ANTIOXIDANTS & REDOX SIGNALING
Volume 4, Number 4, 2002

Original Research Communication

Heparin-Binding EGF-Like Growth Factor (HB-EGF) Decreases Oxygen Free Radical Production *In Vitro* and *In Vivo*

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ABSTRACT

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) has been shown to protect intestinal epithelial cells from anoxia/reoxygenation *in vitro*, and to protect the intestines from ischemia/reperfusion (I/R) injury *in vivo*. The goal of the present study was to determine whether the cytoprotective effects of HB-EGF were due, in part, to its ability to decrease reactive oxygen species (ROS) production. Human whole blood, polymorphonuclear leukocytes, and monocytes, as well as rat intestinal epithelial cells, were exposed to stimuli designed to produce an oxidative burst in these cells. Treatment of the cells with HB-EGF led to a significant decrease in oxidative burst production. *In vivo*, total midgut I/R injury in rats led to increased ROS production, which was markedly decreased by HB-EGF treatment. Histochemically, I/R injury led to increased ROS production, which was significantly decreased with HB-EGF treatment. HB-EGF cytoprotection is due, in part, to its ability to decrease ROS production. Future studies will determine the mechanisms by which HB-EGF exerts these effects. *Antioxid. Redox Signal.* 4, 639–646.

INTRODUCTION

THE GASTROINTESTINAL TRACT has been shown to be susceptible to many forms of injury and has gained recognition as a vulnerable shock organ possibly exacerbating the shock state through humoral activators such as endotoxins, lipid-derived products, and cytokines (19, 43). Ischemia/reperfusion (I/R) injury, an important form of intestinal injury, has been demonstrated to be a factor in the development of several disease states, including necrotizing enterocolitis, systemic inflammatory response syndrome, and multiple organ failure (10, 26). The intestinal mucosa is extremely sensitive to ischemia and subsequent reperfusion. One of the earliest manifestations of the mucosal damage produced by I/R is enhanced capillary permeability that results in tissue edema and fluid movement into the intestinal lumen. Morphological studies indicate that this injury is produced at the time of reperfusion rather than during the period of ischemia (43). The development of intestinal injury following I/R appears to be related to the production of inflammatory mediators such as nitric oxide (NO) (24, 46) and reactive oxygen species (ROS) (28). ROS [superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-)] are formed predominantly in the reperfusion period of I/R, consistent with the previously mentioned morphologic studies of mucosal damage. The site of ROS production in the gut is uncertain; likely candidates include leukocytes (4, 6) or endothelial cells (14), in addition to intestinal epithelial cells (IEC).

The gastrointestinal tract is well endowed with the enzymatic machinery necessary to form large amounts of ROS in postischemic tissues. The major sources of ROS are derived from mucosal xanthine oxidase (XO) (17) and leukocyte-derived NADPH oxidase (15). The subsequent reaction of NO with superoxide produces the potent oxidant peroxynitrite ($ONOO^-$) (8). $ONOO^-$ formation can lead to sequelae including enterocyte apoptosis as a result of changes in mitochondrial function with activation of the caspase cascade (16, 34, 48), as well as entorocyte necrosis, with eventual intestinal barrier

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failure. Ultimately, the occurrence of gut barrier failure and bacterial translocation allow bacteria normally contained in the gut to reach the systemic and portal circulations.

Growth factors can modulate the inflammatory response after intestinal injury (33). Transforming growth factor-α (TGF-α), interleukin-1β, interferon-γ, and epidermal growth factor (EGF) have been shown to be important factors for normal intestinal cell renewal and healing after injury (11). Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family, has been shown to play a role in the healing of various injured tissues, including kidney, liver, brain, and skin (23, 27, 41, 47). HB-EGF is a 22-kDa glycoprotein that was initially recognized as a product of cultured human macrophages (2, 20). As with other members of the EGF family, HB-EGF exerts its biological effects by binding to the *erb* B class of EGF receptors. HB-EGF differs from other members of the EGF family in its high affinity for heparin (3, 21), which potentiates the binding of HB-EGF to EGF receptors and modulates its biologic effects, including cell migration and proliferation (21). HB-EGF has been shown to be mitogenic for smooth muscle cells, fibroblasts, and epithelial cells (2, 31). We have previously demonstrated a role for HB-EGF in protection against intestinal injury. *In vitro*, we have shown that HB-EGF decreases inducible nitric oxide synthase (iNOS) and NO production in IEC (25), and protects IEC from hypoxic necrosis (38) and cytokine-induced apoptosis (29). *In vivo*, we have shown that HB-EGF preserves intestinal integrity and decreases mortality after I/R injury (39), in part, by its ability to decrease iNOS and NO production (49). In addition, HB-EGF preserves intestinal crypt cell proliferation and decreases bacterial translocation after intestinal I/R injury (50). The purpose of the present study was to address specifically the ability of HB-EGF to affect ROS production in cells indigenous to the gastrointestinal tract *in vitro* and in intestine *in vivo*, as evidence of an additional mechanism of HB-EGF-related cytoprotection.

MATERIALS AND METHODS

Materials

The HB-EGF used in all experiments was a highly purified form of recombinant mature human HB-EGF (corresponding to amino acids 74–148 of the HB-EGF precursor), which was produced in our laboratory using an *Escherichia coli* expression system (9). The BioRad Protein assay was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was from Molecular Probes (Eugene, OR, U.S.A.). Phorbol myristate acetate (PMA) was from GIBCO (Baltimore, MD, U.S.A.). The bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) was from Sigma (St. Louis, MO, U.S.A.). Rat intestinal epithelial (RIE) cells were a generous gift from Dr. Ken Brown (Cambridge University, U.K.).

In vitro *cell studies*

The effect of HB-EGF on ROS production *in vitro* was determined using human leukocytes and RIE cells as the target cells. ROS formation in leukocytes was studied both in whole blood, to minimize baseline stimulation of the cells, and in purified leukocyte subpopulations.

Whole blood. Human whole blood (2 ml) was diluted with 48 ml of erythrocyte lysing solution (155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, 0.1 m*M* EDTA) and mixed for 10 min at room temperature. Leukocytes were collected by centrifugation at 350 *g* for 10 min at 4°C and washed twice in phosphate-buffered saline (PBS) gel (0.2 m*M* EDTA, 0.5 m*M* dextrose, 1% gelatin, 95 ml of PBS). Cells were suspended in Hanks' balanced salt solution to a concentration of 2×10^6 cells/ml; some cells then received HB-EGF (100 ng/ml) for 30 min, and all cells were then loaded with DCF-DA (20 μ*M*) [which passively diffuses into cells and is oxidized by H_2O_2 to yield fluorescent DCF (40)] for 15 min in the dark at 37°C. Cells were stimulated with PMA (10^{-5} *M*) to induce an oxidative burst, after which analysis by gated flow cytometry every 10 min for a total of 40 min using a Coulter Epics EL Flow Cytometer with excitation of 488 nm and emission of 535 nm was begun. Baseline relative fluorescence readings are represented by the 0-min time point in cells that were not stimulated with PMA.

Purified leukocytes. Peripheral blood mononuclear cells (PBMC) and polymorphonuclear leukocytes (PMN) were isolated from human whole blood by layering onto a Histopaque-1077 and -1199 step gradient and centrifuging at 700 *g* for 30 min. PMN and PBMC were collected from different interphases of the gradient, and further purified using the MACS Magnetic Cell Separation System (30). PMN and PBMC were separately incubated with MACS magnetic MicroBeads conjugated to monoclonal antibodies (CD15 for PMN and CD14 for monocytes) for 15 min at 6°C, and loaded onto magnetic columns. The cell type of interest was retained by the column, and was recovered by washing upon removal of the magnet. Final cell populations were 99% and 96% pure for PMN and monocytes, respectively, as assessed by flow cytometry. The viability of these purified cell populations, as assessed by propidium iodide staining with flow cytometry, was 70–90%. Purified cells were plated at 1×10^6 cells/well, with some wells receiving HB-EGF (100 ng/ml) for 60 min at 37°C, and all wells were loaded with DCF-DA (20 μ*M*) for 15 min. PMN were then stimulated with PMA (10^{-5} *M*) and monocytes were stimulated with FMLP (10^{-3} *M*) to induce an oxidative burst, after which optical density (OD) was measured every 5 min using a Perkin Elmer HTS Bio Assay Reader spectrofluorometer with excitation of 492 nm and emission of 535 nm. Baseline OD readings are represented by the 0-min time point in cells that were not stimulated with PMA or FMLP.

IEC. RIE cells were plated at 1×10^5 cells/well, and 24 h later received TNF-α (100 ng/ml), with or without HB-EGF (100 ng/ml). After an additional 24 h, cells were loaded with DCF-DA (20 μ*M*) for 15 min and fluorescence was measured using a Perkin Elmer HTS Bio Assay Reader spectrofluorometer with excitation of 492 nm and emission of 535 nm.

Rat model of intestinal I/R injury

All animal experiments utilized a rat model of intestinal I/R involving 90 min of total superior mesenteric artery

(SMA) occlusion followed by reperfusion. Animal care and experimentation conformed to the standards listed in the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*. The experimental protocol was evaluated and approved by the Institutional Animal Care and Use Committee of Children's Hospital (protocol 01496AR). Adult, male Sprague–Dawley rats weighing 200–300 g were used. Animals were fasted for 12 h prior to operation, but were allowed water *ad libitum*. Anesthesia was induced and maintained with ketamine (70 mg/kg) and xylazine (6 mg/kg) given intraperitoneally. All operative procedures were performed aseptically, and body temperature was maintained at 37°C using a water-circulating heating pad. The rat abdomen was shaved, sterilized, and covered with sterile towels. Through a midline abdominal incision, the SMA was isolated using an operating microscope. Two atraumatic microvascular clamps were placed at the origin of the SMA from the aorta, and the midgut blood supply was completely occluded for 90 min. During ischemia, the entire intestine was replaced into the peritoneal cavity and the abdominal wall temporarily closed. Forty-five minutes after the onset of ischemia, the intestine was filled with either 5 ml of PBS (I/R group; $n = 10$) or 5 ml of PBS supplemented with 600 μg/kg of HB-EGF (I/R + HB-EGF group; $n = 10$) by intraluminal injection at three sites (proximal, middle, and distal parts of the small bowel). Six rats served as normal controls. After 90 min of ischemia, clamps were removed and the intestine was reperfused, as evidenced by return of arterial pulsations and color of the intestine. Rats were housed in individual cages postoperatively. Animals were killed after 4 h or 24 h of reperfusion by CO_2 asphyxiation, and intestinal segments were excised. Intestine harvested 4 h after reperfusion was analyzed for ROS production by luminol-enhanced chemiluminescence (LCL), and intestine harvested 24 h after reperfusion was analyzed for histochemical detection of ROS.

Detection of oxidative burst by DCF flow cytometry

Whole blood was analyzed for ROS production by gated flow cytometry every 10 min for a total of 40 min using a Coulter Epics EL Flow Cytometer with excitation of 488 nm and emission of 525 nm. In whole-blood studies, gating of cell populations allowed study of ROS production in red blood cells, lymphocytes, monocytes, and PMN.

Detection of oxidative burst by DCF spectrofluorometry

Purified PMN and monocytes, as well as RIE cells, were analyzed for ROS production by spectrofluorometry, with OD measured every 5 min using a Perkin Elmer HTS Bio Assay Reader spectrofluorometer with excitation of 492 nm and emission of 535 nm.

LCL

This technique is based on the affinity reaction of luminol with ROS whereby light is emitted (6). It has been used to measure ROS levels in biological samples including intestine (22). Superoxide anions, H_2O_2, hypochlorous acid, or hydroxyl radicals can initiate oxidative processes and generate chemiluminescence. LCL was used to measure ROS levels in RIE cells and in jejunal mucosa that was removed by scraping and immediately assayed after 4 h of the reperfusion phase of I/R injury. Jejunal mucosa was immediately placed in a scintillation vial containing 1 ml of preoxygenated Krebs–Henseleit bicarbonate buffer. After 15 min, 1 ml of the same buffer plus 200 μ*M* luminol was added. Samples were assayed by counting for 5 min using a liquid scintillation analyzer (Beckman Instruments LS7500, Beckman Instruments, Inc., Fullerton, CA, U.S.A.) operating in an out-of-coincidence mode. Protein content was determined by BioRad Protein assay, and results expressed as counts per minute per milligram of protein.

Histochemical detection of ROS

The histochemical technique used to detect ROS depends on a manganese-dependent oxidation of 3,3′-diaminobenzidine to an insoluble, osmiophilic polymer, resulting in the deposition of an electron-dense reaction product in ROS-positive cells, as described by Dijkstra *et al.* (12). Ileum from control rats ($n = 6$), I/R rats ($n = 10$), and I/R + HB-EGF-treated rats ($n = 10$) was harvested, snap-frozen in liquid nitrogen, and stored at −80°C. Cryostat sections were placed in washing buffer (0.1 *M* HEPES/1 m*M* azide/5% sucrose) for 5 min and incubation buffer (washing buffer plus 0.5 m*M* $MnCl_2$/1 mg/ml 3,3′-diaminobenzidine) for 10 min. Sections were washed in washing buffer, fixed in acetone, counterstained in hematoxylin, dehydrated in gradient ethanol, cleared in xylene, and examined by light microscopy. Control sections incubated in the absence of Mn^{2+} served as negative controls. ROS-positive cells located in mucosal and submucosal layers were counted. A total of 1,600 fields (from 32 tissue sections) were counted in control rats, 3,000 fields (from 60 tissue sections) were counted in I/R rats, and 2,400 fields (from 48 tissue sections) were counted in I/R + HB-EGF rats. Each slide was counted by two independent investigators, one of whom was "blinded" as to the origin of the tissues.

Statistical analyses

DCF fluorescence and LCL data were analyzed by paired *t* test performed on the SPSS 10.0 statistical program (SPSS, Inc., Chicago, IL, U.S.A.). Histochemistry data were analyzed using the Mann–Whitney test.

RESULTS

Ability of HB-EGF to decrease ROS production in leukocytes and IEC

Whole blood. When whole blood was stimulated with PMA to produce an oxidative burst, gated flow cytometry studies revealed oxidative burst production in PMN. HB-EGF significantly suppressed ROS production in these cells. After 40 min of PMA stimulation, relative fluorescence increased from 229 ± 88 nm to 555 ± 38 nm ($p < 0.001$, paired *t* test).

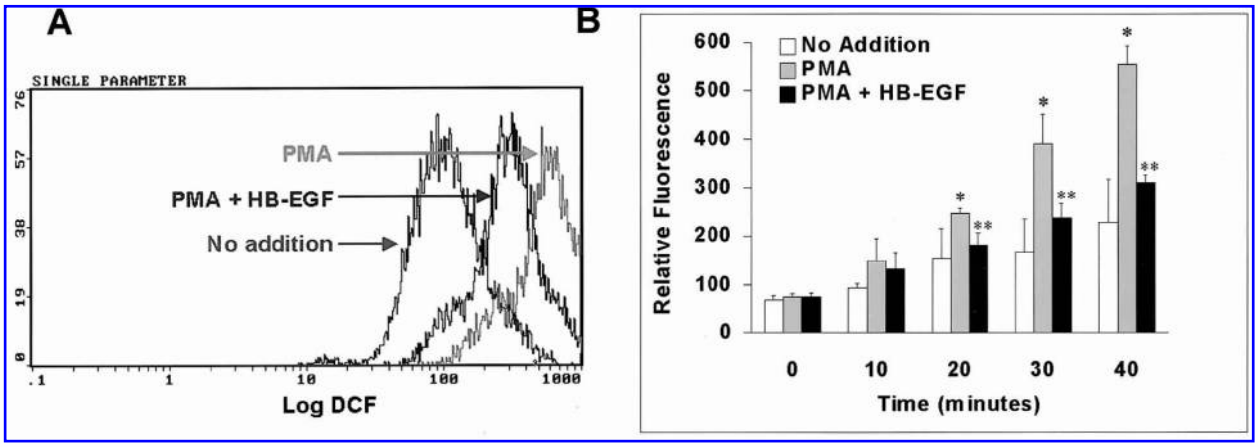

FIG. 1. Suppression of ROS production in human whole blood by HB-EGF. ROS production in whole blood stimulated with PMA was detected by DCF fluorescence as measured by flow cytometry. **(A)** A representative histogram of DCF fluorescence in gated PMN population of whole blood. **(B)** DCF fluorescence in whole blood, where each column represents the mean ± SD of three separate experiments, $n = 2$ for each experiment, $*p < 0.001$ compared with control; $**p < 0.001$ compared with PMA alone (paired t test).

Treatment with HB-EGF decreased relative fluorescence to 310 ± 17 nm ($p < 0.001$ compared with PMA-stimulated, non–HB-EGF-treated cells; paired t test) (Fig. 1). Red blood cells, lymphocytes, and monocytes did not produce a significant oxidative burst upon PMA stimulation in these studies (data not shown).

PMN and monocytes. Purified PMN stimulated with PMA, and purified monocytes stimulated with FMLP, demonstrated significant oxidative burst production. HB-EGF significantly suppressed ROS production in purified PMN and monocytes. For PMN, after 60 min of stimulation with PMA, mean OD increased from 982 ± 21 nm to 1,834 ± 86 nm ($p < 0.001$, paired t test). Treatment with HB-EGF decreased the mean OD to 1,111 ± 55 nm ($p < 0.001$ compared with PMA-stimulated, non–HB-EGF-treated cells; paired t test) (Fig. 2A). Similarly, for monocytes, after 60 min of stimulation with FMLP, mean OD increased from 1,014 ± 46 nm to 1,688 ± 212 nm ($p < 0.001$, paired t test). Treatment with HB-EGF decreased mean OD to 1,100 ± 55 nm ($p < 0.01$ compared with FMLP-stimulated, non–HB-EGF-treated cells; paired t test; Fig. 2B).

IEC. In general, IEC produced lower levels of ROS than leukocytes; however, ROS were produced upon stimulation of RIE cells with TNF-α for 24 h. HB-EGF completely eliminated ROS production in cytokine-stimulated RIE cells, decreasing the % OD from 155.5 ± 28.3 to 77.3 ± 41.0 ($p < 0.05$, paired t test) (Fig. 3). These results were reproduced when LCL was used to assess ROS production (data not shown). In addition to decreasing cytokine-stimulated ROS production in these cells, HB-EGF decreased baseline ROS production when the cells were grown in medium alone (Fig. 3).

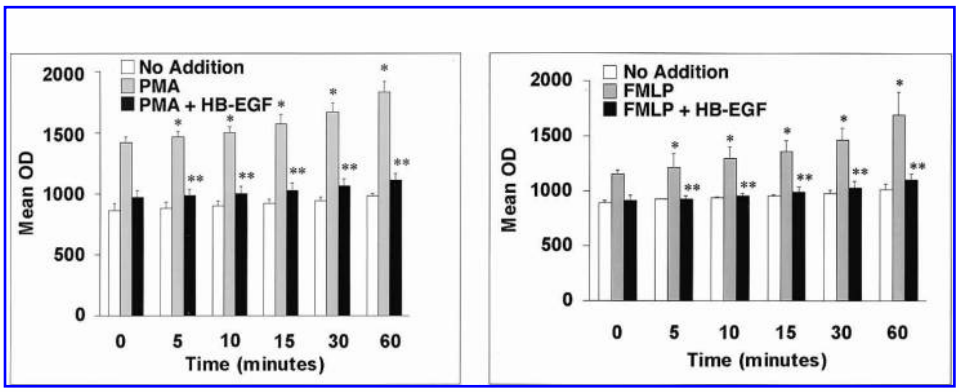

FIG. 2. Suppression of ROS production in purified leukocytes by HB-EGF. ROS production in purified leukocyte subpopulations was detected by DCF fluorescence as measured by spectrofluorometry. **(A)** Purified PMN stimulated with PMA, where each column represents the mean ± SD of four separate experiments, $n = 4$ for each experiment. $*p < 0.001$, compared with control; $**p < 0.001$, compared with PMA alone (paired t test). **(B)** Purified monocytes stimulated with FMLP, where each column represents the mean ± SD of four separate experiments, $n = 4$ for each experiment. $*p < 0.001$, compared with control; $**p < 0.001$, compared with FMLP alone (paired t test).

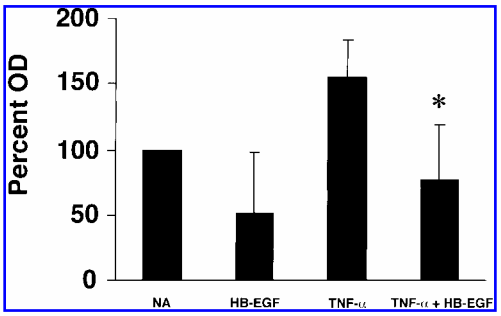

FIG. 3. Suppression of ROS production in RIE cells by HB-EGF. ROS production in RIE cells stimulated with TNF-α was detected by DCF fluorescence as measured by spectrofluorometry. Each column represents the mean ± SD of five separate experiments, $n = 2$ for each experiment. *$p < 0.001$, compared with TNF-α alone (paired t test). NA, no addition.

Ability of HB-EGF to decrease ROS production in injured intestine

LCL measurement of ROS. ROS production, quantified using a liquid scintillation counter, was increased in animals exposed to intestinal I/R injury compared with normal control animals (502,177 ± 80,563 counts/mg of protein vs. 65,371.79 ± 12,958.34; $p < 0.05$, paired t test). ROS production was decreased in animals exposed to I/R injury that were treated with HB-EGF compared with animals exposed to I/R injury alone (175,512 ± 93,004 counts/mg of protein vs. 502,177 ± 80,563 counts/mg of protein, $p < 0.05$) (Fig. 4). These results were reproduced in separate experiments when quantification was performed using a luminometer (data not shown).

Histochemical detection of ROS. Histochemical ROS detection revealed an increased number of ROS-positive cells in animals exposed to I/R injury (Fig. 5B) compared with normal control animals (Fig. 5A). In animals exposed to I/R injury that were treated with HB-EGF (Fig. 5C), significantly fewer ROS-positive cells were present compared with animals exposed to I/R injury alone. In normal intestine, ROS-positive cells are mainly located in the tips of the villi, the most metabolically active site. In I/R intestine, ROS-positive cells are prominent in villi, mucosa, submucosa, and even the underlying muscle layers. In I/R + HB-EGF intestine, ROS-positive cells are mainly located in the basal mucosal layer and submucosa, with many fewer ROS-positive cells in the villi.

Quantification of ROS-positive cells revealed 143 ± 12 cells/mm^2 in the normal control group, 423 ± 19.9 cells/mm^2 in the I/R group, and 203 ± 10.8 cells/mm^2 in the I/R + HB-EGF group ($p < 0.01$ for I/R vs. I/R + HB-EGF, Mann–Whitney test) (Fig. 5D). Control sections incubated in the absence of Mn^{2+} were completely negative (data not shown).

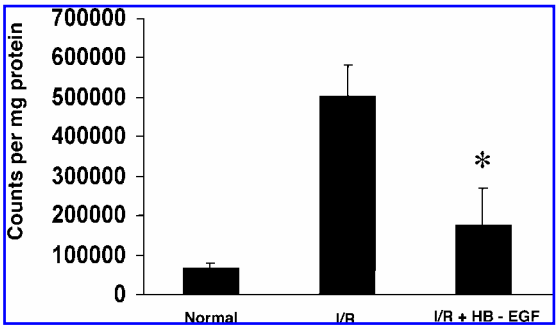

FIG. 4. LCL detection of ROS after intestinal I/R injury. ROS production in rat jejunum was detected by LCL as measured using a liquid scintillation counter. Each column is the average ± SD of two separate experiments, n = two for each experiment. *$p < 0.05$, compared with I/R.

DISCUSSION

The small intestine is recognized as an important organ in the progression of shock, and is a known source of cytokines, lipid-derived products, and endotoxin (19, 43). All of these products may potentiate the release of ROS with resultant tissue damage after I/R injury (42, 43, 45). Increased neutrophil infiltration occurs in ischemic intestinal mucosa (4), and this infiltration represents a rich source of free radicals in the ischemic small bowel. Activated neutrophils produce and release toxic oxygen metabolites, including superoxide, H_2O_2, hydroxy radical ($\cdot OH$) and singlet oxygen ($\cdot O_2$) (35). There is a large body of evidence to suggest that reactive oxygen metabolites (O_2^-, H_2O_2, $\cdot OH$) mediate the increased microvascular permeability and mucosal ulceration produced by reperfusion of ichemic intestine (18, 36, 37). Recent work indicates that neutrophil infiltration into the ischemic small bowel is initiated by XO-derived reactive oxygen metabolites (17, 35). XO may contribute to host defense by triggering a microvascular inflammatory response, leading to recruitment of neutrophils (44).

It is known that the intestinal mucosa is sensitive to I/R injury (28) and that growth factors contribute to the proliferation and repair processes of the small intestine. HB-EGF is a growth factor that is produced by IEC and is a potent mitogen and chemotactic factor for these cells. *In vitro*, HB-EGF mRNA levels are rapidly induced by a variety of stimuli, including oxidative stress (32). The effect of oxidative stress on HB-EGF expression was investigated in rat gastric mucosal RGM1 cells (32). In response to stimulation with H_2O_2, HB-EGF gene expression was immediately increased and could be blocked by antioxidants. The immediate up-regulation of HB-EGF mRNA in response to oxidative stress suggests that HB-EGF may be involved in repair following oxidative injury. *In vivo*, HB-EGF mRNA is rapidly up-regulated in response to tissue injury, including wounding and ischemia (7). HB-EGF is an immediate early gene that plays a pivotal role in mediating the earliest cellular responses to proliferative stimuli and cellular injury (5).

We have previously demonstrated that HB-EGF decreases iNOS and NO production in IEC (25), and protects IEC from necrosis and apoptosis *in vitro* (29, 38). In IEC exposed to hypoxia, HB-EGF restores intracellular ATP levels and preserves cytoskeletal integrity (38). *In vivo*, HB-EGF has been demonstrated to preserve intestinal integrity and decrease

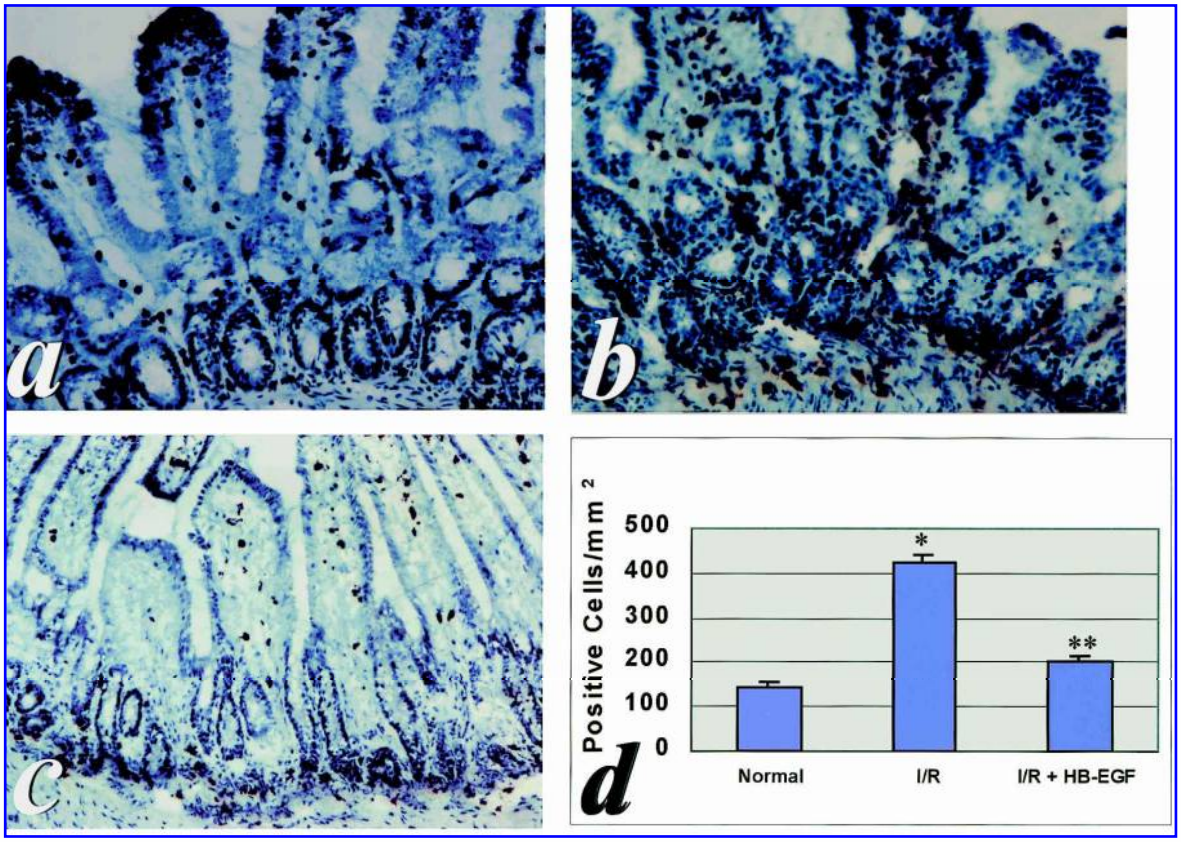

FIG. 5. Histochemical detection of ROS after intestinal I/R injury. **(a)** Normal rat. **(b)** I/R rat. **(c)** I/R + HB-EGF-treated rat. **(d)** Quantitative assessment of ROS-positive cells per mm^2. *$p < 0.01$, compared with control; **$p < 0.01$, compared with I/R. (Mann–Whitney test).

mortality in a rat model of segmental intestinal I/R injury (39). These beneficial effects of HB-EGF *in vivo* were also associated with decreased iNOS, NO, and $ONOO^-$ production (49). In subsequent studies, we have also demonstrated that HB-EGF decreases selectin and integrin expression in intestinal endothelia after intestinal I/R injury, and decreases neutrophil infiltration after intestinal I/R injury (51). Furthermore, we have demonstrated that HB-EGF preserves mucosal integrity after intestinal I/R injury in part by increasing crypt cell proliferation, and that it also decreases bacterial translocation after intestinal I/R injury (50).

We now demonstrate for the first time that HB-EGF can decrease ROS production in cells indigenous to the intestinal mucosa and in I/R-injured rat intestine. Importantly, HB-EGF is able to decrease ROS production in stimulated leukocytes, a rich source of ROS during intestinal I/R injury, as well as in IEC themselves. This illustrates an important additional mechanism by which HB-EGF may protect the intestine after I/R injury. Future studies will address the mechanisms by which HB-EGF decreases ROS production. The current findings support the rationale for further studies of the effects of HB-EGF on ROS-mediated injury pathways.

Many critically ill patients, both adult and pediatric, are susceptible to intestinal I/R injury. We have accumulated numerous lines of evidence, both *in vitro* and *in vivo*, supporting an intestinal cytoprotective role for HB-EGF. Our long-range goal is the use of HB-EGF, both therapeutically and prophylactically, to treat patients with or at risk of developing intestinal I/R injury.

Perspectives

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that participates as an early response factor in circumstances of environmental stress for the cell, including the oxidative stress known to follow I/R episodes (52). A wide variety of agents are able to activate NF-κB, including ROS (1) and $ONOO^-$ (13). This results in the induction of many cellular genes that determine cell fate, including acute phase and inflammatory mediators such as iNOS, adhesion molecules, and cytokines. Although the focus of a separate manuscript, we have recently demonstrated that HB-EGF decreases NF-κB activation by inhibiting IκB phosphorylation and degradation, thus interfering with nuclear translocation of NF-κB and NF-κB–DNA binding. Our data suggest that HB-EGF uses the mitogen-activated protein kinase pathway to inhibit NF-κB activation. Future studies will further explore the cellular signal transduction pathways utilized by HB-EGF.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (GM50905), the Children's Hospital Re-

search Foundation (292699), the Ohio State University Medical Research Development Fund (G.E.B.), and the E. Thomas Boles Pediatric Surgery Fellowship (G.X.).

ABBREVIATIONS

DCF-DA, dichlorofluorescin diacetate; EGF, epidermal growth factor; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HB-EGF, heparin-binding EGF-like growth factor; H_2O_2, hydrogen peroxide; IEC, intestinal epithelial cells; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; LCL, luminol-enhanced chemiluminescence; NF-κB, nuclear factor-κB; NO, nitric oxide; OD, optical density; $ONOO^-$, peroxynitrite; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; RIE, rat intestinal epithelial cells; ROS, reactive oxygen species; SMA, superior mesenteric artery; TNF-α, tumor necrosis factor-α; XO, xanthine oxidase.

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Received for publication January 18, 2002; accepted April 27, 2002.

This article has been cited by:

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