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HB-EGF PROTECTS THE LUNGS FROM INTESTINAL ISCHEMIA/REPERFUSION INJURY

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Abstract

Background—Acute respiratory distress syndrome continues to be a major source of morbidity and mortality in critically-ill patients. Heparin binding EGF-like growth factor (HB-EGF) is a biologically active protein that acts as an intestinal cytoprotective agent. We have previously demonstrated that HB-EGF protects the intestines from injury in several different animal models of intestinal injury. In the current study, we investigated the ability of HB-EGF to protect the lungs from remote organ injury after intestinal ischemia/reperfusion (I/R).

Methods—Mice were randomly assigned to one of the following groups: 1) sham-operated; 2) sham + HB-EGF (1200 µg/kg in 0.6 mL administered by intra-luminal injection at the jejuno-ileal junction immediately after identification of the superior mesenteric artery); 3) superior mesenteric artery occlusion for 45 min followed by reperfusion for 6 h (I/R); or 4) I/R+HB-EGF (1200 µg/kg in 0.6 mL) administered 15 min after vascular occlusion. The severity of acute lung injury was determined by histology, morphometric analysis and invasive pulmonary function testing. Animal survival was evaluated using Kaplan-Meier analysis.

Results—Mice subjected to intestinal I/R injury showed histological and functional evidence of acute lung injury and decreased survival compared to sham-operated animals. Compared to mice treated with HB-EGF (I/R + HB-EGF), the I/R group had more severe acute lung injury, and decreased survival.

Conclusion—Our results demonstrate that HB-EGF reduces the severity of acute lung injury after intestinal I/R in mice. These data demonstrate that HB-EGF may be a potential novel systemic anti-inflammatory agent for the prevention of the systemic inflammatory response syndrome (SIRS) after intestinal injury.

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Keywords

acute lung injury; systemic inflammatory response; multiple organ dysfunction syndrome; heparin-binding EGF-like growth factor; HB-EGF; intestine; lungs

INTRODUCTION

Intestinal ischemia/reperfusion (I/R) injury leads to tissue hypoxia and activation of circulating leukocytes that trigger a local followed by a systemic microcirculatory inflammatory response. Animal models and clinical data support the concept that intestinal injury results in increased gut permeability, which serves as the major inciting event leading to the systemic inflammatory response syndrome (SIRS) (1,2,3). The activated leukocytes that are trapped in remote organs following intestinal injury generate oxidants and proteases that result in increased microvascular permeability and endothelial injury. The lung appears to be the first remote organ that is affected by this process (1). Multiple organ dysfunction syndrome (MODS) can develop after generalized SIRS and is the major cause of death in patients with acute respiratory distress syndrome (ARDS) (4). ARDS remains a major source of morbidity and mortality in critically ill patients (5).

Heparin binding EGF-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family that was initially identified in the conditioned medium of cultured human macrophages (6). It is initially synthesized as a 208 amino acid biologically active transmembrane precursor protein (proHB-EGF) that undergoes extracellular proteolytic cleavage to yield a 14–20 kDa soluble growth factor (sHB-EGF) (7). HB-EGF is produced in multiple cell types and acts as a potent mitogenic and chemoattractant protein (7,8). Expression of HB-EGF is significantly increased in response to hypoxia (9) and tissue damage (10). We have shown that endogenous HB-EGF is increased in intestinal epithelial cells (IEC) in response to anoxia/reoxygenation and in intestine in response to I/R injury (11). We have also shown that HB-EGF knockout (KO) mice have increased intestinal injury in animal models of intestinal I/R (12), hemorrhagic shock and resuscitation (HS/R) (13) and necrotizing enterocolitis (NEC) (14), and that HB-EGF transgenic mice have decreased intestinal injury after HS/R (15) and improved intestinal anastomotic wound healing (unpublished data). Lastly, administration of exogenous HB-EGF protects the intestines from injury in animal models of I/R, HS/R and NEC (16,17,18).

Several therapeutic innovations for the prevention of intestinal I/R-induced lung injury have been proposed with minimal to moderate effects. These include fibrin-stabilizing factor (19), elastase inhibitors (20), antioxidants (21), nutritional agents (22), inhibitors of nuclear factor κ B (NF- κ B) activation and pro-inflammatory cytokine production (23) and nitric oxide synthase (NOS) inhibitors (24). In addition to the beneficial trophic effects of HB-EGF based on its mitogenic and chemoattractant properties, we have also shown that HB-EGF decreases NF- κ B activation (25), decreases the production of reactive oxygen species and pro-inflammatory cytokines (26,27), and decreases the overproduction of inducible NOS (iNOS) (28) after intestinal injury. Based on these findings, we hypothesized that HB-EGF can protect the lungs after intestinal I/R injury by alleviating the inflammatory response.

MATERIALS AND METHODS

Intestinal I/R injury model

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Nationwide Children's Hospital Research Institute (Protocol 00903 AR). Eight to ten week old male C57BL/6 mice (Charles River, Boston, MA) weighing 20–25g were fasted

for 10–12 hours with access to water only. Mice were randomized to four groups: 1) sham-operated mice (sham); 2) sham-operated mice that received HB-EGF (sham+HB-EGF); 3) mice exposed to superior mesenteric artery occlusion (SMAO) for 45 min followed by reperfusion for 6 h (I/R), or 4) mice exposed to I/R that received HB-EGF (I/R+HB-EGF). Animals were anesthetized with 3% isoflurane and the abdomen was shaved and rinsed with 70% isopropanol solution. Anesthesia was maintained using 1% isoflurane and body temperature was maintained at 37°C using a heating pad.

A midline laparotomy was made and using a dissecting microscope the superior mesenteric artery (SMA) as well as the collateral branch from the celiac axis were identified and occluded with non-traumatic vascular clamps. Intestinal ischemia was confirmed by paleness of the small intestine and lack of pulsation in the mesentery. After 15 min of ischemia, mice in the I/R+ HB-EGF group received a 0.6mL intraluminal injection of HB-EGF (1200 µg/kg) diluted in 0.1% bovine serum albumin (BSA) in PBS at the jejuno-ileal junction to ensure that the entire small bowel was filled with the HB-EGF solution. Animals in the I/R only group received 0.6mL of 0.1% bovine serum albumin (BSA) in PBS solution only. The vascular clamps were removed after 45 min of ischemia and the intestine was allowed to reperfuse for 6 h. In the sham-operated group, the SMA and collateral branches from the celiac axis were identified but were not occluded. Animals in the sham + HB-EGF group received a 0.6mL intraluminal injection of HB-EFD (1200 µg/kg) immediately after identification of the superior mesenteric artery, with no subsequent vascular occlusion.

Lung histology/morphometric analyses

Left lungs were used for histologic analyses. Tracheas were cannulated with PE-50 tubing (BD Pharmigen, Franklin Lakes, NJ), right main stem bronchi were ligated, and right lungs were excised and snap frozen for other analyses. Left lungs were fixed by inflation with 10% formalin at 25cm H₂O pressure. Lung tissues were embedded in paraffin, and tissue sections from the entry of the left main bronchus distally for 8–12 mm were stained with hematoxylin and eosin (H&E) and examined using light microscopy. The degree of lung injury was assessed using the scoring system described by Matute-Bello *et al.* that graded congestion of alveolar septae, intra-alveolar cell infiltrates and alveolar hemorrhage (29). Each parameter was graded on a scale of 0–3, as follows: *Alveolar septae*: 0: septae thin and delicate, 1: congested alveolar septae in < 1/3 of the field, 2: congested alveolar septae in 1/3 – 2/3 of the field, 3: congested alveolar septae > 2/3 of the field; *Intra-alveolar cell infiltrates*: 0: < 5 intra-alveolar cells per field, 1: 5 to 10 intra-alveolar cells per field, 2: 10 to 20 intra-alveolar cells per field, 3: >20 intra-alveolar cells per field; *Alveolar hemorrhage*: 0: no hemorrhage, 1: at least 5 erythrocytes per alveolus in 1 to 5 alveoli, 2: at least 5 erythrocytes in 5 to 10 alveoli, 3: at least 5 erythrocytes in > 10 alveoli. Histological analyses were performed by two blinded observers.

Lung morphometric analyses were conducted as follows. For each H&E stained lung section, 10 images were captured at 200× magnification under identical lighting conditions and optical settings. Images were analyzed using digital image analysis software (Image Pro Plus 6.2.1; Media Cybernetics, Silver Spring, MD). A custom macro was written for automated analysis of alveolar surface area. Manual measurements of alveolar septae thickness were performed using the “manual measurement” function of Image Pro Plus software. Pulmonary diffusion capacity was calculated as alveolar surface area divided by alveolar septae thickness.

Immunofluorescent staining of lung inflammatory cells

Paraffin-embedded tissue sections were deparaffinized and dehydrated with 3–5% xylene and 95–100% ethanol. Antigen retrieval was performed by boiling slides in 0.01M citric

acid, pH 6 in a pressure cooker at 15 psi for 20 min. Non-specific binding was blocked by incubating the slides with 5% donkey serum and 5% BSA in 1× PBS in a humid chamber for 60 min at RT. Tissue slides were incubated with a primary anti-CD11b/c antibody (1:100 dilution in blocking buffer; Abcam INC, Cambridge, MA) overnight at 4°C. Slides were then incubated with a secondary Cy3-conjugated AffinPure goat anti-mouse IgG antibody (1:500 dilution in blocking buffer) (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT in a dark room. Detection was performed by mounting slides with Prolong Gold antifade reagent containing DAPI (Invitrogen, Eugene, OR). An average of the number of positively stained cells in four optical fields viewed at 200× magnification was quantified and presented as the number of inflammatory cells per high power field (HPF).

Myeloperoxidase assay

Myeloperoxidase activity in the lung was measured as described by Netea *et al.* (30). Briefly, 150–200 mg of lung tissue was homogenized for 30 sec in 4 mL of potassium phosphate buffer (20 mM), pH 7.4. The homogenate was centrifuged at 20,800g for 45 min at 4°C. The pellet was resuspended in 4 mL of potassium phosphate buffer (50 mM), pH 6 containing cetrimonium bromide (0.5 g/dl) (Sigma-Aldrich, St. Louis, MO) and sonicated for 90 sec at full power (Sonics Vibra Cell VCX 130, Sonics & Materials, Inc, USA). The suspension was incubated in a 60°C water bath for 2 h and centrifuged for 10 min at 20,000g. Twenty-five µl of the supernatant were added to 725 µl of phosphate buffer (50 mM), pH 6 containing o-dianiside (0.167 mg/ml) (Sigma-Aldrich, St. Louis, MO) and 0.0005% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ). Absorbance was measured at 460 nm at 1 min and 3 min. Myeloperoxidase activity was calculated as: MPO activity [units per gram of tissue] = ($\Delta A_{460} \times 13.5$)/tissue weight [in grams], where ΔA_{460} equals the change in absorbance from 1 min and 3 min. The coefficient 13.5 was empirically determined such that one-unit of MPO activity was the amount of enzyme that reduced 1 µmol peroxide per minute.

TUNEL staining

Apoptotic cells in the lung were identified by terminal deoxynucleotidyl transferase dUTP nick end labeling using an ApopTag Red in situ apoptosis detection kit (Chemicon International, Temecula, CA, US) following the manufacturer's protocol as follows: paraffin-embedded tissue sections were deparaffinized and pre-treated with freshly diluted protein digesting enzyme for 15 min at RT. Seventy-five µL/5 cm² equilibration buffer was applied directly to tissue sections and incubated for 10 sec at RT. Excess liquid was gently tapped off the tissue sections and a working strength of Tdt enzyme (55 µL/5 cm²) was applied to the sections and allowed to incubate in a humidified chamber at 37°C for 1 h. Slides were washed in 3 changes of 1× PBS and incubated with anti-digoxigenin conjugate for 30 min at RT in a dark room. Slides were washed in 4 changes of 1× PBS for 2 min at RT and counterstained with a mounting media containing DAPI (Invitrogen, Eugene, OR). Positively-stained apoptotic cells in the lung were counted randomly in ten 400× view fields.

Western Blotting for Akt

Total Akt and phospho-Akt levels in the lungs were determined by Western blotting. Protein lysates were prepared from lung tissues using immunoprecipitation lysis buffer containing 20mM HEPES, pH 7.4, 2 mM EGTA, 10mM NaF, 1% Triton-100, 50mM beta-glycerophosphate, 1mM DDT, 1mM sodium orthovanadate (Na₃VO₄), 10% glycerol and the following inhibitors: saturated PMSF in isopropanol, 100µM leupeptin, 10µg/ml aprotinin and 10nM okadaic acid. Samples were centrifuged at 13,000 rpm × 5 min at 4°C, and protein concentration was assayed using the BioRad protein assay reagent (BioRad Laboratories Inc, Hercules, CA). Samples (100µg protein per sample) were separated on 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis prior to transfer to

nitrocellulose membranes. Membranes were blocked with 5% skim milk at RT $\times$ 1 h and then incubated in primary phospho-Akt or Akt antibodies (1:1000 dilution; Cell Signaling Technologies, Beverly, MA) overnight at 4°C. Membranes were washed for 10 min three times with 20 mmol/L Tris buffer, pH 7.4 containing 0.1% Tween 20, incubated in 2.5% skim milk containing a 1:10,000 dilution of secondary anti-rabbit HRP IgG antibody for 1 h (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), and washed again for 10 min three times with 20 mmol/L Tris buffer, pH 7.4 containing 0.1% Tween 20. Protein bands were developed by enhanced chemifluorescence and scanned with a Typhoon variable mood imager 9400 (Amersham Biosciences, Piscataway, NJ).

Evans blue dye assay for pulmonary vascular permeability

Pulmonary vascular permeability was measured using the Evans blue dye (EBD) assay. Mice were injected with EBD (30 mg/kg) through the external jugular vein at 5 h 30 min of reperfusion, followed by an intraperitoneal (i.p.) injection of 0.4 mL of 1,000 Units heparin. The presence of EBD in the circulation was confirmed by blue discoloration of the paws and nose of the mouse. Thirty minutes after injection of EBD, mice were perfused with warm 1 $\times$ PBS (40 mL) containing 5mM EDTA via the beating left ventricle. The lungs were excised en bloc, blotted dry, snap-frozen and stored in -80°C . Lungs were homogenized in cold 1 $\times$ PBS (0.05 g/2mL) and incubated with 2 volumes of formamide (Fisher Scientific, Fair Lawn, NJ) for 18 h at 60°C. The homogenate was then centrifuged for 30 min at 20,000g. Dye concentration was measured spectrophotometrically at 620 nm.

Pulmonary function tests

Lung compliance and resistance were measured using the SCIREQ® Flexivent System (SCIREQ, Montreal, Canada) at the 6 h reperfusion time point. Mice were anesthetized with an i.p. injection of ketamine (100 mg/ml) and xylazine (20 mg/ml) mixture (2:1, 0.3mL). After induction of anesthesia, the trachea was cannulated with a 20 gauge Luer-Tube adapter #427564 (INTRAMEDIC, Franklin Lakes, NY) that was connected to a computer controlled small animal ventilator (FlexiVent, SCIREQ, Montreal, Canada). Mice were ventilated with a tidal volume of 10 ml/kg at a frequency of 350 breaths/min and a positive end-expiratory pressure of 2 cmH₂O. After stabilization, lung mechanics were measured twice using a forced oscillation technique. Pulmonary resistance and compliance were captured using a single compartment model for measuring respiratory mechanics. The single compartment model produced the widely used dynamic resistance (R) and compliance measurements (C) that quantitatively assess the value of the parameter for the entire lung (31). Forced oscillation perturbation “Quick Prime-3” gave measurements of airway resistance (R_n), total lung capacity (A), lung tissue resistance (G) and lung tissue elasticity (H) simultaneously. A coefficient of determination (COD) of 0.95 was the lower limit for accepting a measurement.

Animal survival study

Animal survival was determined over a two week time period. Animals were monitored every 2 h for the initial 6 h after SMAO; every 12 h for the next 24 h, and every 24 h until the end of the two week study period.

Statistical analyses

Data are expressed as mean $\pm$ SEM. Distribution analysis for all variables were performed to test for skewing. Statistical analyses were performed using the nonparametric Kruskal-Wallis test with statistical significance accepted at *p*-value equal or less than 0.05. SAS 9.1.3 software (SAS Institute, Cary, NC) or SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL) was used for all statistical evaluations. For survival determination, the Kaplan-Meier

survival analysis with Log-Rank significance test was used with p -value accepted at equal or less than 0.05. The statistical power for the Kaplan-Meier analysis was calculated using the proportion surviving method Logrank test (PASS 2008 software, NCSS LLC., Kaysville, Utah). Multiple comparisons between the experimental groups were also performed using the Holm-Bonferroni correction method.

RESULTS

HB-EGF reduces the severity of acute lung injury after intestinal I/R injury

Compared with the sham or the sham+HB-EGF groups, mice subjected to intestinal I/R injury showed histological evidence of acute lung injury based on a grading system that assessed congestion of septae, intra-alveolar cellular infiltration and hemorrhage (Figure 1A–D). Mice that were subjected to I/R injury that received HB-EGF demonstrated lower injury scores (3.41 ± 1.58 vs. 5.43 ± 2.4 ; $p = 0.05$) compared with mice subjected to I/R injury that did not receive HB-EGF (Figure 1E). The lung injury scores of the I/R+HB-EGF mice were not statistically different than the scores of the Sham+HB-EGF mice. Although mice that were subjected to sham surgery with administration of HB-EGF demonstrated slightly increased injury scores (2.75 ± 0.02 vs. 1.04 ± 0.01) compared with sham operated mice, these relatively low injury scores were not indicative of levels of injury likely to have clinical manifestations.

HB-EGF improves pulmonary diffusion capacity after intestinal I/R

Lung morphometric analyses were conducted in the sham, I/R and I/R + HB-EGF groups. The alveolar surface area was not significantly changed in these experimental groups (Figure 2A). There was a significant increase in alveolar septae thickness in mice subjected to I/R compared with sham-operated mice (7.35 ± 0.69 mm vs. 3.07 ± 0.1 mm; $p = 0.008$) (Figure 2B). Mice subjected to I/R injury that were treated with HB-EGF had a significant decrease in alveolar septae thickness compared to mice subjected to I/R injury that did not receive HB-EGF (3.05 ± 0.24 mm vs. 7.35 ± 0.69 mm; $p = 0.002$). Pulmonary diffusion capacity was significantly decreased in mice subjected to intestinal I/R injury (Figure 2C). Mice that were subjected to I/R injury that received HB-EGF treatment had significantly increased diffusion capacity compared with mice subjected to I/R injury that did not receive HB-EGF (49.24 ± 4.39 vs. 20.26 ± 2.64 ; $p = 0.002$).

HB-EGF decreases lung inflammatory cell infiltration after intestinal I/R

Compared with the sham or the sham+HB-EGF groups, mice subjected to intestinal I/R had increased infiltration of macrophages and polymorphonuclear leukocytes in the lungs as demonstrated by both immunofluorescent staining (Figures 3 A–E) and myeloperoxidase (MPO) levels (Figure 3F). Mice that were subjected to I/R injury that received HB-EGF demonstrated decreased inflammatory cell infiltration compared with mice subjected to I/R injury that did not receive HB-EGF (196.70 ± 125.70 cells per HPF vs. 323 ± 112.72 cells per HPF; $p = 0.03$) (Figure 3E). Mice subjected to intestinal I/R had increased lung MPO activity compared with sham-operated mice, whereas mice subjected to I/R but treated with HB-EGF had significantly decreased lung MPO levels compared with mice subjected to I/R injury that were not treated with HB-EGF (6.32 ± 2.63 U/g wet lung vs. 8.70 ± 3.90 U/g wet lung; $p = 0.003$) (Figure 3F).

HB-EGF inhibits cellular apoptosis in the lungs after intestinal I/R

Apoptotic cells in the lungs were evaluated using TUNEL staining. There were significantly increased apoptotic cells observed in the lungs of mice subjected to I/R compared with sham or sham+HB-EGF mice. Mice subjected to I/R but treated with HB-EGF had significantly

decreased lung cell apoptosis compared with mice subjected to I/R injury that were not treated with HB-EGF (1.17 ± 0.17 TUNEL-positive cells / HPF vs. 3.22 ± 0.61 TUNEL-positive cells/HPF; $p = 0.02$) (Figure 4). In addition, the number of apoptotic cells was significantly decreased in mice subjected to sham surgery that were treated with HB-EGF compared with mice subjected to sham surgery alone.

HB-EGF does not affect Akt activation in the lungs after intestinal I/R

In order to determine whether activation of Akt in the lungs was responsible, in part, for the ability of HB-EGF to protect the lungs after intestinal I/R, the expression level of activated Akt was measured in the lungs by Western blotting at 1 h and 6 h after the initiation of reperfusion. The expression of activated Akt was not significantly changed in the sham surgery, sham+HB-EGF, I/R or I/R+HB-EGF experimental groups (Figure 5).

HB-EGF reduces pulmonary vascular permeability after intestinal I/R

Pulmonary vascular permeability was evaluated using the Evan's blue dye assay. Pulmonary vascular permeability was significantly increased in mice subjected to I/R compared with sham operated mice (Figure 6). Mice subjected to I/R but treated with HB-EGF had significantly reduced pulmonary vascular permeability compared with mice subjected to I/R injury that were not treated with HB-EGF (0.025 ± 0.002 vs. 0.05 ± 0.01 ; $p = 0.05$).

HB-EGF improves pulmonary resistance after intestinal I/R

Pulmonary resistance was significantly increased in mice subjected to I/R compared with sham-operated mice (Figure 7A). Pulmonary resistance was significantly decreased in mice subjected to I/R but treated with HB-EGF compared with mice subjected to I/R injury that were not treated with HB-EGF (0.75 ± 0.03 cmH₂O.s/mL vs. 1.06 ± 0.18 cmH₂O.s/mL $p = 0.004$). Mice in the I/R group had significantly decreased pulmonary compliance compared to sham-operated mice ($p = 0.002$) (Figure 7B). The addition of HB-EGF had no significant effect on pulmonary compliance.

HB-EGF prolongs survival after intestinal I/R

Twelve mice were subjected to SMAO for 45 min (I/R group). Three of these mice died at 4 h of reperfusion, 1 died at 6 h of reperfusion, 6 died at 24 h and 1 died at 96 h, giving an overall mortality of 91%. There were 10 mice in the treatment group (I/R + HB-EGF). Three died at 18 h and 2 died at 72 h, giving an overall mortality of 50%. There was no mortality in the sham-operated group (sham) (Figure 8). Treatment with HB-EGF significantly decreased mortality and prolonged survival in mice subjected to intestinal IR injury ($p = 0.003$). The statistical power comparing the sham vs. the I/R group was 100% while the power comparing the I/R and the I/R+ HB-EGF group was 71%.

DISCUSSION

Acute respiratory failure is the single most important component of the MODS that follows intestinal injury, and continues to be a leading source of morbidity and mortality in critically ill patients, with an estimated incidence of 10–14 per 100,000 people and a mortality rate of 36–52%, respectively (32). There is evidence that intestinal I/R results in a generalized systemic inflammatory response and subsequent MODS, beginning with acute lung injury (1,33). In the gut hypothesis of MODS, intestinal injury leads to epithelial barrier dysfunction with subsequent release of bacteria and endotoxin into the systemic circulation. This leads to activation of pro-inflammatory cytokines, circulating leukocytes and the coagulation cascade (1). The tissue hypoperfusion that occurs during ischemic injury also results in activation of circulating leukocytes and up-regulation of endothelial cell surface

adhesion molecules. The interaction between activated leukocytes and endothelial cells leads to emigration of leukocytes and production of reactive oxygen species and proteases that cause further tissue damage and subsequent end organ damage if the ongoing inflammatory response is not controlled.

The surgical critical care practitioner is challenged with patients that develop SIRS secondary to an array of intestinal injuries including abdominal trauma, hemorrhagic shock and resuscitation, pancreatitis, mesenteric ischemia and necrotizing enterocolitis. Irrespective of the cause of SIRS in these patients, the disease follows a predictive course, usually beginning in the lungs in a patient without any previous medical conditions (1,33).

In the current study, we tested the hypothesis that HB-EGF could protect the lungs from remote organ injury after intestinal I/R. Our data demonstrate that HB-EGF significantly decreases the severity of acute lung injury, decreases the infiltration of macrophages and polymorphonuclear leukocytes in the lungs, decreases lung myeloperoxidase activity, decreases pulmonary vascular permeability, inhibits cellular apoptosis in the lungs, decreases pulmonary resistance, improves pulmonary diffusion capacity, and prolongs survival after intestinal I/R injury. Our findings are most likely due to the ability of HB-EGF to protect the intestines from damage following intestinal I/R injury thereby alleviating the initiation of subsequent SIRS. Indeed, we have previously shown in our *in vivo* model of intestinal I/R injury that HB-EGF down-regulates the expression of adhesion molecules in the intestine (34), decreases intestinal neutrophil and macrophage infiltration (34), and decreases systemic pro-inflammatory cytokine production (27). HB-EGF also decreases intestinal permeability and promotes early recovery of intestinal function by restoring the gut permeability barrier after intestinal I/R injury (16).

HB-EGF protects the intestine, in part, via early activation of Akt and ERK. ERK is involved in cell migration and is activated during healing of gastrointestinal epithelium both *in vivo* and *in vitro* (35,36,37). Akt is a serine-threonine protein kinase that is one of the downstream substrates of PI3K, and is a critical mediator of growth factor-induced survival in many cells types including intestinal epithelial cells. Activation of Akt increases the tolerance of organs such as the intestine, brain and heart to I/R injuries (38,39,40) via inhibition of several apoptotic pathways that appear to be a main mechanism of intestinal cell death during I/R (41,42). We have previously demonstrated that HB-EGF leads to early activation of Akt in the intestine followed by a significant reduction in LDH levels after I/R injury in rats, suggesting a higher intestinal tolerance to reperfusion injury (16).

To our knowledge, there have been no reports on the role of PI3K/Akt activation in protecting the lungs from intestinal I/R-induced injury. In a model of ventilation associated lung injury (VALI), some authors have demonstrated a protective role of several PI3K/Akt pathways (43,44) in mouse lungs. On the other hand, in a model of endotoxemia in which mice were injected with LPS intraperitoneally, activation of PI3K/Akt in lung neutrophils worsened acute lung injury, and PI3K knock-out mice were protected from acute lung injury in this model (45). In the current study, we examined whether the ability of HB-EGF to protect the lungs from distant organ injury following intestinal I/R was associated with Akt activation. Although we previously reported that administration of HB-EGF leads to increased Akt activation in the intestines as early as 30 minutes after intestinal I/R, with peak levels at 1h after intestinal I/R injury (16), we found no significant changes in Akt activation in the lungs in any of our experimental groups at either 1h or 6h after reperfusion of the intestines. Future experiments will be designed to investigate the signaling mechanisms utilized by HB-EGF in protection of the lungs after intestinal I/R injury.

The tissue hypoperfusion that occurs during ischemic injury results in activation of circulating leukocytes and up-regulation of endothelial cell surface adhesion molecules. The interaction between activated leukocytes and endothelial cells leads to emigration of leukocytes and production of reactive oxygen species and proteases that cause further tissue damage. Previous studies from our laboratory have demonstrated that HB-EGF decreases human neutrophil-endothelial cell interactions and neutrophil transendothelial electrical resistance in cultured endothelial cells subjected to anoxia/reoxygenation (A/R) injury (46). We have also shown that HB-EGF decreases the production of pro-inflammatory cytokines after intestinal I/R in rats (27). These results together suggest that HB-EGF may be exerting its protective effect by minimizing the activation of circulating leukocytes or interrupting the neutrophil-endothelial cell interactions that are necessary for leukocyte emigration and further tissue damage.

In summary, we have previously demonstrated that HB-EGF protects the intestines from injury using several diverse animal models of intestinal injury. The current study demonstrates that HB-EGF protects a remote distant organ (the lungs) from injury after intestinal I/R. We conclude that HB-EGF may be a novel therapeutic agent that not only protects the intestines, but also protects the lungs, from the sequelae of intestinal I/R-induced injury.

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Figure 1.

Lung histologic injury scoring. Shown are representative photomicrographs of hematoxylin and eosin stained lung sections from: A) a sham operated mouse, B) a sham operated mouse that was treated with HB-EGF, C) a mouse subjected to intestinal I/R, and D) a mouse subjected to intestinal I/R but treated with HB-EGF. Panel E represents quantification of lung injury scores, based on septae congestion, intra-alveolar infiltration and alveolar hemorrhage. Bars represent sham animals ($n = 6$), sham+HB-EGF animals ($n = 5$), I/R animals ($n = 11$), and I/R+HB-EGF animals ($n = 11$). Scores represent the averaged results of two blinded observers.

Figure 2.

Lung morphometric analysis. A) Alveolar surface area and B) alveolar septae thickness of H & E stained lung tissue were determined using Image Pro digital image analysis software. C) Pulmonary diffusion capacity was calculated as the alveolar surface area divided by the alveolar septae thickness. Bars represent sham animals ($n = 4$), I/R animals ($n = 7$), and I/R + HB-EGF animals ($n = 7$).

Figure 3.

Lung inflammatory cell infiltration. Shown are representative photomicrographs of tissue sections immunohistochemically stained for CD11b/c to indicate infiltration of macrophages and polymorphonuclear leukocytes from: A) a sham operated mouse, B) a sham operated mouse that was treated with HB-EGF, C) a mouse subjected to intestinal I/R, and D) a mouse subjected to intestinal I/R but treated with HB-EGF. Arrows indicate positively stained cells. Panel E represents quantification of positively stained cells, represented by the number of inflammatory cells counted in four optical fields viewed at 200 $\times$ magnification. Bars represent sham animals ($n = 6$), sham+HB-EGF animals ($n = 5$), I/R animals ($n = 11$),

and I/R + HB=EGF animals ($n = 11$). Panel F represents MPO levels where $n = 8$ animals per group.

Figure 4.

Lung apoptosis. TUNEL-positive cells stained in red are indicated by arrows. Nuclei were counterstained with Prolong Gold antifade reagent containing DAPI. Shown are representative images of: A) a sham operated mouse, B) a sham operated mouse that was treated with HB-EGF, C) a mouse subjected to intestinal I/R, and D) a mouse subjected to intestinal I/R but treated with HB-EGF. Panel E represents quantification of TUNEL-positive stained cells. The apoptotic cells in lung were scored randomly in 10 optical fields viewed at 400× magnification. Bars represent sham animals ($n = 6$), sham+HB-EGF animals ($n = 5$), I/R animals ($n = 10$), and I/R + HB-EGF animals ($n = 10$).

Figure 5.

Akt activation. Shown are representative Western blots demonstrating phosphorylated-Akt and total Akt levels in the lungs from animals exposed to: A) intestinal ischemia for 45 min followed by reperfusion for 1 h and B) intestinal ischemia for 45 min followed by reperfusion for 6 h. Panels C and D represent densitometry results for the 1 h and 6 h reperfusion time points respectively. The densitometry results are expressed as activated Akt normalized to total Akt levels from three independent experiments that used separate groups of mice. There were no statistically significant differences in expression levels of activated Akt in the lungs at either the 1 h or 6 h reperfusion time points.

Figure 6. Pulmonary vascular permeability. Pulmonary vascular permeability was determined using the Evans blue dye assay. Bars represent $n = 4$ in each group.

Figure 7. Pulmonary function tests. A) Lung resistance and B) lung compliance were measured using the SCIREQ Flexivent system at the 6 h reperfusion time point. Tracheas were cannulated, and mice were ventilated using a computer controlled small animal ventilator. Two sets of measurements of pulmonary resistance and compliance were obtained using a single compartment model ($n = 6$ animals in each group).

Figure 8.

Kaplan-Meier survival analysis. Mice were subjected to 45 min of ischemia (I/R, $n=12$); 45 min of ischemia with administration of HB-EGF (I/R + HB-EGF, $n=10$) or sham-surgery (sham, $n = 5$), and were monitored every 2 h for 6 h, every 12 h for the next 24 h, and every 24 h until the end of the two-week study period.