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EFFECTS OF OXIDATIVE STRESS ON INTESTINAL TYPE I INSULIN-LIKE GROWTH FACTOR RECEPTOR EXPRESSION

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Abstract

Introduction—Oxidative stress activates multiple signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3-K), in injured intestine such as necrotizing enterocolitis (NEC). We have previously shown that H_2O_2-induced PI3-K activation is significantly enhanced with exogenous insulin-like growth factor (IGF)-1 in intestinal epithelial cells. However, the effects of oxidative stress on IGF receptor type I (IGF-IR) activation and expression in neonatal intestine during NEC are unknown.

Material and Methods—Intestinal sections from neonates undergoing bowel resections (control=3, NEC=20) were analyzed for IGF-IR expression. NEC was induced in newborn mouse pups using hypoxia and hyperosmolar feeds, and distal small bowel segments were analyzed for IGF-IR expression (control=3, NEC=7). H_2O_2 was used to induce oxidative stress in rat (RIE-1) and fetal human (FHs74 Int) intestinal epithelial cells. Phosphorylation of IGF-IR, Akt, a downstream effector of PI3-K, and IGF-IR levels were determined by Western blotting. Flow cytometry, immunofluorescence, immunohistochemistry, IGF-IR tyrosine phosphorylation array, Cell Death ELISA and Western blotting were used to determine the IGF-IR expression.

Results—An increased IGF-IR expression was noted in intestinal sections from NEC as well as murine model of NEC. H_2O_2 treatment rapidly activated IGF-IR and increased expression in RIE-1 and FHs74 Int cells. Inhibition of IGF-IR resulted in significant RIE-1 cell apoptosis during oxidative stress. IGF-IR tyrosine phosphorylation array showed recruitment of several key SH2 domain-containing proteins and oncogenes to IGF-IR tyrosine kinase domain in H_2O_2-treated RIE-1 cells.

Conclusion—IGF-IR-mediated activation of intracellular signaling may play a critical role during oxidative stress-induced apoptosis during NEC.

Keywords

PI3-K/Akt; IGF-IR; oxidative stress; intestinal injury

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INTRODUCTION

Oxidative stress plays an important role in the pathogenesis of various disease processes. Effects of oxidative stress on tissues can range from reversible cellular damage to overwhelming cellular injury leading to apoptosis or necrosis. The underlying mechanisms responsible for reactive oxygen species (ROS)-mediated cell death are complex, and involve activation or dysfunction of various cellular components, receptors, signal transduction pathways and DNA damage. Some of the ROS species are extremely unstable, degrade quickly, and may not pose a significant injury to a cell; however, others, such as hydrogen peroxide (H_2O_2), are easily diffusible with a long half-life, and are potentially harmful even at low concentrations. At very low concentrations, ROS activate mitogenic response in cells and induce growth factor-mediated ROS production [1].

Necrotizing enterocolitis (NEC) is a devastating disease of premature infants with significant morbidity and mortality; the exact pathogenesis of NEC remains largely unknown [2]. ROS, generated as a result of ischemia-reperfusion injury to the gut, have been linked to the development of NEC. Moreover, the effects of oxidative stress on intestinal epithelial cell homeostasis remain poorly understood, and the exact cellular signal transduction pathways involved in this disease process remain unclear. Our recent studies have shown that ROS activate both cell survival and stress pathways in intestinal epithelial cells *in vitro* [3,4]. Phosphatidylinositol 3-kinase (PI3-K), a major cellular protective pathway, becomes activated during oxidative injury and mediates cell survival. We have demonstrated that the PI3-K pathway can further be stimulated with exogenous insulin-like growth factor (IGF)-1, a potent inducer of PI3-K/Akt pathway, during *in vivo* NEC and oxidative stress in intestinal epithelial cells, and can ameliorate mitochondrial apoptotic signaling *in vitro* [4-6].

IGF-1/IGF type I receptor (IGF-IR)-triggered signaling is essential for the regulation of tissue formation and remodeling, prenatal growth, brain development, and muscle metabolism. Cellular growth, proliferation, differentiation, and survival against apoptosis are among the well-described biological actions of IGF-1 signaling, and when impaired, may contribute to the pathogenesis of human diseases and reduced fetal growth in-utero [7-10]. IGF-1 has been shown to promote cell survival through PI3-K pathway-dependent signaling in various cell types, and plays a protective role during inflammation, cytokine-induced apoptosis and in gut homeostasis [11-13]. Hence, enhanced IGF-1 signaling during oxidative stress can significantly protect cells from apoptosis. We have also shown improved survival of mouse pups with exogenous IGF-1 administration, and enhanced activation of PI3-K/Akt signaling pathway during NEC [5].

The IGF-1/IGF-IR signaling may play a critical role in survival of the premature neonates who are shown to have low levels of circulating serum IGF-1 associated with increased risk of multiple disabilities, including retinopathy of prematurity and NEC [14]. Therefore, the purpose of our study was to demonstrate that oxidative stress significantly alters IGF-IR expression levels in intestinal epithelial cells, in order to maximize IGF-1 binding capacity, and that enhanced IGF-IR activation is critical to intestinal epithelial cell survival signaling during NEC.

MATERIALS AND METHODS

Cell lines, reagents and antibodies

Rat intestinal epithelial (RIE)-1 cells (a gift from Dr. Kenneth D. Brown; Cambridge Research Station, Cambridge, U.K.) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and cultured at 37°C under an atmosphere containing 5% CO_2. Human fetal intestinal epithelial (FHs74 Int) cells were purchased from American Type Culture Collection (ATCC), maintained in Hybri-Care Medium (Modified Dulbecco's medium) supplemented with 30 ng/ml epidermal growth factor (EGF) and 10% FBS. Tissue culture media and reagents were obtained from Mediatech, Inc (Herndon, VA). H_2O_2, mouse monoclonal anti-β-actin antibody and other reagents were purchased from Sigma (St. Louis, Mo). AlexaFluor® 488-labeled goat anti-rabbit IgG and Höechst 33342 (Molecular Probes, Eugene, OR) were used for immunofluorescent studies. All primary antibodies, including IGF-IR_β, were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal fluorescein-conjugated mouse anti-human IGF-IR antibody used for flow cytometry evaluation of cell surface receptor density was purchased from R&D Systems Inc. (Minneapolis, MN). Polyvinylidene difluoride (PVDF) membranes were from Millipore Corp. (Bedford, MA). Enhanced chemiluminescence $(ECL)^{Plus}$ system was purchased from Amersham Biosciences (Piscataway, NJ).

Human intestinal sections

Paraffin-embedded intestinal sections from 20 neonates with NEC and 3 neonates with non-inflammatory condition (intestinal atresia) of the gastrointestinal tract (control) undergoing bowel resection were analyzed. Intestinal tissues were fixed and paraffin-embedded for further analysis. Control and NEC sections (5 μm) were prepared for immunohistochemical analysis. Sections were incubated with rabbit anti-IGF-IR overnight at 4°C, then incubated with an anti-rabbit secondary antibody and stained with DAB chromogen (Dako Cytomation EnVision®+ System-HRP (DAB) kit, Carpinteria, CA). Slides were washed, counterstained with hematoxylin, dehydrated and cover slipped. For negative control, sections were stained with rabbit IgG.

In vivo NEC model

Timed-pregnant Swiss Webster mice were purchased (Charles River Labs, Pontage, MI), and pup littermates were obtained from litters at birth and randomized to either control (n=3) or NEC (n=7) group. Control pups were maternally reared. All murine pups in NEC group were hand-fed KMR liquid milk replacer formula (0.3 cc/g/day; q 3 h). To induce NEC, pups were stressed twice daily with hypoxia by placing them in a plexi-glass chamber, breathing 5% oxygen for 10 min. After 96 h, all surviving mice were sacrificed and distal ileal sections were harvested and snap frozen in liquid nitrogen for protein analysis. Animal care was in accordance with approved institutional IACUC protocol.

Protein extraction and Western blot analysis

Tissue lysates prepared from full-thickness mouse intestines were clarified by centrifugation (13000×*g* for 20 min at 4°C) and analyzed for IGF-IR protein. RIE-1 cells were treated with H_2O_2 (0.5 mM) for 1, 3, 6 and 24 h. FHs74 Int cells were treated with H_2O_2 in dose- (0.05-1 mM) and time-dependent (5-60 min) manner. Cell lysates were clarified by centrifugation and stored at −80°C. Protein concentrations were determined using Bradford method [15]. Equal amounts of total protein (100 μg for tissues; 30 μg for cells) were loaded onto NUPAGE 4-12% Bis-Tris Gel and transferred to PVDF membranes, incubated in a blocking solution for 1 h (Tris-buffered saline containing 5% nonfat dried milk and 0.1 % Tween 20), and then incubated with primary antibody overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody. Anti-β-actin antibody and total Akt were used for protein loading control. IGF-IR_β, phospho-IGF-IR, Akt and phospho-Akt antibodies were used to probe the membranes. The immune complexes were visualized by ECL^{Plus}. Densitometric analyses of all blots were performed using ImageJ software (National Institutes of Health, Maryland).

Cell Death ELISA

RIE-1 cells were transfected with either IGF-IR small interference (si) RNA targeted at IGF-IF or non-targeting control (NTC) and plated in 24-well plates for 24 h before H_2O_2 treatment for 3 h. DNA fragmentation assay was evaluated by examination of cytoplasmic histone-associated DNA fragments using Cell Death Detection $ELISA^{plus}$ kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions.

IGF-IR fluorescence and flow cytometry

To evaluate for oxidative stress-induced changes in IGF-IR expression, RIE-1 and FHs74 Int cells treated with H_2O_2 for 3 h. Localization of intracellular IGF-IR was determined by indirect immunofluorescence method using anti-IGF-IR_β primary antibody followed by Alexa-Fluor® 488-conjugated anti-rabbit secondary antibody. The fluorescence was monitored using a Nikon fluorescence microscope. To quantify changes in cell surface receptor density, RIE-1 cells were treated with H_2O_2 for 1 and 3 h, then washed and incubated with the fluorescein-labeled monoclonal IGF-IR antibody according to manufacturer's instructions. Cell surface expression of IGF-IR was determined by FACScan flow cytometric analysis using 488 nm wavelength laser excitation. Geometric means were used to quantify the fluorescent intensity of the cell surface IGF-IR expression in RIE-1 cells.

IGF-IR tyrosine phosphorylation array

To further analyze IGF-IR transactivation and signal transduction activation during H_2O_2-induced oxidative stress in intestinal epithelial cells, we used TranSignal™ IGF-IR tyrosine phosphorylation array (Panomics, Redwood City, CA) to determine which specific SH2 domain-containing molecules are recruited to H_2O_2-stimulated IGF-IR in the absence of IGF-1. This array allows for determining specific "fingerprints" of tyrosine phosphorylation of proteins in cells using SH2 domain binding specificity. Briefly, RIE-1 cells were plated and grown for 24 h, serum-starved overnight and then treated with H_2O_2 for 3 h. Cell lysates

were clarified by centrifugation and stored at −80°C. Protein concentration was determined using Bradford method, and assay was completed according to the manufacturer's instructions.

In vitro siRNA transfection

For *in vitro* siRNA delivery, non-targeting control (NTC) and *SMART* pool IGF-IR siRNA duplexes were purchased from Dharmacon (Lafayette, CO). RIE-1 cells (3×10^6) were transfected with siRNA duplexes using electroporation method (400V; 500μF) and plated onto dishes in growth medium for 72 h. Cells were treated with H_2O_2 for 3 h, and protein was analyzed by Western immunoblotting.

Statistical analysis

Results are expressed as the mean ± SEM. All effects and interactions were analyzed using the Kruskal-Wallis and assessed at the 0.05 level of significance. The data was analyzed with PROC MIXED with LSMEANS option and Satterthwaite approximation for the denominator degrees of freedom in SAS®, Release9.1 (SAS Institute Inc., SAS/STAT® 9.1 User's Guide, Cary, NC: SAS Institute Inc., 2004).

RESULTS

Intestinal IGF-IR expression during NEC and in FHs74 Int cells

IGF-IR expression was noted in the mucosal tips of healthy human neonatal intestines, as assessed by immunohistochemical analysis (Fig.1A, *a*). Notably, there was no evidence of IGF-IR staining in submucosa, myenteric plexus and muscular layers (Fig. 1A, *c*, *e*). In contrast, neonatal intestinal sections from NEC showed dramatic transmural (Fig. 1A, *b* and *d*) as well as myenteric plexus (Fig. 1A, *f*) increases in IGF-IR expression. These findings may represent compensatory increase in intestinal IGF-IR expression during NEC in order to promote intestinal survival signaling during severe injury. Interestingly, we also noted a dramatic staining of enteric nervous system (ENS), represented as enhanced enteric neuronal expression of IGF-IR during NEC (Fig. 1A, *f*). This is an important finding that indicates a potential role for ENS in pathologies associated with intestinal barrier dysfunction. The gastrointestinal tract is a highly innervated organ, where enteric neurons control various gut functions, including motility, microcirculation, and epithelial secretory and intestinal barrier functions. Enteric neuropathy is thought to be an emerging central feature in pathologies associated with intestinal barrier dysfunction such as inflammatory bowel disease, NEC, irritable bowel syndrome, diabetes, autoimmune disease and neurotrophic virus infection of the gut [16]. Future studies focusing on enteric glial and/or ganglion cell injury, survival and regulation of intestinal mucosal barrier function during NEC are necessary to elucidate this potential mechanism in the gut.

Next, we examined IGF-IR expression levels *in vitro* using H_2O_2-induced oxidative stress in human fetal intestinal epithelial cells (FHs74 Int). Phosphorylation of Akt, a downstream effector of PI3-K, increased in a dose-dependent fashion after H_2O_2 treatment, suggesting a mechanism of upregulation of PI3-K/Akt survival signaling during oxidative stress in FHs74 Int cells (Fig. 1B). A significant, rapid increase in IGF-IR expression was also noted in a

time-dependent manner after oxidative stress. A rapid IGF-IR phosphorylation occurred at 10 min after oxidant exposure; this effect was sustained for 60 min. Interestingly, the PI3-K/Akt pathway was activated within 5 min following H_2O_2 treatment in FHs74 Int cells (Fig. 1C). IGF-IR expression increased significantly at 30 min time point of oxidant injury. Immunofluorescent study also demonstrated sustained increase in intracellular IGF-IR levels at 3h after oxidative injury (Fig. 1D), suggesting a brisk cellular response to oxidative stress. Collectively, these findings demonstrate enhanced tissue expression of IGF-IR in human intestinal NEC sections and corresponding fetal intestinal epithelial cells *in vitro*, indicating that NEC-induced injury activates an important balancing protective cellular mechanism such as IGF-IR/PI3-K signaling pathway. Further studies focusing on exact mechanisms of H_2O_2 interaction with IGF-IR and activation of its downstream signaling system during gut injury are necessary.

Oxidative stress induces increases in IGF-IR expression *in vivo* and in RIE-1 cells

To validate our neonatal intestinal section data, we examined the effects of oxidative stress on IGF-IR expression both *in vivo* and *in vitro* models. NEC was induced in neonatal mouse pups as previously described [5]. Intestinal sections (distal small bowel) were harvested for tissue and protein analyses. Immunohistochemical analysis revealed findings similar to those seen in human NEC sections (data not shown). Protein analysis of NEC intestinal tissues demonstrated significant upregulation in tissue IGF-IR expression in all intestinal samples from murine NEC group (Fig. 2A).

The immunofluorescent analysis of RIE-1 using our NEC *in vitro* model also demonstrated an increased intracellular IGF-IR expression after 3 h of H_2O_2-induced oxidative stress (Fig. 2B, *green fluorescence*). To determine the effects of prolonged oxidant exposure on IGF-IR expression, we treated RIE-1 cells with H_2O_2 over a time course (1, 3, 6 and 24 h) and assessed for expressions of IGF-IR, phospho-IGF-IR and phospho-Akt relative to β-actin (Fig. 2C). H_2O_2 treatment increased IGF-IR expression at 1 h in RIE-1 cells; this effect leveled off by 6 h and was sustained to 24 h time point. Activation of IGF-IR/PI3-K signaling pathway occurred by 1 h after oxidant injury and remained significantly stimulated for 24 h.

Next, we sought to quantify the cell surface membrane expression of IGF-IR after oxidative stress in RIE-1 cells (Fig. 2D). Flow cytometry analysis of treated and stained cells revealed increased expression of IGF-IR at 1 h (16%) and 3 h (17%) after H_2O_2 treatment. These findings demonstrate that RIE-1 response to oxidative stress involves activation of IGF-IR/PI3-K survival signal transduction pathway along with rapid increase in intracellular and cell-surface levels of IGF-IR. Taken together, our data support human NEC tissue staining, and FHs74 Int *in vitro* data, thus demonstrating an overall increase in intestinal IGF-IR expression during NEC, and in intestinal epithelial cells in particular.

H_2O_2 induces IGF-IR tyrosine phosphorylation-mediated recruitment of SH2 domain-containing molecules in RIE-1 cells

In order to demonstrate IGF-IR transactivation during oxidative stress in intestinal epithelial cells, we used IGF-IR tyrosine phosphorylation array designed to elucidate which SH2

domain-containing molecules become recruited to the tyrosine kinase domain of IGF-IR during H_2O_2-induced injury. After serum starvation, RIE-1 cells without exogenous growth factor stimulation were analyzed after 3 h of oxidative stress. The array results revealed binding of a number of SH2 domain-containing molecules to IGF-IR tyrosine kinase domain, including p85α/p85β subunits of PI3-K, Shc, Src, RASGAP & RaLP. Recruited SH2 domain-containing molecules included various important mediators of both stress and survival signal transduction pathways (Fig. 3).

The SH2 domains are found in enzymes, adaptor proteins, regulatory subunits of signaling proteins, scaffold proteins, transcription factors and oncogenic proteins. These proteins play a critical role by acting as adaptors between receptors and downstream signaling molecules, transmitting signals within a cell and regulating the kinase activity of specific proteins in different cell types [17,18]. The SH2 domain-containing molecules recruited to H_2O_2-activated IGF-IR in RIE-1 cells fall into the category of enzymes, kinases (*HCK, LCK, MATK, ZAP70*), adaptor proteins (*GRB2, GRAP, NCK*), and regulatory subunits of signaling proteins (*p85α/p85β*), scaffold proteins, transcription factors and oncogenic proteins (*Src, TNS, YES, FGR*). Most notably, there was nearly 15-20 fold increase in adaptor proteins *NCK1/2* are implicated in organization of actin cytoskeleton and cell movement [19], whereas PI3-K p85 subunits are critical in activating major survival signaling pathway. The rest of SH2 domain containing molecules demonstrated nearly 2-5 fold increase relative to quiescent control conditions and serve important physiological functions.

IGF-IR inhibition enhances RIE-1cell apoptosis during oxidative stress

We have previously demonstrated that H_2O_2 induces significant RIE-1 cell death, and IGF-1 enhances survival signaling during oxidative stress in rat intestinal epithelial cells; however, the effect of oxidative stress on RIE-1 survival with IGF-IR silencing during NEC are not known. IGF-IR was targeted silenced in RIE-1 cells (Fig. 4A), and then treated with H_2O_2 or vehicle. We found a significant increase in cell death during oxidative stress in RIE-1 cells with silenced IGF-IR (Fig. 4B). IGF-IR silencing-mediated cell death was increased by nearly two-fold when compared to H_2O_2-induced RIE-1 cell apoptosis, thus suggesting that IGF-IR anti-apoptotic signaling plays a critical role in intestinal epithelial cell survival during *in vitro* NEC. Interestingly, enhanced recruitment of PI3-K p85 subunits (*p85α/p85β*) to IGF-IR in H_2O_2–treated RIE-1 cells observed in IGF-IR tyrosine kinase phosphorylation assay data (Fig. 3) provides important clues to anti-apoptotic balance and attenuated apoptotic impact of IGF-IR silencing in intestinal epithelial cells *in vitro* during oxidative stress.

DISCUSSION

In this study, we demonstrate significant upregulation of IGF-IR expression in the gut during NEC. We also show correlative IGF-IR expression in neonatal intestinal NEC sections, murine *in vivo* NEC model, as well as in human fetal and rat intestinal epithelial cell *in vitro* models of oxidative stress. We also show rapid and sustained activation of IGF-IR/PI3-K survival signaling over a time course in RIE-1 cells, and demonstrate a significant protective effect of IGF-IR signaling, as shown by increased apoptosis with targeted

silencing of IGF-IR. Mechanisms underlying modulation of IGF-IR expression by H_2O_2 in intestinal epithelial cells and during NEC have not been elucidated. Future studies exploring transcriptional and translational alterations, phosphatase activity and possible ENS regulation of intestinal barrier integrity via enhanced IGF-IR expression during oxidative stress are necessary.

Enhanced IGF-IR upregulation and signaling have been described in various cancer cell lines; however, the exact role of IGF-IR has not been elucidated in NEC. Previous *in vivo* NEC studies have examined IGF-1 binding protein family to determine whether availability of the specific binding protein is altered, and whether using this modality to deliver exogenous IGF-1 can enhance neonatal survival and growth, and reduce the risk and incidence of NEC [20-22]; however, the results were limited and this area remains largely unexplored.

IGF-IR is a tetramer with extracellular ligand-binding and intracellular autophosphorylating tyrosine kinase domains which becomes activated with IGF-1 ligand binding. Activated receptor phosphorylates tyrosines within its kinase domain and tyrosines outside of the kinase domain, creating high-affinity docking sites for the binding of a number of intracellular signaling proteins that share highly conserved SH2 phosphotyrosine-binding domains, enabling the proteins that contain them to bind to activated IGF-IR tyrosine kinases. The IGF-IR and insulin receptor (IR) are highly homologous; however, the complexity of IGF signaling is further increased by the formation of the hybrid receptors that result from dimerization of IGF-IR and IR hemireceptors. This poses a significant challenge in studying receptor-specific signaling pattern; therefore, we have used IGF-IR-specific antibodies and IGF-IR-specific tyrosine phosphorylation array.

We elucidated the H_2O_2-induced IGF-IR tyrosine phosphorylation “fingerprint” in rat intestinal epithelial cells using IGF-IR tyrosine phosphorylation array. This array provided us with important insight into specific types of SH2 domain containing molecules to IGF-IR during oxidative stress. We observed nearly a 20-fold increase in phosphorylation levels of *p85α* and *p85β* regulatory subunits of PI3-K pathway during H_2O_2 stimulation of IGF-IR in RIE-1 cells, thus clearly demonstrating enhanced IGF-IR/PI3-K survival signaling in absence of a high affinity ligand, IGF-1, and with IGF-IR silencing. This response is supportive of our previous findings of survival signal activation during oxidative stress in intestinal epithelial cells *in vitro* and in neonatal mouse pup intestine *in vivo* [3-5]. Recruitment of SH2 domain-containing molecules responsible for integrin-mediated cell signaling and cytoskeletal alterations in shape and motility, intracellular calcium level regulation and activation of GTPase-dependent signaling molecules, including downstream effectors of IGF-IR such as oncogene *Src* and proto-oncogene *CrkL*, directly link IGF-IR to integrin-mediated signaling and cytoskeleton alterations. Furthermore, this array also demonstrates recruitment of *PLCγ* responsible for increasing intracellular calcium levels and indirectly activating second messenger IP_3/DAG signaling pathway.

Adaptor proteins recruited to phosphorylated IGF-IR tyrosine kinase domain during oxidative stress in RIE-1 cells included *GRB2* (*Growth factor receptor-bound protein 2*), *GRAP* (*GRB2* adaptor protein), and *NCK1/2* (*NCK* adaptor proteins 1 and 2) and are thought

to play an important role in activation of cell proliferation and survival signaling. Activation of *SHC2* (*SHC* transforming protein 2,*Sck*) molecule in particular demonstrated an important role it plays in recruitment of *GRB2*, leading to *Ras* (*Ras p21* protein activator) recruitment and activation of Raf-1/MEK/ERK pathway and downstream nuclear factors, and resulting in an induction of cell proliferation. *GRB2* protein is crucial in linking activated receptor tyrosine kinase to *Ras*, whereas *Shc* is known to compete with insulin receptor substrate (IRS)-1 for a limited cellular pool of *GRB2* and it represents the predominant mechanism in activation of the Ras/ERK signaling pathway in response to IGF-1 [10,23-25].

Dramatic IGR-IR staining of myenteric plexus in human NEC sections and *in vivo* is a novel finding that suggests a potential role for ENS in the pathogenesis of NEC. Enteric neuropathy has become an emerging central feature of many gut diseases, with specific focus on neuronal dysfunction. However, astrocyte-like enteric glial cells (EGC) are also thought to play an important role in pathogenesis of a wide range of gut disease, as they provide trophic and cytoprotective functions toward enteric neurons and may possibly regulate neuronal activity [16,26,27]. Future studies focusing on neuronal and EGC dysfunction and their potential contribution in the pathogenesis of NEC may help elucidate underlying signaling and regulatory mechanisms involving IGF-IR and other growth factor receptor activation during oxidative stress.

Oxidative stress plays an important role in cellular homeostasis and pathology. It exerts significant effect on various cellular components, organelles, membrane structures and signal transduction pathways, and contributes to the pathogenesis of various diseases, including ageing. In an environment of mild to severe oxidative stress, cellular adaptation may vary; however, once overwhelming, oxidative stress can lead to cellular death. Although the pathogenesis of NEC in premature neonates remains unclear, ROS are thought to be the product of hypoxia/ischemic injury to premature gut, leading to devastating outcome. Many growth-factor receptors have been shown to undergo enhanced phosphorylation in response to oxidant stimulation [28,29]. The mechanism involved in this effect is thought to be oxidant-mediated inactivation of target phosphatases required to “turn off” or dephosphorylate the growth-factor receptors [28]. Oxidants seem to stimulate growth-factor receptors, mimicking the action of natural ligands. Therefore, transactivation of growth-factor receptors via activation of receptor tyrosine kinases is thought to be ligand-independent. H_2O_2 either derived exogenously or produced endogenously after growth-factor stimulation, can reversibly inactivate the “turn off” switch protein-tyrosine phosphatase 1B in cells [29]. Although H_2O_2-induced transactivation of growth factor receptors, such as EGF and platelet-derived growth factor receptors, have been well described in fibroblasts, endothelial and cancer cells, mechanisms involved in oxidative stress-mediated EGF receptor and IGF-IR transactivation in intestinal epithelial cells are yet to be characterized [30,31].

CONCLUSIONS

Notable upregulation in intestinal IGF-IR expression during NEC and *in vitro* oxidative stress is an important indicator of upregulated downstream survival signaling in injured gut. Comprehensive approach aimed at understanding signaling mechanisms involved in

oxidative stress-mediated intestinal epithelial cell injury and activation of IGF-IR with subsequent recruitment of important SH2 domain containing proteins during NEC may provide necessary insight for development of effective therapeutic modalities targeting intestinal IGF-IR activation/upregulation in premature neonates with NEC.

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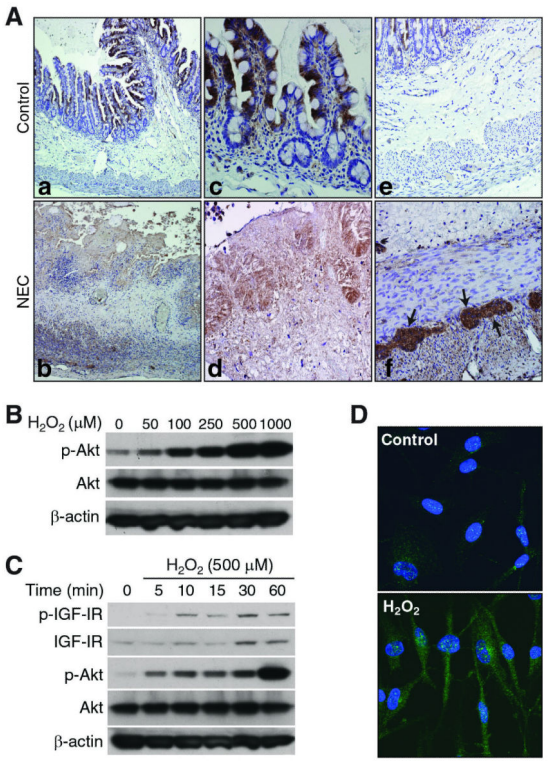

Fig 1. Intestinal IGF type 1 receptor (IGF-IR) expression in human NEC sections and in FHs74 Int cells

(A) Immunohistochemical analysis of human neonatal intestinal sections (control; non-inflammatory conditions) revealed predominantly mucosal localization for IGF-IR (*a*). During NEC, obliterated mucosal lining and diffuse transmural increase in IGF-IR expression was noted (*b*); significant increase in IGF-IR expression in submucosa and crypts was seen in NEC sections (*d*). In controls, the most intense staining was localized to intestinal epithelial cells (*c*); myenteric plexus revealed no IGF-IR localization *(e).* In contrast, intense staining of IGF-IR in NEC myenteric plexus was noted *(f; arrows).* **(B)** FHs74 Int cells were treated with H_2O_2 for 30 min, and protein was extracted for Western blot analysis. H_2O_2 treatment increased phosphorylation of Akt in dose-dependent fashion. **(C)** FHs74 Int cells were treated with H_2O_2 over a time course. H_2O_2 induced Akt phosphorylation at 5 min and this activation sustained till 60 min. IGF-IR phosphorylation occurred slightly delayed at 10 min after H_2O_2, whereas total IGF-IR protein levels peaked after 30 min. Equal β-actin and Akt levels indicate even loading. **(D)** FHs74 Int cells (1×10^4 cells/well) were treated with H_2O_2 (500 μM) for 3 h, and then incubated with IGF-IR antibody and Höechst nuclear stain (*blue fluorescence*). Cells were visualized using Zeiss LSM 510 UV Meta laser scanning confocal microscopy. H_2O_2 treatment induced significant increase in intracellular IGF-IR protein levels (*pseudo green fluorescence*).

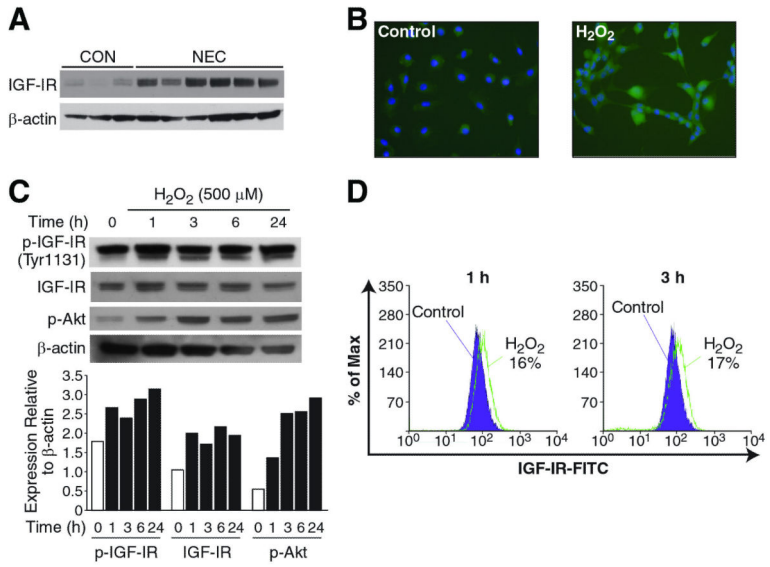

Fig 2. Intestinal IGF-IR expression *in vivo* and in RIE-1 cells

(A) Intestinal tissue protein from pups was analyzed for IGF-IR expression by Western blotting. NEC induced significant increase in IGF-IR expression levels when compared with control. **(B)** RIE-1 cells (1×10^4 cells/well) were treated with H_2O_2 (500 μM) for 3 h, and then incubated with IGF-IR antibody and Höechst nuclear staining. Immunofluorescent staining showed increased intracellular IGF-IR levels (*green fluorescence*), similar to those of FHs74 Int cells in Fig. 1D. **(C)** RIE-1 cells were treated with H_2O_2 (500 μM) over a time course, and protein was extracted for Western blot analysis. H_2O_2 induced Akt phosphorylation at 1 h; this effect was sustained throughout a time course. H_2O_2 induced a rapid increase in IGF-IR expression and receptor phosphorylation 1 h. **(D)** The density of cell surface IGF-IR after H_2O_2 treatments (500 μM) was measured using FACScan flow cytometry at 488 nm wavelength laser excitation at 1 and 3 h. RIE-1 cells were stained with fluorescein-labeled monoclonal IGF-IR antibody. Cell surface expression of IGF-IR increased at both time points after induction of oxidative stress by 16 and 17%, respectively (*green color*).

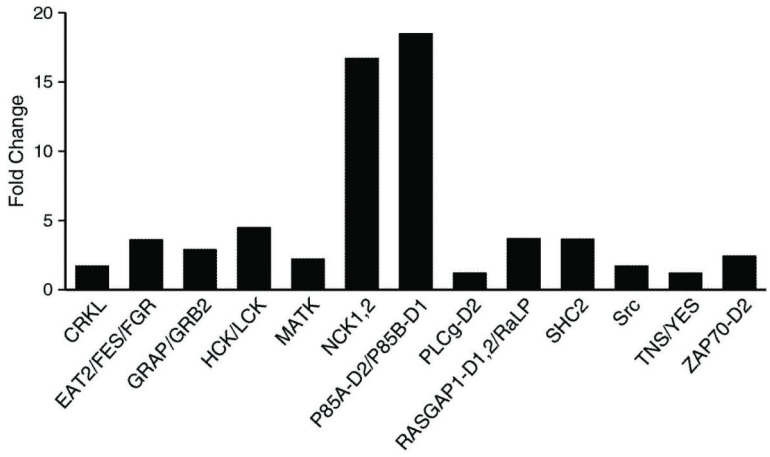

Fig 3. Oxidative stress induces IGF-IR tyrosine phosphorylation in RIE-1 cells

RIE-1 cells (1×10^6) were serum-starved overnight and treated with H_2O_2 (500 μM) for 3 h. Protein was harvested for IGF-IR tyrosine phosphorylation array and processed according to the manufacturer's instructions. Several key SH2 domain proteins involved in activation of IGF-IR phosphotyrosine signaling were detected in H_2O_2-treated RIE-1 cells. The several-fold increase in binding levels of SH2 domain proteins, including key intracellular signaling molecules, PI3-K subunits, oncogenes and various adaptor proteins, were analyzed relative to their respective controls using *ImageJ* densitometry software.

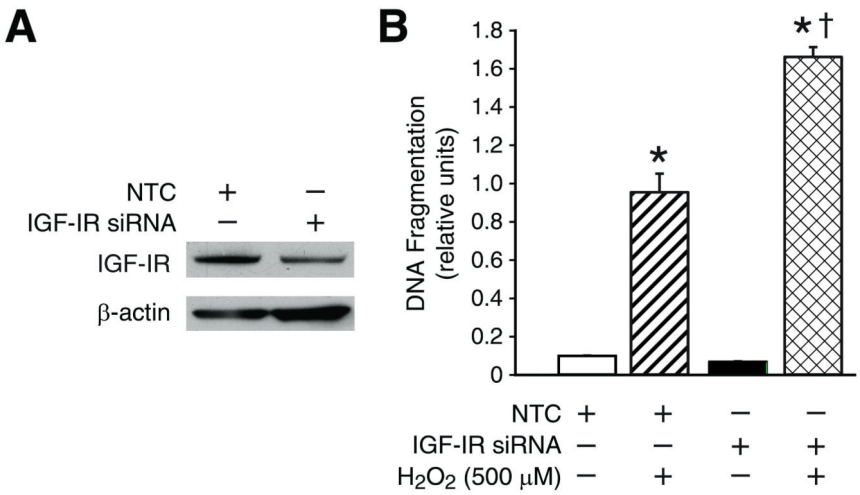

Fig 4. IGF-IR inhibition impairs cell survival in RIE-1 cell during oxidative stress

(A) RIE-1 cells (1×10^4) were transfected with non-targeting control (NTC) and IGF-IR siRNA, and grown for 72 h. Cells were lysed and IGF-IR protein was analyzed by Western blotting. IGF-IR silencing resulted in significant attenuation in receptor expression levels. Equal β-actin levels indicate even loading. **(B)** Transfected RIE-1 cells were treated with H_2O_2 and analyzed for cell death using DNA fragmentation ELISA kit. Oxidative injury induced significant cell death (*=p<0.05 vs. NTC siRNA) in RIE-1 cells. This effect was significantly enhanced with targeted IGF-IR silencing (†=p<0.05 vs. NTC siRNA+H_2O_2).