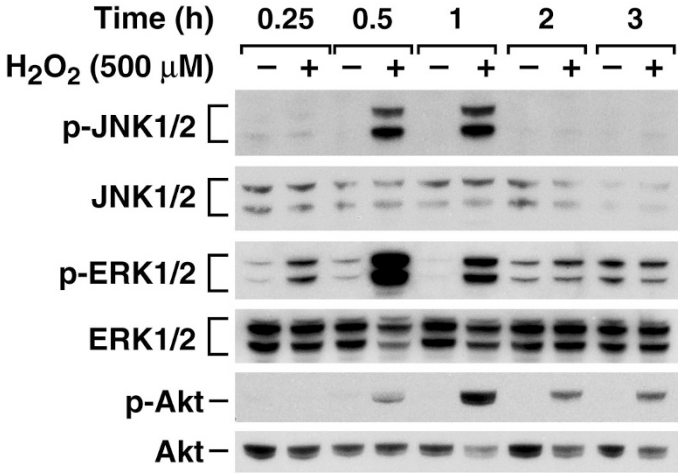

Figure 2. Activation of signal transduction pathways by H_2O_2. RIE-1 cells were treated with H_2O_2 over a time course for Western blot analysis. A representative blot from three separate experiments is shown here.

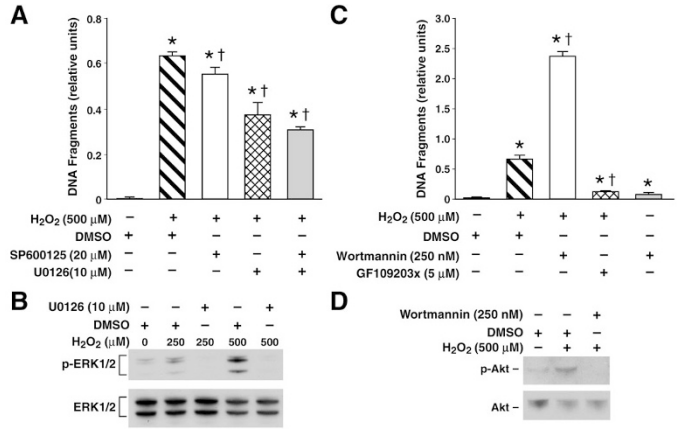

Figure 3. Effects of kinase inhibitors on H_2O_2-induced RIE-1 cell death. (*A, C*) RIE-1 cells were pretreated with kinase inhibitors [JNK inhibitor SP600125, the MEK1 inhibitor U0126 or combination (*A*) and PI3-K inhibitor wortmannin or PKC inhibitor GF109203x (*C*)] for 30 min before treatment with H_2O_2 for 3 h. Apoptosis was estimated by a DNA fragmentation ELISA (data represent triplicate determinations ± SEM; * $p < 0.05$ *vs* control; † $p < 0.05$ *vs* H_2O_2 alone). Experiments were repeated twice. (*B*) RIE-1 cells were pretreated with the U0126 for 30 min before treatment with H_2O_2 for 30 min and Western blotting performed. (*D*) RIE-1 cells were pretreated with the wortmannin for 30 min before treatment with H_2O_2 for 3 h and Western blotting performed. Representative blots from three separate experiments are shown as (*B*) and (*D*).

and this response was completely blocked by U0126 compound (Fig. 3*B*).

To delineate the role of PI3-K/Akt and PKC signaling pathways involved in H_2O_2-mediated apoptosis, RIE-1 cells were pretreated for 30 min with Akt upstream kinase PI3-K inhibitor, wortmannin or PKC inhibitor GF109203x, before addition of H_2O_2 (500 μM). Wortmannin significantly enhanced H_2O_2-mediated RIE-1 cell death at 3 h (Fig. 3*C*). In contrast, GF109203x markedly attenuated H_2O_2-induced RIE-1 cell death. The inhibitory effect of wortmannin on the PI3-K/Akt pathway was also confirmed by demonstrating the inhibition of H_2O_2-induced Akt phosphorylation by wortmannin in Figure 3*D*. Collectively, these results suggest a proapoptotic role for the MEK/ERK, JNK, and PKC pathways and an antiapoptotic role for PI3-K/Akt pathway in H_2O_2-induced RIE-1 cell death.

PKC negatively regulates Akt activity in RIE-1 cells. To further determine the molecular mechanisms involved in the effects of PKC or PI3-K inhibition on H_2O_2-induced apoptosis, we next determined activation of caspase-3. As expected, treatment with H_2O_2 resulted in activation of caspase-3; this activation was attenuated by treatment with GF109203x (an inhibitor of PKC) as demonstrated by decreased cleavage of pro-caspase-3 (Fig. 4*A*). In contrast, pretreatment with wortmannin significantly enhanced cleavage of pro-caspase-3.

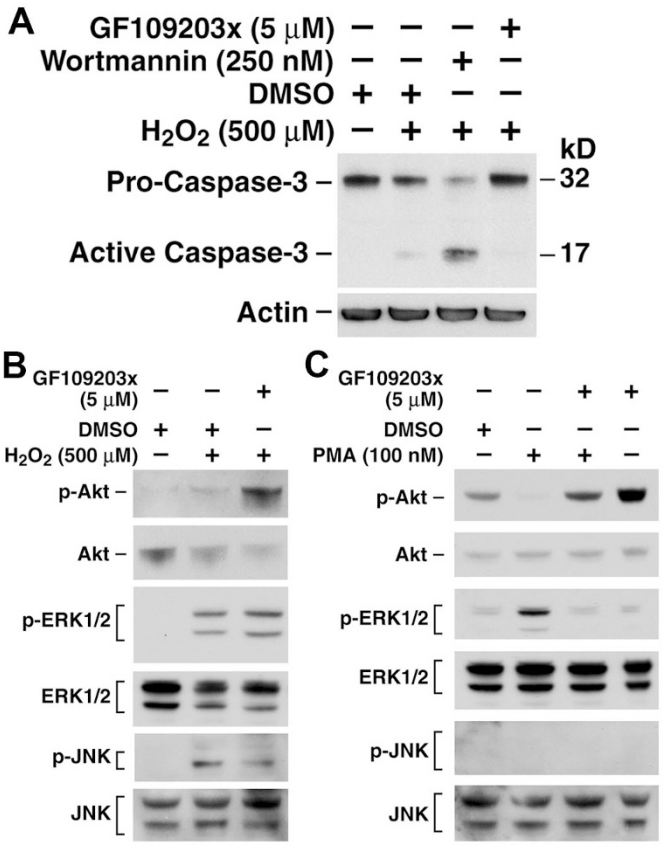

Figure 4. Inhibition of PKC results in the activation of Akt and blocks H_2O_2-induced caspase-3 cleavage. (*A*) RIE-1 cells were pretreated with the PI3-K inhibitor wortmannin or the PKC inhibitor GF109203x for 30 min before treatment with H_2O_2 for 3 h for Western blot analysis. (*B, C*) RIE-1 cells were pretreated with the GF109203x for 30 min before treatment with H_2O_2 (*B*) or PMA (*C*) for Western blot analysis. Representative blots from three separate experiments are shown here.

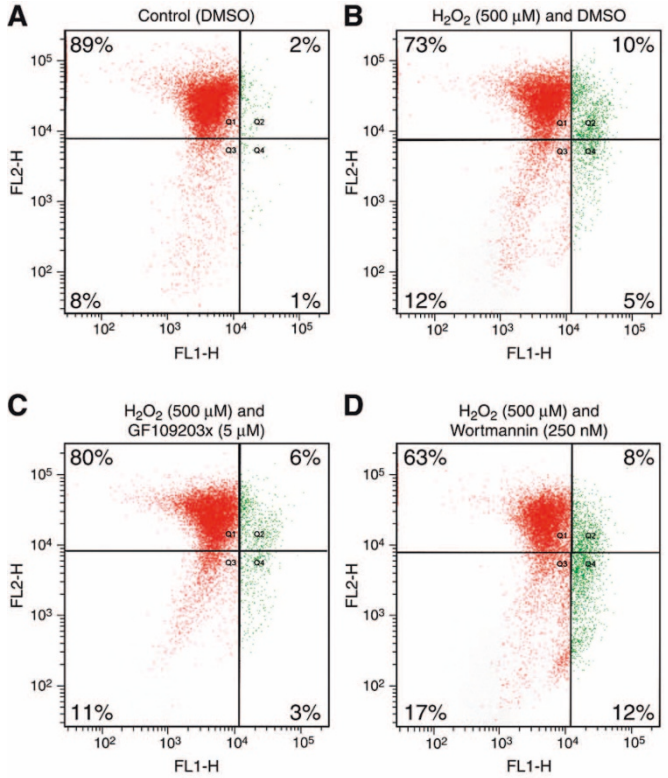

Figure 5. Inhibition of PKC attenuates, whereas inhibition of PI3-K enhances, H_2O_2-induced mitochondrial depolarization. RIE-1 cells were pretreated with the PI3-K inhibitor wortmannin or the PKC inhibitor GF109203x for 30 min before treatment with H_2O_2 for 3 h. Cells were labeled with 2 μM JC-1 for 15 min at room temperature, and intensities of FL-1 and FL-2 fluorescence were measured. JC-1 fluorescence in the FL-1 channel increases as the mitochondrial membrane potential drops while its fluorescence in FL-2 channel decreases. Percentage numbers in Q1 and Q2, Q4 indicate proportion of cells with normal and depolarized mitochondria, respectively. Representative data from three separate experiments are shown here.

Because PI3-K/Akt appears to be an important anti-apoptotic pathway during H_2O_2-induced RIE-1 cell death and inhibition of PKC significantly prevents H_2O_2-induced cell death, we next determined the regulation of Akt phosphorylation by PKC. As shown previously, treatment with H_2O_2 induced Akt phosphorylation (Fig. 4*B*). Interestingly, the treatment with GF109203x resulted in synergistic increase in H_2O_2-induced activation of Akt, suggesting that significant attenuation of H_2O_2-mediated apoptosis with PKC inhibition may act through the enhanced PI3-K/Akt activation. Pretreatment with GF109203× has no effect on H_2O_2-induced ERK1/2 or JNK phosphorylation, suggesting that H_2O_2 induces ERK1/2 or JNK phosphorylation in a PKC-independent fashion. To further delineate the regulation of Akt phosphorylation by PKC, RIE-1 cells were pretreated with the PKC inhibitor GF109203x (5 μM) for 30 min before treatment with PKC activator, PMA (100 nM) for additional 30 min. As shown in Figure 4*C*, PMA reduced Akt phosphorylation. GF109203x alone increased Akt phosphorylation and attenuated PMA-reduced Akt phosphorylation. Treatment with PMA induced ERK1/2 but not JNK phosphorylation and this induction was blocked by the combination treatment with GF109203x, suggesting that PKC isoform(s) involved in PMA-induced ERK phosphorylation is different from that involved in H_2O_2-induced cell death.

Inhibition of PKC attenuates, whereas inhibition of PI3-K enhances, H_2O_2-induced mitochondrial depolarization. Mitochondrial membrane depolarization, which increases permeability and releases pro-apoptotic factors into the cytosol, is an early event of apoptosis. Recently, decreased mitochondrial membrane potential was found to be the early signs of apoptosis in human intestinal epithelial cell line SW-480 (21). To determine whether the mitochondria-initiated pathway is involved in H_2O_2-induced apoptosis, we measured H_2O_2-induced alterations in mitochondrial membrane potential and the effect of PKC or PI3-K inhibition. In healthy cells, lipophilic cation JC-1 exists as a monomer in the cytosol (FL-1 positive; green) and also accumulates as aggregates in the mitochondria (FL-2 positive; red). In apoptotic and necrotic cells, JC-1 exists exclusively in monomer form and produces a green cytosolic signal (22). As shown in Figure 5, treatment with H_2O_2 resulted in a decline in the mitochondrial membrane potential (Fig. 5, *A* and *B*), implicating altered mitochondrial membrane permeability as an important event for H_2O_2-induced apoptosis in RIE-1 cells. Pretreatment with PKC inhibitor GF109203x attenuated H_2O_2-induced mitochondrial depolarization (Fig. 5*C*). In contrast, pretreatment with wortmannin significantly enhanced H_2O_2-induced mitochondrial

depolarization (Fig. 5*D*). These results suggest that regulation of H_2O_2-induced apoptosis by PKC or PI3-K may lie upstream of mitochondria.

DISCUSSION

In this study, we found that H_2O_2 treatment induces the phosphorylation of ERK, JNK, and Akt, resulting in RIE-1 cell apoptosis *via* the caspase pathway. The activation of ERK and JNK may be involved in the H_2O_2-induced intestinal epithelial cell apoptosis since the inhibition of either pathway resulted in attenuation of apoptotic response. In contrast, the activation of Akt may be important as the cell survival signal given the fact that inhibition of PI3-K significantly enhanced H_2O_2-induced cell death. Moreover, inhibition of PKC, which resulted in activation of Akt, was found to attenuate the apoptosis induced by H_2O_2.

NEC is the most common gastrointestinal tract surgical emergency in neonates. Despite recent advances, the overall mortality rate remains high, especially in extremely small premature infants. The numerous risk factors for the pathogenesis of NEC have been well described; however, the exact cellular mechanisms involved in intestinal epithelial cell injury during NEC are still not clearly elucidated. Transient mesenteric ischemia-reperfusion injury resulting in the production of ROS has been implicated to contribute to NEC (3–5). Additionally, extensive apoptosis occurs in enterocytes in the apical villi of infants with NEC (23); however, the exact role of ROS on the intestinal epithelial cell fate during NEC as well as the cellular mechanisms that are involved in this process are largely unknown. Therefore, in the present study, we attempted to investigate the role of intracellular signal transduction pathways involved in oxidative stress–induced intestinal epithelial cell apoptosis.

Oxidative stress can trigger the activation of multiple signaling pathways that influence the cytotoxicity observed in affected cells, including the activation of the MAPK cascade. The MAPK signaling, which is stimulated by growth factor receptors, is also linked to the protection of various cell types against apoptosis. Having established that H_2O_2 triggers ERK1/2, JNK1/2, and PI3-K activation in RIE-1 cells, we then investigated role of these protein kinase pathways in H_2O_2-induced cell death using a range of pharmacological inhibitors. Our results show that pretreatment with the selective MEK1 inhibitor U0126 (10 μM) or JNK inhibitor SP600125 (20 μM) partially reduced H_2O_2-induced apoptosis in RIE-1 cells. Similar results were found with the use of PD98059 compound, another MEK inhibitor (data not shown). These data clearly suggest that an inhibition of ERK1/2 and JNK pathways protect RIE-1 cells from H_2O_2-induced cell death.

Our results are consistent with the findings from other cell-type studies. For example, ERK1/2 is involved in neuronal cell death triggered by oxidative stress (24), and inhibition of the JNK pathway improves cell viability in response to oxidant injury (25). Furthermore, inhibition of MEK PD98059 attenuates H_2O_2-induced cell death of the human colon cancer cell line HT-29 through inhibition of JNK (16). However, several studies have also shown the opposite role of ERK1/2 pathway during oxidative stress-induced cell injury. Treatment of primary cortical neurons with the MEK1/2 inhibitors, U0126 and PD98059, have been shown to significantly increase neuronal cell loss induced by H_2O_2 and hypoxia, respectively (14). Therefore, the role of ERK1/2 in determining the fate of cells (survival or death) after oxidative stress injury is cell-type specific. Moreover, several reports have also demonstrated that H_2O_2-induced activation of ERK is mediated by both PKC-dependent and -independent mechanisms (26,27). In our study, treatment with PKC inhibitor, GF109203x, which attenuated H_2O_2-induced RIE-1 cell death, blocked PMA- but not H_2O_2-mediated ERK1/2 phosphorylation, suggesting that H_2O_2 may activate ERK1/2 *via* PKC-independent pathway in RIE-1 cells.

Apoptotic signals by H_2O_2 also up-regulated antiapoptotic signaling, such as the PI3-K/Akt pathway. PI3-K/Akt pathway plays a critical role in human intestinal epithelial cell survival and differentiation (28,29). Furthermore, the role of the PI3-K/Akt pathway in protecting against ROS-induced cell death has been extensively documented (6,11,30). In this study, we show that H_2O_2 stimulates Akt phosphorylation in RIE-1 cells *via* a PI3-K-dependent pathway suggesting that H_2O_2-induced Akt activation may be involved in protecting RIE-1 cells against oxidative stress-induced cell death. To investigate this possibility, we used the PI3-K inhibitor, wortmannin, and found that it significantly increases cell death triggered by H_2O_2. Our findings suggest that the ultimate fate of intestinal epithelial cells in response to oxidative stress–induced injury is determined by the balance between activation of apoptotic and antiapoptotic signals.

The ERK and JNK inhibition did not completely block H_2O_2-induced cell death, suggesting the involvement of multiple pathways in H_2O_2-induced apoptosis in RIE-1 cells. Indeed, our results show that inhibition of PKC using GF109203x maximally attenuated H_2O_2-induced apoptosis, indicating that H_2O_2-mediated RIE-1 cell death also requires PKC activation. The involvement of various isoforms of PKC in the regulation of H_2O_2-mediated processes has been documented in a number of cell types (31,32); however, its role in the regulation of H_2O_2-induced intestinal epithelial cell death is undefined. The activation of selective PKC isoforms, δ and ϵ, have been implicated in tumor necrosis factor (TNF)-α-induced intestinal epithelial cell death as proapoptotic signals (33). Further studies are required to determine the role of PKC isoforms that are involved in H_2O_2-induced intestinal epithelial cell death.

Another important finding identified in our study is that PKC may play a role in H_2O_2-mediated apoptosis through the regulation of PI3-K/Akt, a critical pathway in cell survival. Our results indicated that the H_2O_2-induced apoptosis required PKC activation. Moreover, we show that inhibition of PKC contributed to the activation of Akt and in contrast, activation of PKC by PMA reduced Akt activity (Fig. 4, *B* and *C*). Consistent with our findings, recent studies have shown that nonselective PKC inhibitors stimulate and PMA attenuates Akt phosphorylation in A549 and HEK293 cells (34) and PKC negatively modulates the hepatocyte growth factor-induced migration, integrin expression, and PI3-K activation in human hepatoma cells (35).

Lastly, intracellular signaling pathways directly and indirectly regulate mitochondrial membrane stability and function to ultimately dictate apoptotic fate of a cell. Activation of PKC in some cell types initiates an apoptotic pathway that involves alteration of mitochondrial membrane potential (36). Moreover, the major mechanism involved in the antiapoptotic effect of PI3-K/Akt is the phosphorylation of BAD, which is then sequestered in the cytosol and prevented from interacting with Bcl-2/Bcl-x_L and from disrupting the mitochondrial membrane (37). Inhibition of PI3-K/Akt signaling potentiates cytochrome *c* release from mitochondria and induces mitochondrial transmembrane potential decrease in bovine carotid artery endothelial cells (38). In agreement with these mechanisms, we observed that activation of PKC decreases Akt phosphorylation whereas inhibition of PKC increases Akt phosphorylation and attenuates H_2O_2-induced apoptosis and mitochondrial depolarization. In contrast, inhibition of PI3-K/Akt signaling significantly potentiated H_2O_2-induced apoptosis and induced significant mitochondrial membrane potential decrease. Our results suggest that the regulation of H_2O_2-induced apoptosis by PKC may act through down-regulation of PI3-K/Akt and this regulation may occur upstream of mitochondria.

In conclusion, our study demonstrates that H_2O_2-induced apoptosis involves the activation of ERK, JNK, and PKC signaling pathways in intestinal epithelial cells, and that inhibition of PI3-K enhances apoptosis. Moreover, PKC may play an important role in H_2O_2-induced intestinal epithelial cell death through negative regulation of PI3-K/Akt and this may occur upstream of mitochondria. Further studies will be directed toward identifying the isoform(s) of PKC and the downstream mediators responsible for intestinal cell death. A better understanding of the signal transduction pathways involved in H_2O_2-induced intestinal cell death will potentially allow us to develop novel therapy in the treatment of oxidative stress–mediated gut injury.

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