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Original Research Communication

Heparin-Binding EGF-Like Growth Factor Decreases Inducible Nitric Oxide Synthase and Nitric Oxide Production After Intestinal Ischemia/Reperfusion Injury

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ABSTRACT

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) has been shown to protect intestine from ischemia/reperfusion (I/R) injury *in vivo* and to down-regulate inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production in intestinal epithelial cells *in vitro*. The present study was undertaken to investigate whether HB-EGF could modulate the iNOS/NO axis after total midgut I/R injury in rats. I/R injury induced a significant increase in iNOS gene expression (quantified by real-time RT-PCR) and protein production (detected by western blots), as well as elevation of serum NO levels (measured by chemiluminescence assay). Nitrotyrosine (NT) and iNOS production colocalized immunohistochemically, with positive staining found mainly in villous and crypt epithelial cells, as well as ganglion cells. Intraluminal administration of HB-EGF 45 min after the start of a 90-min ischemic interval significantly decreased I/R-induced iNOS gene expression and protein production, as well as serum NO levels. Immunohistochemically, HB-EGF administration led to elimination of iNOS and NT staining in crypt epithelial cells and ganglion cells, with only weak staining that remained in villous epithelial cells. Thus, HB-EGF protects the intestine from I/R injury, at least partially, through down-regulation of the iNOS/NO/NT pathway, a mechanism that is central to I/R injury in multiple organ systems. Antioxid. Redox Signal. 5, 919–930.

INTRODUCTION

INTESTINAL ISCHEMIA/REPERFUSION (I/R) injury contributes to the pathophysiology of various clinical conditions, including shock, sepsis, neonatal necrotizing enterocolitis, volvulus, and intestinal transplantation (42). Importantly, I/R is not only injurious to the intestine itself, but also to remote organs, including the lungs. Intestinal injury can lead to breakdown of gut barrier function, translocation of enteric bacteria, and/or absorption of endotoxin, followed by systemic sepsis and even multiple-system organ failure (22). Thus, high morbidity and mortality are associated with this injury. Although the mechanism of I/R is not completely understood, several pathogenic factors have been implicated in its development, including the overproduction of reactive nitrogen and oxygen species, the expression of adhesion molecules in vascular endothelia, neutrophil infiltration into the wounded tissue, and the expression of proinflammatory cytokines (22, 42).

The development of intestinal injury following I/R appears to involve overproduction of

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nitric oxide (NO; 21, 39). NO, one of the smallest biologically active molecules, is produced as a by-product of the conversion of arginine to citrulline. Its production is regulated by three isoforms of nitric oxide synthase (NOS), all of which [neural, endothelial and inducible (iNOS) NOS] are present in the gastrointestinal tract (36). NO plays an essential role in normal intestinal function, serving as a signal transduction factor (generated by the endothelial isoform) and a neurotransmitter (generated by the neural isoform). However, dramatic upregulation of iNOS is induced by inflammatory cytokines during intestinal injury, leading to a very rapid and quantitatively massive generation of NO, which participates in the generation of tissue injury.

Although a weak free radical by itself, NO is quickly oxidized to a series of potent reactive nitrogen species such as peroxynitrite ($ONOO^-$), NO_2, N_2O_3, NO_2^+, NO^+, and NO^- (2, 7). These highly reactive nitrogen oxides play important roles in host defense, including the killing of microbes and tumor cells; however, they can also modify the structure and function of various biological molecules through reactions such as nitration, oxidation, and nitrosation, contributing to cellular injury in pathological conditions (2, 7, 28, 36). Peroxynitrite, formed by the reaction of NO with superoxide (7), can nitrate mitochondrial proteins and inhibit cellular respiration (31). The resultant changes in mitochondrial function can lead to activation of the caspase cascade followed by enterocyte apoptosis (13), or can lead to cellular necrosis (41). This may lead to gut barrier failure (19) followed by multiple-system organ failure. There is evidence that I/R injury is associated with increased iNOS and sustained NO production in several organs, including heart, brain, muscle, and pancreas. Similar pathways are likely to be involved in intestinal I/R injury, as supported by a recent report of resistance to I/R-induced bacterial translocation and mucosal injury in iNOS knockout mice (38).

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a member of the EGF family that was first recognized as a secreted product of cultured human macrophages (4) and later recognized to be a member of the EGF family of growth factors (16). It is a 22-kDa glycoprotein that is produced in a membrane-anchored precursor form that is processed to a soluble, secreted mature form (sHB-EGF). HB-EGF exerts its biological effects by binding to cell-surface EGF receptor type 1 and type 4 (3, 11). In addition, HB-EGF binds strongly to heparin and elutes from heparin-affinity chromatography columns with 1.0 *M* NaCl (4, 5). Heparin potentiates binding of HB-EGF to the EGF receptor (1, 17) and also modulates its biologic effects on target cells, including cell migration and proliferation (17). HB-EGF is mitogenic for smooth muscle cells, fibroblasts, and epithelial cells (4, 15, 16) and is produced by many different cell types, including epithelial cells, for which it acts as an autocrine growth factor (15).

In vitro, HB-EGF mRNA levels are rapidly induced by a variety of stimuli, including oxidative stress in rat gastric epithelial cells (29) and scrape-wounding of rat intestinal epithelial cells (12). *In vivo*, HB-EGF mRNA is rapidly upregulated in response to tissue injury, including wounding or ischemia of the kidney (18, 35), brain (40), liver (20), and skin (26). HB-EGF is an immediate early gene that plays a pivotal role in mediating the earliest cellular responses to proliferative stimuli and cellular injury (6).

We have previously shown that HB-EGF protects intestine from severe histologic damage and decreases mortality associated with segmental intestinal I/R injury in rats (34). *In vitro*, we have shown that treatment of intestinal epithelial cells (IEC) with exogenous HB-EGF protects cells from hypoxic necrosis (33) and from proinflammatory cytokine-induced apoptosis (27). Further examination of the mechanisms by which HB-EGF protects IEC from injury *in vitro* revealed that HB-EGF suppresses cytokine-induced iNOS and NO production (24). Our hypothesis is that HB-EGF may mediate its potent cytoprotective effects during I/R injury to different tissues by down-regulating iNOS and NO production. We report here for the first time that human HB-EGF down-regulates intestinal I/R-induced iNOS and NO production in rats, and discuss the implications of these findings.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS) and diaminobenzidine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Histochoice was from Amresco (Solon, OH, U.S.A.). Trizol reagent and the SuperScript First-Strand Synthesis System were from Life Technologies (Grand Island, NY, U.S.A.). Rabbit anti-iNOS antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit anti-nitrotyrosine (NT) antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Biotinylated BioGenex Multilink* antibody was from BioGenex (San Ramo, CA, U.S.A.). Alkaline phosphatase anti-rabbit IgG was from Vector Laboratories (Burlingame, CA, U.S.A.). 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium was from Promega Co. (Madison, WI, U.S.A.).

Animals

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 experimental protocol was approved by the Institutional Animal Care and Use Committee of the Children's Research Institute (Protocol 01496AR) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHHS publication 85-234).

Recombinant human HB-EGF

Human mature HB-EGF, corresponding to the processed secreted protein sHB-EGF, was produced in *E. coli* using the maltose-binding protein fusion system (8). In brief, human HB-EGF cDNA corresponding to amino acids 74–148 of the HB-EGF precursor molecule was cloned into plasmid pMAL-c2, and recombinant plasmid was then transformed into *E. coli* BL21. After expression of the fusion protein, bacteria were lysed with a French press. Maltose-binding fusion protein was purified by amylose column chromatography and enzymatically cleaved into HB-EGF and maltose-binding protein using factor Xa. HB-EGF was further purified using heparin-affinity FPLC, as well as reverse-phase HPLC, and dried through a refrigerated condensation trap system. Amino acid sequencing, western blotting, and functional assays were used to identify the purity and function of recombinant HB-EGF. The highly purified and fully functional HB-EGF was stored at −20°C until use.

Total midgut I/R injury

After induction of anesthesia, the rat abdomen was shaved, sterilized, and covered with sterile towels. Through a midline abdominal incision, the superior mesenteric artery was isolated using an operating microscope. Two atraumatic microvascular clamps were then placed at the origin of the superior mesenteric artery from the aorta, and the midgut blood supply was completely occluded as evidenced by macroscopic examination (loss of vessel pulsation and pallor of the intestine) and confirmed by vital dye injection in selected animals. The range of ischemia included ~1 cm of distal duodenum, the entire jejunum, ileum, and cecum, and 2–3 cm of proximal ascending colon. Total ischemia time was 90 min. During ischemia, the entire intestine was replaced into the peritoneal cavity and the abdominal wall temporary closed. Forty-five minutes after the onset of ischemia, the intestine was filled with either 5 ml of phosphate-buffered saline (PBS) (I/R group) or 5 ml of PBS supplemented with 600 μg/kg HB-EGF (I/R + HB-EGF group) by intraluminal injection at three sites (proximal, middle, and distal parts of small bowel). 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. The abdominal wall was closed with two layers of sutures. Animals were kept warm until fully awake and housed in individual cages. Sham-operated rats served as controls, and received all operative procedures except for occlusion of the superior mesenteric artery.

All surgical procedures were performed aseptically, and no antibiotic was administered. Except for saline-moistened gauze, which was used to wrap the intestine during exteriorization, saline resuscitation was not used during or after operation.

Experimental protocol

A total of 54 rats were used and randomized into four groups: I/R group ($n = 20$), I/R + HB-EGF group ($n = 16$), sham-operation group ($n = 8$), and naive normal control group ($n = 10$). Six additional rats were injected intraperitoneally with LPS and killed for examination at 6 h after injection. For plasma NO analysis, heparinized whole blood (0.5 ml) was withdrawn from direct heart puncture at different time points from the same sham-operated ($n = 5$), I/R-injured ($n = 8$), and I/R + HB-EGF treated rats ($n = 6$). Blood was sampled prior to operation (−1.5 h), at the end of ischemia, prior to reperfusion (0 h), and after reperfusion (+2, +4, +8, +12, and +24 h). Whole blood samples were quickly centrifuged, and plasma was collected and stored at −20°C. Twenty-four hours after I/R, animals were killed for examination. Segments of proximal, middle, and distal ileum (1.5–2 cm in length) were harvested, flushed with Histochoice tissue fixative to remove feces, fixed in Histochoice at room temperature for 12 h, and then transferred to 70% ethanol and stored at 4°C until embedding. Additional tissue segments were flushed with cold PBS, rinsed into Trizol reagent, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Measurement of nitrate/nitrite in the plasma

NO is very short-lived and is rapidly oxidized to nitrate and nitrite in the plasma, making it impossible to measure directly the level of NO present in the plasma. However, nitrate/nitrite levels can be measured by reducing them to NO using 0.1 *M* vanadium chloride and 1.0 *M* HCl at 95°C. NO generated by reduction of nitrate and nitrite ions was delivered by vacuum to a Sievers NO analyzer (Sievers Instruments, Boulder, CO, U.S.A.), where it was reacted with analyzer-generated ozone in a chemiluminescent reaction that was detected in a photomultiplier tube in the analyzer. Based on this principle, a standard curve was first established by running pure samples of nitrate ion, and then unknown plasma samples (2 μl of a 1:1 dilution of each sample) were injected in duplicate into the analyzer. NO levels were calculated based on a least squares method and expressed as micromolar.

Analysis of iNOS mRNA by real-time reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated from whole intestine using Trizol reagent, and cDNA was generated by reverse transcription of 1–5 μg of total RNA using the SuperScript First-Strand Synthesis System for RT-PCR with random hexamer primers. The cDNA samples were used as a template for real-time PCR. Real-time PCR was performed using rat iNOS-specific primers and probes that we designed using the published rat iNOS mRNA sequence (accession no. NM-012611). Ribosomal RNA (rRNA) primers and probe were obtained from published sequences from a PE Applied Biosystems protocol (TaqMan Ribosomal RNA control reagents bulletin). rRNA was measured in the same samples as the iNOS gene as an internal control, and was used to ensure the quality of the RNA preparation, to account for the efficiency of reverse transcription, and to control for any loading variation of the initial amount of cDNA. Reactions were performed in duplicate wells in a MicroAmp Optical 96-well reaction plate (PE Applied Biosystems, Foster City, CA, U.S.A.) using 900 n*M* iNOS forward primers (5′-AGCGGCTCCATGACTCTCTCA-3′) and reverse primers (5′-TGCACCCAAACACCAAGGT-3′), 200 n*M* FAM (6-carboxyfluorescein) labeled iNOS probe (ATGCGGCCTCCTTTGAGCCCTCT), 50 n*M* concentration of the internal control forward and reverse primers and labeled probe for rRNA, and 2× PCR Master Mix [8% glycerol, 1× TaqMan buffer A, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 0.05 U/μl AmpErase uracil *N*-glycosylase, 5 m*M* $MgCl_2$, 0.01 U/μl

Gold AmpliTaq DNA polymerase (PE Applied Biosystems)]. PCR amplification of iNOS and rRNA was performed in the same reaction. The reaction was performed using the following amplification scheme: one cycle of 2 min at 50°C (AmpErase UNG activation); one cycle of 10 min at 95°C (activation of Gold AmpliTaq, and inactivation of AmpErase UNG); followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C.

All reactions were carried out using a 7700 Sequence Detector thermocycler (PE Applied Biosystems) linked to a Macintosh computer using the Sequence Detector software (PE Applied Biosystems) to run the PCR reaction. The PCR cycle at which the reporter fluorescent dye was cleaved from the target gene (iNOS or rRNA) to generate a detectable specific fluorescent signal that reaches a fixed threshold was defined at C_T (threshold cycle). The larger the starting cDNA copy number, the earlier the specific signal is detected and the lower the C_T value (see Fig. 2A and B). To perform the relative quantification of each duplicate sample, the iNOS PCR product expressed as the C_T value was normalized to the C_T of rRNA. To perform the relative quantitative analysis among samples, a calibrator (normal rat intestine) was used for comparison. The relative amount of target mRNA in the experimental samples was expressed as folds of change relative to the calibrator sample.

Detection of iNOS by western blotting

Protein was extracted from Trizol lysates of rat intestine after removal of RNA, quantified using the Laemmli method, and 10 μg of protein was loaded into each lane of a 10% polyacrylamide gel for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoresed and transferred to a nitrocellulose membrane. Blots were blocked with Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) for 30 min followed by overnight incubation with a 1:10,000 dilution of rabbit anti-iNOS antibody. Blots were then washed in TBS with 0.1% Tween 20 and incubated with a 1:1,000 dilution of alkaline phosphatase anti-rabbit IgG for 60 min. Blots were washed again and incubated with the substrates 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 20 min. Blots were then washed in 0.5 *M* EDTA/TBS, dried, and scanned for analysis.

Immunohistochemical localization of iNOS and NT

Ileal specimens were dehydrated, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin for standard microscopic evaluation. For immunohistochemistry, paraffin sections were deparaffinized in xylene and rehydrated through graded ethanols to distilled water. All incubations below were performed at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2/PBS for 15 min. Nonspecific binding was blocked by incubation in 1% BSA for 1 h, followed by 5% normal goat serum in 2% BSA/PBS for 30 min. Sections were then incubated separately in either rabbit anti-iNOS antibody (4 μg/ml) or rabbit anti-NT antibody (2 μg/ml) diluted in 2% BSA for 1 h. Sections were incubated in purified rabbit IgG (4 μg/ml for iNOS staining or 2 μg/ml for NT staining) as a negative control. Sections were washed three times in PBS prior to incubation with biotinylated BioGenex Multilink* antibody diluted 1:100 in 2% BSA for 20 min. After washing twice in PBS, BioGenex Label (biotin-streptavidin) diluted 1:100 in 2% BSA was added with incubation for 20 min. Free label was washed away, and slides were incubated with freshly prepared diaminobenzidine chromogen for 2–5 min. Finally, sections were counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, mounted in Permount* medium, and imaged using a digital camera. The percentage of immunoreactive crypts was calculated as a ratio of positively stained crypts to total crypts, counted microscopically.

Statistical analyses

Data were analyzed using Mann-Whitney or Student's *t* test to determine significant differences between groups.

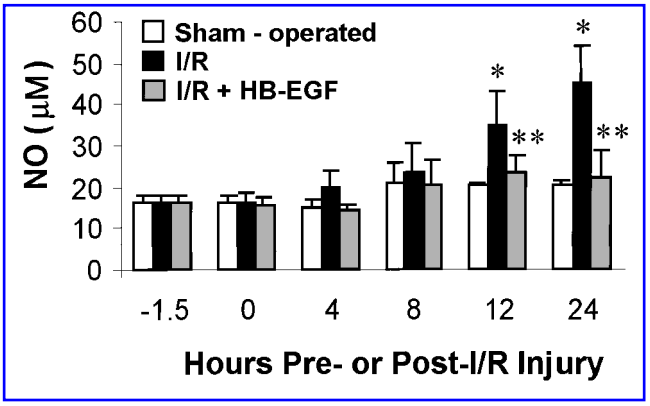

FIG. 1. Effect of HB-EGF on serum nitrate/nitrite levels after intestinal I/R injury. Serum nitrate/nitrite levels were measured in sham-operated ($n = 5$), I/R-injured ($n = 8$), and I/R + HB-EGF treated rats ($n = 6$). I/R-injured rats had significantly elevated levels of NO at 12 and 24 h after injury compared with sham-operated rats (*$p < 0.01$, Mann–Whitney test). HB-EGF treatment of I/R-injured rats completely eliminated elevation of serum NO levels (**$p < 0.01$, Mann–Whitney test).

RESULTS

HB-EGF prevented elevation of plasma nitrate/nitrite concentration after I/R injury

Normal naive rats had low baseline levels of NO (16.1 ± 2 μM) that were unaffected by sham operation (20.1 ± 1.3 μM) (Fig. 1). Animals subjected to intestinal I/R injury had significant elevation of plasma NO levels at 12 and 24 h (35 ± 8.1 and 45 ± 9 μM, respectively) after injury. Intraluminal administration of HB-EGF after 45 min of ischemia completely eliminated the elevation of serum NO levels seen in non–HB-EGF-treated rats, with NO levels at 12 and 24 h (23.5 ± 4.4 and 22.3 ± 6.5 μM, respectively) equivalent to those in sham-operated rats.

HB-EGF down-regulated transcription of iNOS mRNA after I/R injury

The highly quantitative technique of real-time PCR was used to determine whether HB-EGF was able to modify I/R-induced iNOS transcription. Figure 2A shows a representative amplification plot of real-time RT-PCR products, depicting the difference in iNOS mRNA expression in normal, I/R-injured, and I/R-injured, HB-EGF-treated rats. Figure 2B shows rRNA amplification in the same samples as Fig. 2A (internal control). Figure 2C shows the fold increase in iNOS expression relative to a calibrator (normal uninjured rat intestine). I/R injury induced a 12.5 ± 4.3-fold increase in iNOS mRNA expression relative to the calibrator. There were no significant differences in iNOS mRNA expression between normal and sham-operated rats (data not shown), indicating that sham operation itself was not able to induce iNOS mRNA transcription. In I/R-injured rats that were treated with HB-EGF, iNOS mRNA expression was significantly decreased by 2.4-fold (a 58.3% reduction) compared with I/R-injured, non–HB-EGF-treated rats.

HB-EGF blocked translation of iNOS protein after I/R injury

As demonstrated by western blotting, iNOS protein was not detectable in intestinal samples from normal or sham-operated rats (Fig. 3). I/R injury resulted in significantly increased iNOS protein production, similar to that observed in LPS-stimulated rats. LPS is a well known iNOS inducer and was given by intraperitoneal injection 6 h prior to tissue harvesting (positive control). In animals that were exposed to I/R injury that were treated with HB-EGF, there was no detectable iNOS protein, indicating that HB-EGF efficiently blocked I/R-induced iNOS translation.

HB-EGF modified production and distribution of iNOS after I/R injury

In normal rat ileum, there was no iNOS staining in villous or crypt epithelial cells, or in the ganglion cells present in Meissner's and Auerbach's plexuses, whereas scattered inflammatory cells in the lamina propria showed weak reactivity for iNOS (Fig. 4A and B). Intestine harvested from sham-operated rats showed the same staining pattern as normal intestine. I/R injury caused villous structural damage, accompanied by strong reactivity for iNOS in villous epithelial cells and inflammatory cells located in the lamina propria. Moreover, ganglion cells and $91 \pm 4\%$ of crypts showed strong reactivity for iNOS (Fig. 4C and D). In contrast, intestine from animals that underwent I/R injury and were treated with HB-EGF

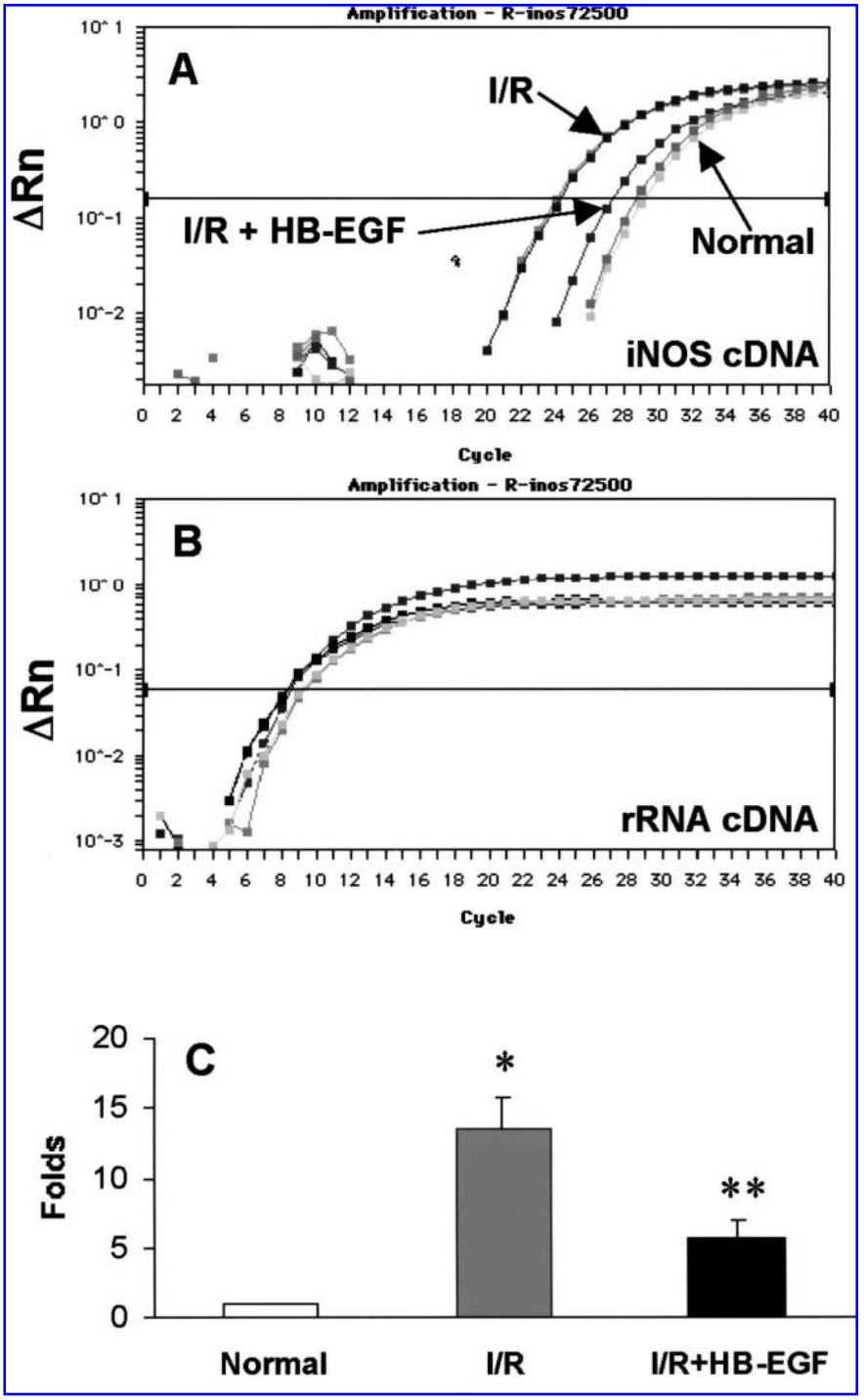

FIG. 2. Effect of HB-EGF on iNOS mRNA expression after intestinal I/R injury. (**A**) Representative amplification plot depicting the difference in intestinal iNOS expression in normal rats, I/R-injured rats, and I/R-injured, HB-EGF-treated rats. (**B**) rRNA amplification in the same samples (internal control). (**C**) Fold increase in iNOS expression in I/R-injured animals treated with PBS ($n = 5$) or HB-EGF ($n = 4$) relative to a calibrator (normal rat intestine, $n = 3$). Each column shows the mean ± SEM. There was significantly increased expression of iNOS in I/R-injured intestine compared with normal intestine (*$p < 0.01$, Student's t test). HB-EGF treatment of I/R-injured rats significantly decreased I/R-induced iNOS expression (**$p < 0.05$, Student's t test).

showed only faint reactivity for iNOS in villous epithelial cells with no crypt or ganglion cells that stained positively for iNOS (Fig. 4E and F). Sections that were stained with normal rabbit IgG in place of the primary antibody demonstrated no iNOS staining in any of the sections (data not shown).

HB-EGF blocked formation of NT after I/R injury

Immunoreactivity of NT colocalized with that of iNOS. In normal ileum, no staining for NT was observed (Fig. 5A and B). This was also true in intestine from sham-operated rats. I/R injury induced destruction of villous structure

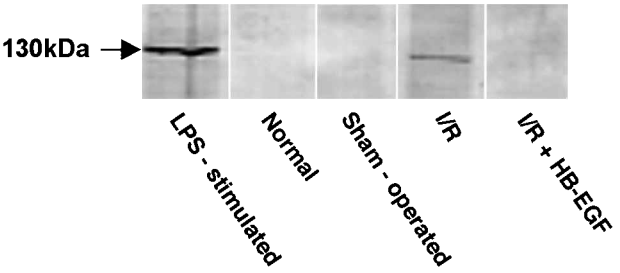

FIG. 3. Effect of HB-EGF on iNOS protein production after intestinal I/R injury. Proteins were extracted from intestine and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting. Lane 1, intestine from LPS-stimulated rats (positive control); lane 2, intestine from normal rats (negative control); lane 3, intestine from sham-operated rats; lane 4, intestine from I/R-injured rats; lane 5, intestine from I/R-injured, HB-EGF-treated rats. A 130-kDa iNOS band was found in intestine from rats subjected to LPS stimulation or I/R injury. HB-EGF treatment of I/R-injured rats resulted in complete elimination of detectable iNOS protein.

and massive production of NT. The reactivity for NT was observed mainly in villous and crypt epithelial cells, as well as in ganglion cells (Fig. 5C and D). HB-EGF completely prevented I/R-induced formation of NT in crypts and ganglion cells, and only very faint reactivity was found in villous epithelia (Fig. 5E and F). Again, sections that were stained with normal rabbit IgG in place of the primary antibody demonstrated no NT staining in any of the sections (data not shown).

DISCUSSION

In the intestine, NO generated by various cell populations of the mucosa, submucosa, and muscle layers can have a dual effect, protective or injurious, based on its concentration. The exact source of this NO production is uncertain, but candidates include macrophages, neutrophils, epithelial cells, endothelial cells, and smooth muscle cells. At low nanomolar concentrations, NO derived from constitutive NOS (endothelial NOS, neural NOS) is protective for blood vessel and neuron activity, as well as for preserving vascular permeability and mucosal barrier integrity. In contrast, at micromolar concentrations, as produced by iNOS, NO is cytotoxic for various cells, including epithelial cells and endothelial cells (28, 30, 36). Targeting iNOS production to prevent formation of high levels of NO and other reactive nitrogen species may provide therapeutic benefits in I/R scenarios. Indeed, several nonselective and selective iNOS inhibitors such as L-arginine analogues and aminoguanidine have been shown to efficiently inhibit iNOS activity and subsequently prevent or improve injury to cells, tissues, or organs in various models (28, 30).

We have now demonstrated that in addition to decreasing cytokine-induced iNOS and NO production in IEC *in vitro* (24), HB-EGF decreases I/R-induced production of iNOS and NO *in vivo*. Our newly designed quantitative technique of rat iNOS real-time PCR demonstrated decreased iNOS mRNA expression, and western blotting confirmed decreased iNOS protein production in animals subjected to intestinal I/R injury that were treated with HB-EGF. In addition, HB-EGF decreased plasma NO levels and intestinal peroxynitrite deposition after I/R injury. Among the nitrogen oxides derived from NO, peroxynitrite formed from the reaction of NO with superoxide is a potent and toxic oxidant. As peroxynitrite is ephemeral and difficult to measure *in vivo*, indirect evaluation of its nitration product NT by immunostaining is a useful marker for its formation. As other reactions, including that of nitrite with hypochlorous acid and myeloperoxidase with hydrogen peroxide, induce tyrosine nitration as well (10), NT staining is a useful marker for "nitrosative stress" after I/R injury. It is important to recognize the protective role of HB-EGF in preventing the formation of toxic nitrogen oxides in epithelial cells and ganglion cells after I/R injury. Protection of these cells from damage may have crucial beneficial effects in the I/R-injured intestine in terms of preservation or recovery of important functions, including gut barrier function, absorption, and motility.

Several growth factors, including transforming growth factor-β, platelet-derived growth factor, and acidic and basic fibroblast growth factors, have been found to inhibit iNOS production and exert protection in various cells and tissues (14, 23, 25, 43). Interestingly, although EGF was shown to decrease iNOS production in human and mouse keratinocytes, lung epithelial cells, and hepatocytes (25), it

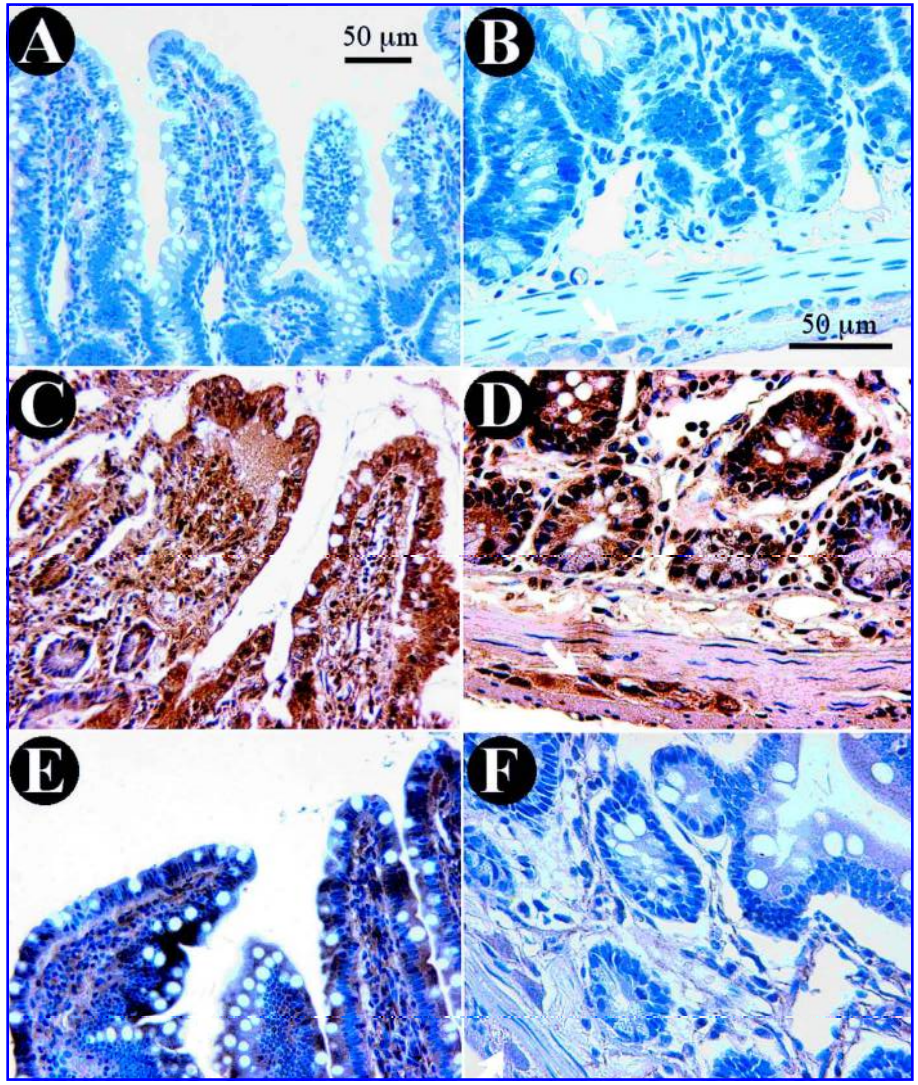

FIG. 4. Effect of HB-EGF on iNOS production after intestinal I/R injury. iNOS immunohistochemistry was performed in (**A, B**) normal rats, (**C, D**) I/R-injured rats, and (**E, F**) I/R-injured rats treated with HB-EGF. A, C, E represent the appearance of the villi (×200 magnification), whereas B, D, and F represent the appearance of the crypts (×400 magnification). Arrows indicate ganglion cells.

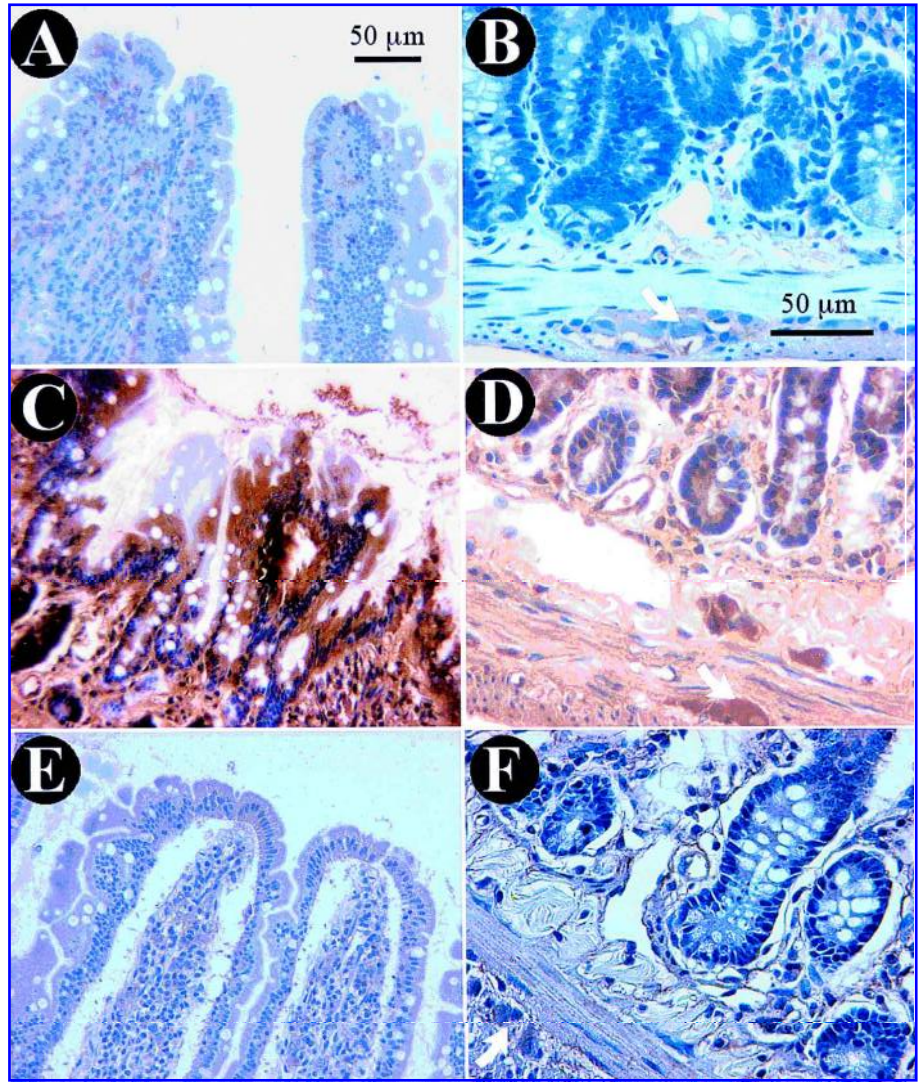

FIG. 5. Effect of HB-EGF on NT formation after intestinal I/R injury. NT immunohistochemistry was performed in (**A, B**) normal rats, (**C, D**) I/R-injured rats, and (**E, F**) I/R-injured rats treated with HB-EGF. A, C, and E represent the appearance of the villi (×200 magnification), whereas B, D, and F represent the appearance of the crypts (×400 magnification). Arrows indicate ganglion cells.

had no effect on iNOS production in DLD-1 cells (37), a human intestinal epithelial cell line in which we were able to demonstrate marked suppression of iNOS production by HB-EGF (24). Although the targeting sites of these growth factors for iNOS inhibition are not yet fully elucidated, modulation of the nuclear transcription factor nuclear factor-κB (NF-κB) has been documented for platelet-derived growth factor and fibroblast growth factor (23). In fact, expression of iNOS requires activation of NF-κB, further implicating the interactions between I/R injury, NF-κB activation, and iNOS expression (9). Similar mechanisms may exist for HB-EGF, and future studies will address this possibility.

It is important to recognize the timing of HB-EGF administration in the present studies. Our model of intestinal I/R injury involved intraluminal administration of HB-EGF after half of the ischemic interval had already occurred, and before reperfusion. Thus, these animals were not "pretreated" with the growth factor *per se*. Likewise, we have used a rat model of segmental intestinal I/R injury to demonstrate that HB-EGF administered after the majority of ischemia had already occurred decreased histologic injury and mortality after reperfusion (34). The present findings are in contrast with our *in vitro* studies, where HB-EGF was only able to decrease iNOS and NO production in IEC when administered prior to cytokine stimulation (24). The fact that HB-EGF exerts beneficial effects *in vivo* even when administered after an insult supports our contention that it may be useful clinically in the future to treat patients with intestinal I/R injury.

In summary, HB-EGF protects the intestine from I/R injury, at least in part, through down-modulation of the iNOS/NO/NT axis, an important pathway involved in I/R injury. In addition, HB-EGF has been shown to be a potent inhibitor of reactive oxygen species production in leukocytes and intestinal epithelial cells *in vitro*, and in I/R-injured intestine *in vivo* (unpublished observations). HB-EGF also decreases adhesion molecule expression in injured endothelia, and leukocyte infiltration into injured intestine (unpublished observations). Together with its inhibitory effects on iNOS and NO production, and its mitogenic and chemotactic effects on epithelial cells, HB-EGF may be a prime therapeutic agent for use in patients with or at risk of developing intestinal I/R injury. Importantly, using a growth factor such as HB-EGF to treat I/R injury may have dual beneficial effects based on its strong mitogenic and chemotactic effects on epithelial cells, on the one hand, and its modulatory effects on pathogenic effectors of I/R injury, including NO, on the other hand. In effect, HB-EGF could potentially treat I/R injury at several different levels.

Perspectives

It is well known that iNOS expression requires activation of NF-κB (44) and that NF-κB is activated after intestinal I/R injury (45). Furthermore, endogenous HB-EGF is expressed after activation of NF-κB (32). Based on the present demonstration that exogenous administration of recombinant HB-EGF efficiently down-regulates iNOS expression, the next step will be to investigate whether this effect is mediated via modulation of NF-κB activity by HB-EGF. Future studies will delineate the relationship between I/R injury, NF-κB, and endogenous HB-EGF expression, as well as the effects of exogenous HB-EGF administration on NF-κB activation.

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ABBREVIATIONS

BSA, bovine serum albumin; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; IEC, intestinal epithelial cells; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; LPS, lipopolysac-

charide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; NT, nitrotyrosine; PBS, phosphate-buffered saline; rRNA, ribosomal RNA; RT-PCR, reverse transcription–polymerase chain reaction; TBS, Tris-buffered saline.

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