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IGF-1 PROTECTS INTESTINAL EPITHELIAL CELLS FROM OXIDATIVE STRESS-INDUCED APOPTOSIS*

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Abstract

Background—Reactive oxygen species (ROS) are involved in the pathogenesis of necrotizing enterocolitis (NEC) in premature infants. We have recently found that activation of multiple cellular signaling transduction pathways occurs during ROS-induced intestinal cell apoptosis; the phosphatidylinositol 3-kinase (PI3-K) pathway plays an anti-apoptotic role during this process. Insulin-like growth factor (IGF)-1 activates PI3-K pathway to promote cell survival; however, the effects of IGF-1 treatment during gut injury are not clearly defined. The purpose of this study was to determine whether IGF-1 protects intestinal cells from ROS-induced apoptosis.

Materials and Methods—Rat intestinal epithelial (RIE)-1 cells were treated with either IGF-1 (100 nM), hydrogen peroxide (H₂O₂; 500 μM) or combination. Western blotting was performed to assess phosphorylation of Akt, a downstream effector of PI3-K. Cell Death Detection ELISA, DCHF and JC-1 assay were performed to demonstrate protective effects of IGF-1. Wortmannin, an inhibitor of PI3-K, was used to show PI3-K-dependent mechanism of action for IGF-1.

Results—H₂O₂ treatment resulted in increased intestinal epithelial cell apoptosis with intracellular ROS generation and mitochondrial membrane depolarization; IGF-1 pretreatment attenuated H₂O₂-induced apoptosis and mitochondrial membrane depolarization without affecting ROS production. H₂O₂-induced phosphorylation of Akt was further increased with IGF-1 treatment; wortmannin abolished these effects in RIE-1 cells.

Conclusions—PI3-K pathway is activated during ROS-induced intestinal epithelial cell injury; IGF-1 exerted an anti-apoptotic effect during this response by stimulation of PI3-K activation. A better understanding of the exact role of IGF-1-mediated activation of PI3-K may

Keywords

IGF-1; PI3-K; Oxidative stress; Apoptosis; Necrotizing enterocolitis

INTRODUCTION

Necrotizing enterocolitis (NEC) is a disease of predominantly premature, very low birth weight infants which results in significant infant mortality and morbidity. Despite identifying numerous, well-described risk factors associated with the onset and progression of the disease, the exact pathogenesis of NEC remains ill-defined and largely unknown. In particular, reactive

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oxygen species (ROS), generated as a result of ischemia-reperfusion injury to the gut, has been linked to the development of NEC in premature infants [1,2]. However, the exact intestinal cellular signals involved during this process remain undefined.

There are multiple signaling pathways implicated during oxidative stress-induced injury in various cell types [3]. Several signaling pathways have been identified either to propagate intestinal epithelial cell injury and apoptosis or to promote cell survival during NEC [2,4]. ROS observed during intestinal cell injury, regardless of the exact mechanisms involved, inevitably lead to activation of critical cellular signaling processes which ultimately regulate the fate of intestinal epithelial cells. ROS are able to initiate cell death programs and are implicated in cellular injury during hypoxia and re-oxygenation [5]. Mitochondrial ROS generation, at low levels, is an inherent cellular function that is thought to be mitogenic, but it can also lead to accelerated oxidative cell damage if that equilibrium is shifted. Oxidative stress can induce mitochondrial DNA damage, further leading to increased release of ROS. Multiple signaling pathways are sensitive to a rise in intracellular ROS levels and can dictate overall cell survival [6]. Hence, ROS-mediated activation of cellular signaling in intestinal epithelial cells may play a critical role in the pathogenesis of NEC.

We have recently demonstrated that activation of multiple intracellular signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3-K), occurs in intestinal epithelial cells during oxidative stress and that inhibition of PI3-K results in increased apoptosis [3]. Growth factors activate multiple signaling pathways to promote cell growth, survival and proliferation in various cell types. In particular, insulin-like growth factor (IGF)-1 has been shown to promote cell survival through PI3-K pathway-dependent signaling in different tissues [7–11]. Strong evidence of such signaling mechanism exists in neuronal cells and in pancreatic islet cells after an acute cytokine insult [12]. These studies established IGF-1 as an important protective factor during inflammation which is capable of blocking cytokine-induced apoptosis; however, the potential beneficial role of IGF-1 during NEC is unknown.

In this study, we sought to determine the role of IGF-1 in H₂O₂-induced intestinal epithelial cell apoptosis. We show that IGF-1 treatment activated PI3-K to attenuate H₂O₂-induced intestinal cell apoptosis by restoring mitochondrial membrane potential without altering intracellular ROS level. These findings suggest that the activation of PI3-K occurs as an anti-apoptotic, cell protective process during oxidative stress-induced intestinal epithelial cell injury.

MATERIALS AND METHODS

Reagents

Wortmannin and hydrogen peroxide (H₂O₂) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal anti-phospho-Akt, and rabbit polyclonal anti-Akt antibodies were purchased from Cell Signaling (Beverly, MA). The anti-actin antibody was purchased from Sigma Chemical Co. Tissue culture media and reagents were obtained from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride (PVDF) membranes were from Millipore Corp. (Bedford, MA). The enhanced chemiluminescence (ECL) system was purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture

Rat intestinal epithelial (RIE)-1 cells (a gift from Dr. Kenneth D. Brown; Cambridge Research Station, Babraham, Cambridge, U.K.) were maintained in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum (FBS) and cultured at 37°C under an atmosphere containing 5% CO₂. All experiments were performed on cells within 6 weeks of culture from

liquid nitrogen stocks and were determined as *Mycoplasma*-free. To eliminate the potential ROS scavenger action of dimethyl sulfoxide (DMSO) in vehicle, we used the same concentrations of DMSO (0.1%) for both inhibitor and vehicle.

Cell Death ELISA

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

DCF fluorescence and flow cytometry

DCF fluorescence was used to determine intracellular ROS based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCFH) to a fluorescent 2',7'-dichlorofluorescein (DCF). Following treatment of RIE-1 cells, the medium was aspirated and cells were washed twice with phosphate-buffered saline (PBS) and then incubated in 1 ml of FBS-free medium. DCFH-DA was added at a final concentration of 10 μ M and incubated for 20 min. The cells were then washed once with PBS and maintained in 1 ml culture medium. The fluorescence was monitored after 30 min of incubation using a Nikon fluorescence microscope equipped with a fluorescein isothiocyanate filter. For the flow cytometry, RIE-1 cells were treated with H₂O₂ or IGF-1 and washed in PBS three times and incubated with 10 μ M DCFH-DA for 15 min at 37°C. To quantify the intensity of fluorescence, the samples were analyzed on a Becton-Dickenson FACScan flow cytometer equipped with a 488nm Argon-ion laser and 525 $\pm$ 10nm bandpass emission filter.

Protein extraction and Western blot analysis

RIE-1 cells were lysed with 80–100 μ l of Cell Lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin]. Lysates were clarified by centrifugation (13000 $\times$ g for 20 min at 4°C), and protein concentrations were determined using method described by Bradford [13]. Equal amounts of total protein (30 μ g) were loaded onto NUPAGE 4–12% Bis-Tris Gel and transferred to PVDF membranes. The membranes were incubated for 1 h at room temperature in a blocking solution (Tris-buffered saline containing 5% nonfat dried milk and 0.1 % Tween 20), followed by incubation with primary antibodies at 4°C overnight, and then with horseradish peroxidase-conjugated secondary antibodies. The immune complexes were visualized by ECL.

JC-1 mitochondrial membrane potential detection

The mitochondrial membrane potential was analyzed using Mito Probe JC-1 Assay kit (Molecular Probes, Eugene, OR). The collapse in the electrochemical gradient across the mitochondrial membrane was measured using 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. Cells (1×10^6) were incubated with 2 μ M JC-1 for 15 min at 37°C in the dark. Cells were washed with PBS, resuspended in 500 μ L PBS, and analyzed on a FACScan flow cytometer.

Statistical analysis

Results are expressed as the mean $\pm$ SEM. The data in the Figure 1 were analyzed using the Kruskal-Wallis and assessed at the 0.05 level of significance.

RESULTS

IGF-1 attenuates H₂O₂-induced apoptosis

We have previously showed that H₂O₂ treatment induces intestinal epithelial cell apoptosis [3]. We hypothesized that IGF-1 may exert an important protective effect on intestinal epithelial cells during H₂O₂-induced injury. To confirm this, we pretreated RIE-1 cells with IGF-1 (100 nM) prior to H₂O₂ treatment. Consistent with our previous finding, H₂O₂ treatment induced RIE-1 cell death by nearly 7-fold when compared to control cells. Importantly, H₂O₂-induced apoptosis was significantly attenuated when cells were pretreated with IGF-1 for 30 minutes prior to H₂O₂ (Fig. 1A). We speculated that IGF-1 exerts its anti-apoptotic action by activation of PI3-K. In addition, we have recently reported that inhibition of PI3-K with wortmannin pretreatment significantly increases H₂O₂-induced intestinal cell apoptosis [3]. Therefore, we next examined the effects of PI3-K inhibition on IGF-1 pre-treated RIE-1 cells before H₂O₂ treatment. Inhibition of PI3-K with wortmannin abolished anti-apoptotic effects of IGF-1 and, in fact, significantly increased apoptosis in RIE-1 cells when compared to H₂O₂ treatment alone, further confirming its cell survival role to be PI3-K-dependent during oxidative stress (Fig. 1B). These findings support our hypothesis that IGF-1 protects intestinal epithelial cells against H₂O₂-mediated intestinal cell injury.

IGF-1 activates PI3-K/Akt pathway in RIE-1 cells

We had found that RIE-1 cells respond to H₂O₂ by activating various intracellular signaling pathways [3]. In particular, H₂O₂ treatment activated PI3-K pathway, demonstrating its critical anti-apoptotic role during oxidative stress-induced gut injury [3]. In this study, we also confirmed that treatment with H₂O₂ alone induces phosphorylation of Akt, a downstream effector of PI3-K, in RIE-1 cells. In addition, we showed that IGF-1 treatment increases phosphorylated Akt protein level, thus demonstrating PI3-K/Akt pathway activation by IGF-1 in RIE-1 cells (Fig. 2). Moreover, combination treatment with both IGF-1 and H₂O₂ resulted in a synergistic increase in phosphorylated Akt expression, suggesting that IGF-1 enhances oxidative stress-induced activation of PI3-K pathway in RIE-1 cells (Fig. 2). These findings further support and delineate a protective role of IGF-1 during H₂O₂-induced apoptosis in intestinal cells.

IGF-1 attenuates H₂O₂-induced mitochondrial depolarization

Decreased mitochondrial membrane potential is an early sign of apoptosis, marked by increased membrane permeability and release of pro-apoptotic factors into the cytosol. To determine the level of mitochondrial dysfunction and the effects of IGF-1 during oxidative stress, we used a mitochondrial membrane sensor kit to perform a JC-1 assay. JC-1 cation fluoresces differently in apoptotic and healthy cells. In healthy cells, it aggregates in mitochondria and exhibits red fluorescence. In apoptotic cells, it fails to accumulate in mitochondrial matrix because of altered membrane potential, instead it is found in cytosol and leads to a green fluorescence. As shown in Figure 3A, treatment with H₂O₂ resulted in significant decline in mitochondrial membrane potential, indicating this mechanism of action for ROS-induced intestinal cell apoptosis (*right upper panel*). Notably, pretreatment with IGF-1 attenuated H₂O₂-induced mitochondrial depolarization (*left lower panel*). Conversely, pretreatment with wortmannin abolished the protective effects of IGF-1 against H₂O₂-induced mitochondrial potential change (*right lower panel*). These findings were further complemented by Western blotting, where activation of PI3-K, as assessed by increased phosphorylation of Akt, occurred with either H₂O₂ alone or H₂O₂ plus IGF-1 treatment; this induction was completely abolished with wortmannin treatment (Fig. 3B). Our findings confirm that IGF-1 exerts its protective effect via the PI3-K/Akt pathway and that inhibition of this pathway induces intestinal cell apoptosis with loss of the protective effect exerted by IGF-1. These

findings indicate that the PI3-K/Akt pathway is a key cell survival pathway utilized by growth factors during oxidative injury in intestinal epithelial cells.

H₂O₂ increases intracellular ROS production

A rise in intracellular levels of ROS can damage various cell components, including mitochondria, and trigger activation of specific signaling pathways. We next sought to determine whether IGF-1 pretreatment affects intracellular ROS level in RIE-1 cells, as a mechanism to protect against apoptosis. As expected, there was a significant rise in DCF fluorescence to indicate increased ROS generation after H₂O₂ treatment in RIE-1 cells when compared to the control cells (Fig. 4A). These findings were confirmed with DCF flow cytometry (Fig. 4B; *green area*). Interestingly, IGF-1 pretreatment did not significantly affect DCF fluorescence after H₂O₂ in RIE-1 cells, indicating a lack of direct effect on intracellular ROS level by IGF-1 during oxidative stress (Figs. 4A; *third column* and 4B; *red area*). Therefore, our findings suggest that the mechanism of action for IGF-1 in protecting intestinal cells against oxidative stress-induced apoptosis is independent of altering levels of intracellular ROS.

DISCUSSION

In this study, we demonstrate attenuation of apoptosis and activation of PI3-K/Akt signaling pathway with IGF-1 pretreatment in RIE-1 cells during H₂O₂-induced oxidative stress. IGF-1 pretreatment dramatically decreased H₂O₂-induced mitochondrial membrane depolarization. Inhibition of this pivotal survival pathway with pharmacologic PI3-K inhibitor, wortmannin, significantly enhanced oxidant-induced mitochondrial membrane depolarization in intestinal epithelial cells. Interestingly, the protective action of IGF-1 that we observed during oxidative stress injury appeared to be independent of intracellular ROS production.

IGF-1 is a single-chain peptide of 70 amino acids with considerable homology to insulin. The main biological effect of IGF-1 is stimulation of cellular growth and differentiation [14–19]. In particular, IGF-1 has been shown to exert beneficial effect on gut homeostasis [20,21]. IGF-1 augmented compensatory gut mucosal hyperplasia and restitution in rats undergoing small bowel resection [20] and decreased bacterial translocation after severe burns by maintaining gut mucosal integrity [21]. IGF-1 also accelerated ileal epithelial cell migration *in vivo* and *in vitro*, mimicking precocious mucosal maturation associated with spontaneous ileal perforation in extremely small premature neonates [22]. Importantly, a recent study demonstrated that IGF-1 attenuates gut mucosal apoptosis in a murine model of NEC, implicating an anti-apoptotic mechanism of action for IGF-1 in the gut during oxidative injury [23]. Consistent with these results, our present study also showed that IGF-1 attenuates intestinal cell apoptosis in response to H₂O₂-induced injury. Since ROS has been shown to play a critical role in the pathogenesis of NEC, our *in vitro* data suggest that IGF-1 may be a novel therapy option to attenuate extensive gut injury that occurs in premature infants with NEC as a result of marked apoptosis. IGF-1 mediates its cellular effects via cell surface tyrosine kinase receptors and is known to activate PI3-K in a variety of cell types [12,16,24–26]. PI3-K has recently been shown to play a critical role in mediating cellular response to inflammation and injury [12]. In the present study, we also found that IGF-1 synergistically stimulates H₂O₂-induced activation of PI3-K signaling to protect RIE-1 cells against oxidative stress-mediated apoptosis.

The role of mitochondria as a main orchestrator of apoptosis is well established [27–33]. Disruption of electron transport in mitochondria has long been recognized as an early feature of cell death. Mitochondria play a key role in oxidative damage and regulate apoptosis through various mechanisms, which include disruption of the mitochondrial membrane potential leading to membrane depolarization, enhanced cytochrome c release, activation of caspases and subsequently, mitochondrial electron transport chain cleavage by caspase [34–37]. The

effects of IGF-1 on mitochondria during direct oxidant insult have not been clearly defined. We, therefore, hypothesized that IGF-1 may act as an anti-apoptotic factor by maintaining mitochondrial membrane potential integrity, thus, preventing activation of early apoptotic signals. Our study results showed that IGF-1 significantly reversed the decrease in mitochondrial membrane potential that occurs with H₂O₂ treatment in RIE-1 cells. Pretreatment with wortmannin abolished the protective effects of IGF-1 and further confirmed an important function of PI3-K as a protective signaling mechanism during oxidative stress-mediated gut injury. These findings suggest that IGF-1 acts as a stabilizer of mitochondrial membrane potential during H₂O₂-mediated oxidative stress, hence reversing apoptotic signals.

ROS is thought to promote apoptosis but can also activate anti-apoptotic pathways [26,38]. Sources of ROS range from dysfunctional mitochondrial electron transport chain to ROS-generating plasma membrane and cytosolic enzymes. The burden of ROS production is counteracted by the intracellular antioxidant system, where maintaining this balance is thought to be critical to normal cellular functions. Mitochondrial DNA is more sensitive to oxidative insult, and when mitochondrial function and integrity are compromised, ROS production and DNA damage are increased leading to cell death [6]. A variety of inducers, such as ultraviolet radiation, chemotherapeutic agents and growth factors, can generate high levels of ROS, perturbing the normal redox balance and shifting cells into a state of oxidative stress [6]. Interestingly, direct treatment with oxidants can activate multiple pathways, including PI3-K, through stimulation of growth factor receptors. In the present study, as expected, intracellular ROS production in RIE-1 cells was markedly increased with H₂O₂ treatment. However, IGF-1 did not significantly decrease intracellular ROS levels during direct oxidant insult on RIE-1 cells, indicating that the protective effects of IGF-1 may not be intimately related to altering intracellular ROS production. It is well known that the oxidants can reversibly inactivate protein-tyrosine phosphatase 1B 9A, a “turn off” switch for tyrosine kinase receptors [6]. This may, in part, explain why we did not observe a significant change in ROS production in IGF-1 pretreated cells during oxidative stress. Recent reports also show a cell pro-survival role of ROS and growth factor-induced ROS production by NADPH oxidase [38–40]. Although the exact mechanisms of this interaction are largely unknown, these studies demonstrate growth factor activation of NADPH oxidase through 5-lipoxygenase independent mechanism [39].

In conclusion, H₂O₂-mediated oxidative stress in RIE-1 cells increases apoptosis and induces PI3-K/Akt survival pathway via activation of growth factor receptors. With IGF-1 pretreatment, intestinal cells are protected from apoptosis and may utilize multiple mechanisms of survival. Along with activation of PI3-K, IGF-1 also demonstrates a benefit with regards to restoring mitochondrial membrane potential, thus, preventing further depolarization and activation of apoptosis. Our findings establish the importance of growth factors (e.g., IGF-1) in modulating apoptosis in injured intestinal epithelial cells. A better understanding of the mechanisms involved in IGF-1-mediated activation of the PI3-K pathway, as a cell protective signaling mechanism, may allow for development of novel therapy in the treatment of infants with NEC.

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Figure 1. RIE-1 cell apoptosis

(A) RIE-1 cells were treated with H₂O₂ for 3h, IGF-1 for 30 minutes, or pretreated with IGF-1 for 30 minutes prior to H₂O₂. IGF-1 pretreated RIE-1 cells showed significant attenuation of H₂O₂-induced apoptosis as measured by DNA fragmentation ELISA (data represent triplicate determinations mean $\pm$ SEM; * p <0.05 vs. control; † p <0.05 vs. H₂O₂ alone). (B) RIE-1 cells were treated with H₂O₂ alone or in combination with wortmannin (250 nM) and IGF-1₂. Wortmannin pretreatment increased apoptosis when compared to H₂O₂ alone (data represent triplicate determinations mean $\pm$ SEM; * p <0.05 vs. H₂O₂ alone). Representative data from three separate experiments are shown here.

Figure 2. pAkt expression after IGF-1 or H₂O₂ treatment

RIE-1 cells were treated with IGF-1 for 30 minutes, H₂O₂ for 3 h, or pretreated with IGF-1 for 30 minutes prior to H₂O₂ for pAkt and β -actin by Western blotting. Either treatment with IGF-1 or H₂O₂ alone increased phosphorylation of Akt (pAkt), a measure of PI3-K activation. Pretreatment with IGF-1 enhanced H₂O₂-induced pAkt expression in RIE-1 cells. Representative blot from three separate experiments is shown here.

Figure 3. Effects of IGF-1 and wortmannin on mitochondrial membrane potential and pAkt expression

(A) Cells were treated with either wortmannin or IGF-1 for 30 minutes, followed by H₂O₂ treatment for 3 h. Cells were labeled with 2 μM JC-1 for 15 minutes at room temperature, and FL-1 and FL-2 fluorescence intensity was measured. JC-1 fluorescence increases as the mitochondrial membrane potential drops, while its fluorescence in FL-2 channel decreases. Percentage numbers in Q1, Q3 and Q2, Q4 represent proportions of cells with normal and depolarized mitochondrial membranes, respectively. H₂O₂ treatment increased mitochondrial membrane depolarization (*right upper panel; Q2, Q4*); this effect was reversed with IGF-1 (*left lower panel; Q2, Q4*). Inhibition of PI3-K with wortmannin increased mitochondrial membrane depolarization (*right lower panel; Q2, Q4*). Representative data from two separate experiments are shown here. (B) Western blot from RIE-1 cells pretreated with IGF-1 alone or in combination with wortmannin and then treated with H₂O₂ for 3 h. pAkt protein expression was increased with H₂O₂ alone and IGF-1 plus H₂O₂; these effects were nearly completely abolished with wortmannin. Representative blot from three separate experiments are shown here.

Figure 4. Intracellular ROS in RIE-1 cells

(A) RIE-1 cells were exposed to 30 minutes of IGF-1 (100 nM) then treated with H₂O₂ (500 μ M) for 3 h. DCF fluorescent microscopic images with corresponding non-fluorescent images are shown here. H₂O₂ treatment increased DCF fluorescence in intestinal epithelial cells compared with control (vehicle treated) cells (*second column*). IGF-1 pretreated cells showed similar DCF fluorescence as the H₂O₂ group (*third column*). Representative images from three separate experiments are shown here. (B) Fluorescence intensity was measured by FACSscan analysis. Filled areas in black represent control cells, green for H₂O₂ treatment alone and red for H₂O₂ plus IGF-1. A composite graph (*third one*) showed no significant difference between H₂O₂ alone (green) vs. IGF-1 with H₂O₂ (red).