The role of RAGE in the pathogenesis of intestinal barrier dysfunction after hemorrhagic shock

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Raman, Kathleen G., Penny L. Sappington, Runkuan Yang, Ryan M. Levy, Jose M. Prince, Shiguang Liu, Simon K. Watkins, Ann Marie Schmidt, Timothy R. Billiar, and Mitchell P. Fink. The role of RAGE in the pathogenesis of intestinal barrier dysfunction after hemorrhagic shock and resuscitation (HS/R). C57Bl/6, rage−/−, or congenic rage++ mice were subjected to HS/R (mean arterial pressure of 25 mmHg for 3 h) or a sham procedure. Twenty-four hours later, bacterial translocation to mesenteric lymph nodes and ileal mucosal permeability to FITC-labeled dextran were assessed. Additionally, samples of ileum were obtained for immunofluorescence microscopy, and plasma was collected for measuring IL-6 and IL-10 levels. HS/R in C57Bl/6 mice was associated with increased bacterial translocation, ileal mucosal hyperpermeability, and high circulating levels of IL-6. All of these effects were prevented when C57Bl/6 mice were treated with recombinant human soluble RAGE (sRAGE; the extracellular ligand-binding domain of RAGE). HS/R induced bacterial translocation, ileal mucosal hyperpermeability, and high plasma IL-6 levels in rage++ but not rage−/− mice. Circulating IL-10 levels were higher in rage−/− compared with rage++ mice. These results suggest that activation of RAGE-dependent signaling is a key factor leading to gut mucosal barrier dysfunction after HS/R.

translocation; HMGB1; sRAGE; s100b

INTESTINAL EPITHELIAL PERMEABILITY is increased in victims of severe trauma (9, 22). By permitting bacteria or microbial products, such as exotoxin A (2), to cross from the lumen into the systemic compartment, posttraumatic gut barrier dysfunction might contribute to the development of multiple organ dysfunction syndrome, a notion that is supported by data from studies using animal models of hemorrhagic shock and resuscitation (HS/R) (4, 12).

The mechanisms responsible for gut barrier dysfunction after HS/R remain to be fully elucidated. Although mesenteric hypoperfusion and cellular ATP depletion on this basis are important factors (47), data obtained using animal models of HS/R indicate that inflammatory pathways, such as secretion of IL-6 (54), are also important. Numerous factors, such as activation of signaling via mitogen-activated protein kinase pathways by angiotensin II (30) or hypoxia (29) and increased production of reactive oxygen species (1), have been implicated as being important for the induction of inflammatory responses in animals subjected to hemorrhage or HS/R. Conceivably, another important factor might be the binding of various ligands to a cell-surface receptor, the receptor for advanced glycation end products (RAGE), that is known to be capable of initiating activation of the proinflammatory transcription factor NF-κB (24) as well as other signaling cascades (24, 43).

RAGE, a member of the Ig superfamily of proteins, is activated by a wide variety of ligands, including advanced glycation end products (21), the amyloid-β peptide cleavage product of β-amyloid precursor protein (52), and the S100/calgranulin family of proinflammatory cytokine-like mediators (16). High-mobility group box 1 (HMGB1), a nuclear protein with cytokine-like properties (28), also binds to RAGE with high affinity (17, 43), and at least some of the proinflammatory and cytopathic effects of HMGB1 appear to be mediated by binding of HMGB1 to RAGE (10, 20, 39, 44).

Administration of recombinant human soluble RAGE (sRAGE), the extracellular ligand-binding domain of RAGE, has been shown to ameliorate some of the pathological features of diabetes mellitus in experimental animals, presumably because it inhibits proinflammatory signaling initiated by advanced glycation end products and other proinflammatory ligands that accumulate in diabetic tissues (34, 48). Treatment of rodents with sRAGE has also been shown to ameliorate experimentally induced colitis, presumably by inhibiting signaling mediated by HMGB1 or various members of the S100/calgranulin superfamily of proinflammatory cytokines (16).

Herein, we investigated the hypothesis that RAGE-dependent signaling is important for the pathogenesis of gut barrier dysfunction after HS/R in mice. To test this hypothesis, we employed both pharmacological and genetic strategies. Specifically, we assessed the extent of bacterial translocation to mesenteric lymph nodes and measured changes in ileal mucosal permeability following HS/R in mice treated with either sRAGE or a control protein, mouse serum albumin (MSA). We also assessed these two indices of gut barrier function in homozygous RAGE-null (rage−/−) mice and appropriate wild-type control animals subjected to HS/R. Additionally, we carried out a parallel series of in vitro experiments using Caco-2 human enterocyte-like cells growing as monolayers

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bicameral diffusion chambers, using transient hypoxia and reoxygenation as a model for HS/R in vivo and using permeation of the monolayers by FITC-labeled dextran (FD4; average molecular mass = 4 kDa) as the measure of epithelial barrier function. Collectively, our findings from these studies support the view that derangements in intestinal epithelial barrier function after HS/R are mediated, at least in part, by interactions involving RAGE.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Recombinant murine sRAGE. sRAGE was prepared in a baculovirus expression system using SF9 cells (Clontech, Palo Alto, CA; Invitrogen, Carlsbad, CA). Serum-free medium containing sRAGE was subjected to FPLC Mono S for purification (Pharmacia). Purified murine sRAGE (a single band of 40 kDa, by Coomassie-stained sodium dodecylsulfate-polyacrylamide gel electrophoresis) was dia- lyzed against phosphate-buffered saline, made free of detectable lipopolysaccharide, based on the Limulus amebocyte assay (Sigma) after passage through Detox-igel columns (Pierce, Rockford, IL) and sterile filtered (0.2 μm). MSA was used as a control at equimolar concentrations compared with sRAGE.

Murine HS/R model. Male C57Bl/6 mice were from the Jackson Laboratory (Bar Harbor, ME). Male rage−/− mice (generated in the 129 strain) were backcrossed six generations into C57Bl/6; mating of heterozygous RAGE null males and females yielded heterozygous mice as well as wild-type RAGE-bearing (rage+/+) and homozygous rage−/− mice. Wild-type littersmates were employed as controls for rage−/− mice, as previously described (50). The animals were main- tained in the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and free access to standard laboratory feed and water. Animals were not fasted before experiments. All animal studies were conducted with the review and approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. The hemorrhagic shock protocol employed has been de- scribed (15). The mice were subjected to hemorrhagic shock by withdrawal of blood (2.25 ml/100 g body wt) over 10 min to achieve a mean arterial pressure of 25 mmHg. Mean arterial pressure was maintained at 25 mmHg for 3.0 h with continuous monitoring of mean arterial pressure and withdrawal and return of blood as needed. Cannulas, syringes, and tubing were flushed with heparin sodium (100 U/ml) before all procedures. The animals were resuscitated to an initial mean arterial pressure of 25 mmHg at the onset of resuscitation and 12 h after resuscitation. The dose of sRAGE employed for these studies (100 μg per dose) has been employed for other recent studies of this protein using mice (6, 50).

In each of the previous experiments except the third and fourth experiments, mice were reanesthetized with intraperitoneal pentobarbital sodium (70 mg/kg) 24 h after resuscitation (or the sham procedure). A segment of terminal ileum was excised for determination of mucosal permeability, the mesenteric lymph node complex was harvested to quantify bacterial translocation, and blood was aspirated from the heart. Plasma was obtained by centrifugation and stored at −80°C for subsequent determinations of IL-6 and IL-10 concentra-
tions by ELISA (R & D Systems, Minneapolis, MN). In the third experiment, mice were reanesthetized with intraperitoneal pentobarbital sodium (70 mg/kg) 24 h after resuscitation (or the sham procedure), and ileal mucosal scrapings were obtained for Western blot analyses. In the fourth experiment, mice were reanesthetized with intraperitoneal pentobarbital sodium (70 mg/kg) 4 h after resuscitation (or the sham procedure) and ileal segments were obtained for determination of mucosal permeability.

Table 1. Brief summary of the experiments performed

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<tr>
<td>Treatments</td>
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<td>None</td>
<td>sRAGE (100 μg per dose) or MSA (100 μg per dose) at the onset of resuscitation and 12 h after resuscitation</td>
<td>sRAGE (100 μg per dose) or MSA (100 μg per dose) at the onset of resuscitation</td>
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HS/R, hemorrhagic shock and resuscitation; MSA, mouse serum albumin; RAGE, receptor for advanced glycation end-products; sRAGE, soluble RAGE.

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and incubated for 30 min on ice. The lysate was centrifuged at 10,000 g for 20 min at 4°C, and then the supernatant was transferred to a new tube. Total protein concentration was determined using the Bio-Rad (Hercules, CA) protein reagent.

Equivalent amounts of mucosal protein were mixed with Laemmli buffer (20% glycerol; 10% 2-mercaptoethanol; 5% SDS; 0.2 M Tris·HCl, pH 6.8; and 0.4% bromophenol blue). After being boiled 5–10 min, the protein samples were centrifuged for 10 s. Samples of the supernatants containing 30 μg of mucosal protein/lane were electrophoresed at 100 mA for 40 min on 7.5% precast SDS-polyacrylamide gels (Bio-Rad). For samples from studies using Caco-2 cells, equal volumes of culture supernatants were mixed with 6× loading buffer [375 mM, Tris·HCl, pH 6.8, 50% glycerol, 0.03% bromophenol blue, and 10% SDS]. After being boiled, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V for 60 min.

The size-fractionated proteins were electroblotted onto a Hybond-P polyvinylidene fluoride membrane (Amersham Biosciences, GE Healthcare, Piscataway, NJ) and blocked with BLOTTO (1× TBS, 5% milk, 0.05% Tween-20, and 0.2% NaN3) for 60 min. The membranes were then incubated at 4°C overnight with a 1:2,000 dilution of rabbit anti-HMGB1 polyclonal antibody (BD Pharmingen, San Jose, CA) or a 1:4,000 anti-β-actin monoclonal antibody (Invitrogen, Carlsbad, CA). The antibodies were diluted using Tris-buffered saline with Tween (1× Tris-buffered saline and 0.1% Tween-20 and 5% bovine serum albumin). After being washed three times in 1× phosphate-buffered saline with Tween (phosphate-buffered saline and 0.2% Tween-20), immunoblots were exposed at room temperature for 1 h to a 1:10,000 dilution of the appropriate horseradish peroxidase-conjugated anti-immunoglobulin secondary antibody. After three washes in phosphate-buffered saline with Tween and two washes in phosphate-buffered saline, the membrane was impregnated with the enhanced chemiluminescence substrate (Amersham Biosciences) and used to expose X-ray film according to the manufacturer’s instructions.

Cell culture. Caco-2 cells were maintained on collagen-1-coated Biocoat tissue culture dishes (BD Biosciences, San Jose, CA) at 37°C in a 8% CO2 humidified atmosphere in Dulbecco’s minimum essential medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (<0.05 LPS U/ml; Hyclone, Logan, UT), penicillin G (100 U/ml), streptomycin (100 μg/ml), pyruvate (2 mM), t-glutamine (4 mM), and nonessential amino acid supplement (2% vol/vol).

Monolayer permeability assays. Permeation of Caco-2 enterocyte-like monolayers by FD4 was assessed as described previously (31). Briefly, Caco-2 cells were plated (100,000 cells/well) onto permeable filters (0.4-μm pore size) in 12-well Transwell chambers (COSTAR, Corning, NY) and fed biweekly. Permeability studies were performed using confluent monolayers during the interval from 21 to 28 days after seeding. For permeability studies, the medium was aspirated from the apical and basolateral aspects of the Transwell chambers. FD4 solution (25 mg/ml; 200 μl) was pipetted into the apical compartments. In some cases, either sRAGE (50 μg/ml) or polyclonal anti-RAGE antibody (75 μg/ml) (24) was added to the medium on the basolateral side of the Transwell chambers. The concentration of sRAGE employed was similar to the concentrations of this reagent that has been used in other in vitro studies (40–60 μg/ml) (32, 37). The concentration of polyclonal anti-RAGE antibody employed was similar to the concentration of this reagent that has been used in other in vitro studies (40–70 μg/ml) (36, 37). The Transwell chambers were incubated at 37°C under normoxic conditions (21% O2, 5% CO2, and 74% N2) or under hypoxic conditions using a modular chamber (Coy Laboratory Products, Grass Lake, MI) containing an hypoxic gas mixture (0.2% O2, 5% CO2, and 94.8% N2). In some cases, after 8 h of incubation, S100b (100 μg/ml) final concentration; Calbiochem, La Jolla, CA) was added to the basolateral side of the Transwell chambers. The concentration of S100b employed was the same as that used in another recent in vitro study (27). Hypoxia was maintained for a total of 16 h, and then normoxia was reestablished for an additional 8 h. After incubation for a total of 24 h under either normoxic conditions or a combination of hypoxia followed by normoxic conditions, 30 μl of medium were aspirated from the basolateral compartments of the Transwell chambers for spectrofluorometric determination of FD4 concentration (31). The permeability of monolayers was expressed as a clearance with units of nanoliters per hour per square centimeter. Concurrent controls were performed with each experiment.

Immunohistochemistry. Immunohistochemistry using polyclonal antibody to S100b (DakoCytomation, Carpenteria, CA) was performed on frozen sections. The nonimmune IgG for the antibody was used at the same concentration as the primary; nonspecific immunostaining was not observed using nonimmune IgG (data not shown). Nuclei were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR).

Colocalization studies were performed using polyclonal antibodies to S100b and myeloperoxidase, a neutrophil and macrophage marker, as well as S100b and F4–80, a macrophage marker (3). The anti-myeloperoxidase antibody was from (DakoCytomation), and the anti-F4–80 antibody was from Serotec (Raleigh, NC).

Statistical methods. Numerical data are presented as means ± SE. Differences among groups were assessed using analysis of variance followed by Fisher’s least significant difference test or Student’s t-test for unpaired samples, as appropriate. The null hypothesis was rejected for P < 0.05.

RESULTS

Posttreatment of mice with sRAGE downregulates the systemic inflammatory response and ameliorates gut barrier dysfunction induced by HS/R. Groups of mice received two 100 μg/mouse doses of either sRAGE or a control protein, MSA. The first dose was injected at the time of resuscitation (or at the end of the sham procedure), and the second dose was injected 12 h later. Subjecting MSA-treated mice to HS/R significantly increased the circulating concentration of IL-6 24 h after resuscitation (Fig. 1A), whereas plasma IL-10 concentration was unchanged (Fig. 1B). When sRAGE-treated mice were subjected to HS/R, the plasma IL-6 concentration at 24 h was significantly lower than in MSA-treated (control) animals subjected to HS/R. Furthermore, the plasma IL-10 level measured at 24 h was significantly greater than that measured in MSA-treated hemorrhaged mice. Interestingly, pre- and posttreatment with sRAGE also significantly increased the circulating concentration of IL-10 in sham-hemorrhaged mice.

Two indices of intestinal barrier function, namely mucosal permeability to FD4 (Fig. 1C) and bacterial translocation to mesenteric lymph nodes (Fig. 1D), were assessed 24 h after resuscitation (or the sham procedure). Subjecting MSA-treated mice to HS/R significantly increased both mucosal permeability and bacterial translocation. In contrast, when mice were pre- and posttreated with sRAGE, neither ileal mucosal permeability nor bacterial translocation increased after HS/R.

HS/R increases expression of the RAGE ligands, S100b and HMGB1. S100b is a member of a family of small (molecular mass = 9–13 kDa) proteins characterized by the presence of two helix-loop-helix Ca2+-binding sites (8). Extracellular S100b has been shown to activate a variety of cell types by binding to RAGE (40). HMGB1 has been shown to inhibit the
migration of monocytes (37) and mesangioblasts (33), upregulate iNOS expression in Caco-2 human enterocyte-like cells (39), and increase expression of adhesion molecules by vascular endothelial cells (45), at least in part, by binding to RAGE.

Twenty-four hours after HS/R in MSA-treated mice, there was a marked increase in staining for S100b that was mostly localized to the muscularis propria (Fig. 2). Colocalization studies revealed S100b staining in cells that also stained with
24 h, ileal mucosa was harvested and analyzed for the presence of HMGB1 by Western blotting. As expected, HMGB1 was present in all of the samples (Fig. 3). However, in samples from mice treated with MSA, HS/R was associated with a consistent increase in HMGB1 expression. Moreover, among mice treated with sRAGE, irrespective of whether the animals were subjected to the sham procedure or HS/R, there was a consistent decrease in HMGB1 expression.

**RAGE knockout mice are protected from the development of systemic inflammation and gut barrier dysfunction after HS/R.** Circulating concentrations of IL-6 were significantly greater in \( \text{rage}^{+/+} \) mice subjected to HS/R than in \( \text{rage}^{+/+} \) mice subjected to the sham procedure (Fig. 4A). Post-HS/R IL-6 levels were significantly lower in \( \text{rage}^{-/-} \) compared with \( \text{rage}^{+/+} \) mice. Circulating concentrations of IL-10 were not increased after HS/R in \( \text{rage}^{+/+} \) mice (relative to the level observed in \( \text{rage}^{-/-} \) mice subjected to the sham procedure; Fig. 4B). In contrast, plasma IL-10 concentrations were significantly greater in \( \text{rage}^{-/-} \) compared with \( \text{rage}^{+/+} \) after HS/R. When assessed 24 h after HS/R, ileal mucosal permeability was significantly greater in \( \text{rage}^{+/+} \) mice than in \( \text{rage}^{-/-} \) mice (Fig. 4C). As expected, FD4 clearance was significantly greater when \( \text{rage}^{+/+} \) mice were subjected to HS/R compared with the sham procedure. As shown in Fig. 4D, bacterial translocation was significantly greater in \( \text{rage}^{+/+} \) mice subjected to HS/R compared with any of the other three groups of mice (i.e., \( \text{rage}^{+/+} \) mice subjected to the sham procedure or \( \text{rage}^{-/-} \) mice subjected to either HS/R or the sham procedure).

**Treatment with sRAGE fails to prevent early changes in ileal mucosal permeability after HS/R.** The data shown in Figs. 1 and 4 support the view that the interaction of RAGE with one or more ligands for this receptor is critical for the development of gut mucosal hyperpermeability 24 h after HS/R. Previously, however, we reported that HS/R increases ileal mucosal permeability as early as 4 h after resuscitation (53, 54). Accordingly, we sought to determine whether treatment with sRAGE would ameliorate HS/R-induced hyperpermeability at this ear-

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**Fig. 3.** Effect of posttreatment with sRAGE on ileal mucosal expression of high-mobility group box 1 (HMGB1). The experimental design was the same as that described in the legend for Fig. 2. Ileal mucosal scrapings were obtained 24 h after HS/R or the sham procedure, and HMGB1 levels were assayed by Western blotting. Results (gel and densitometry) are from a representative experiment. Similar results were obtained in 2 other replicate experiments. A lysate from RAW 264.7 cells was run as a positive control for HMGB1.

**Fig. 4.** Effect of HS/R or the sham procedure on plasma IL-6 concentration (A), plasma IL-10 concentration (B), ileal mucosal permeability to FD4 (C), and bacterial translocation to mesenteric lymph nodes (D) in \( \text{rage}^{+/+} \) or \( \text{rage}^{-/-} \) mice. In all cases, ileal specimens and blood samples were obtained 24 h after HS/R or the sham procedure. *\( P < 0.05 \) for HS/R in \( \text{rage}^{+/+} \) vs. \( \text{rage}^{-/-} \) mice; †\( P < 0.05 \) for HS/R vs. sham in \( \text{rage}^{+/+} \) mice.
lier time point. Groups of mice were subjected to HS/R or the sham procedure. Some mice (control) were injected with MSA (100 µg/dose) at the onset of resuscitation, and other mice (sRAGE) were treated with equivalent doses of sRAGE at the same time point. Permeability was assessed 4 h after HS/R or the sham procedure. *P < 0.05 for control HS/R vs. control sham; †P < 0.05 for sRAGE HS/R vs. sRAGE Sham.

S100b and hypoxia plus reoxygenation synergistically increase the permeability of Caco-2 monolayers. Subjecting Caco-2 human enterocyte-like cells to hypoxia and reoxygenation has been used previously as a reductionist system for studying the effects of trauma and resuscitation or mesenteric ischemia and reperfusion on the structure or function of gut epithelium (5, 51). When Caco-2 monolayers were incubated for 16 h under hypoxic conditions, permeability to FD4 increased by almost threefold (Fig. 6A). When an 8-h episode of reoxygenation was added to the 16-h period of hypoxia, permeability increased by almost fourfold (Fig. 6B). Incubating Caco-2 monolayers under normoxic conditions with 100 µg/ml S100b for 8 or 16 h increased permeability by about two- or fourfold, respectively. The combination of S100b and hypoxia or hypoxia/reoxygenation increased the permeability of Caco-2 monolayers to a significantly greater extent than did either perturbation alone. Adding either sRAGE or anti-RAGE antibody prevented the increase in permeability induced by either 16 h of hypoxia or 16 h of hypoxia and 8 h of reoxygenation plus S100b.

Hypoxia/reoxygenation promotes HMGB1 release from Caco-2 monolayers. We previously reported that incubating Caco-2 cells with HMGB1 increases the permeability of the monolayers and that this effect of HMGB1 was substantially blocked by addition of anti-RAGE IgG (24). More recently, we reported that immunostimulated enterocytes actively secrete HMGB1 (26). Accordingly, we hypothesized that hypoxia/reoxygenation stress might promote the release of HMGB1 from Caco-2 cells. To test this concept, we measured HMGB1 levels in control supernatants and supernatants from cells subject to 16 h of hypoxia and 8 h of reoxygenation. As shown in Fig. 7, hypoxia/reoxygenation clearly promoted the release of HMGB1 from Caco-2 cells. This phenomenon could explain why incubation with sRAGE inhibits hypoxia/reoxygenation-induced hyperpermeability of Caco-2 monolayers (Fig. 6).

DISCUSSION

Treatment with recombinant sRAGE ameliorates bone loss induced by periodontal inflammation in diabetic mice (23), improves wound healing in diabetic mice (11, 49), prevents the

Fig. 6. Effect of hypoxia or hypoxia/reoxygenation on the permeability to FD4 of Caco-2 monolayers. In some cases, either sRAGE (50 µg/ml) or polyclonal anti-RAGE Ab (75 µg/ml) was added to the medium on the basolateral side of the Transwell chambers. The Transwell chambers were incubated at 37°C under normoxic conditions or under hypoxic conditions. In some cases, after 8 h of incubation, S100b (100 µg/ml final concentration) was added to the basolateral side of the Transwell chambers. Hypoxia was maintained for a total of 16 h (A) and then normoxia was reestablished for an additional 8 h (B). After incubation for a total of 24 h under either normoxic conditions or a combination of hypoxia followed by normoxic conditions, monolayer permeability was determined. *P < 0.05 for normoxia vs. the other conditions overdrawn by the bar, †P < 0.05 for hypoxia + S100b vs. the other conditions overdrawn by the bar.
progression of atherosclerosis in diabetic apolipoprotein E knockout mice (6), inhibits the development of glomerulosclerosis in diabetic mice (50), and prevents hepatocellular necrosis in a murine model of liver ischemia and reperfusion (57). In mice with lethal sepsis, treatment with sRAGE slightly but not significantly improves survival (25). Herein, we present data showing that treatment with sRAGE prevented alterations in gut epithelial barrier function induced by HS/R. Moreover, we showed that administration of sRAGE decreased circulating plasma concentrations of the anti-inflammatory cytokine IL-10. The beneficial effects of sRAGE were apparent irrespective of whether the recombinant protein was administered as a pretreatment (i.e., before the induction of hemorrhagic shock) or as a posttreatment (i.e., at the time of resuscitation).

To provide additional confirmation of the importance of RAGE-dependent signaling in the pathogenesis of gut mucosal barrier dysfunction after HS/R, we carried out an additional series of experiments, assessing several physiological and biochemical readouts in rage−/− compared with rage+/+ mice. Previous studies have shown that diabetic rage−/− mice fail to develop thickening of the glomerular basement membrane (50) or neointimal proliferation after arterial injury (38). RAGE null mice are also protected from mortality induced by cecal ligation and perforation (25). Herein, we showed that gut mucosal barrier function was preserved in rage−/− but not rage+/+ mice 24 h after HS/R. In addition, plasma IL-6 levels were increased at 24 h after HS/R in rage−/− but not rage+/+ mice, suggesting that increased secretion of this proinflammatory cytokine after HS/R is mediated through a RAGE-dependent signaling pathway.

In previous studies using the same murine model of HS/R (53, 54), we showed that ileal mucosal permeability to FD4 increases as early as 4 h after resuscitation. Herein, we confirmed that ileal mucosal hyperpermeability is detectable at 4 h after resuscitation and showed that this abnormality persisted until at least 24 h after resuscitation. Whereas the development of mucosal barrier dysfunction at the late time point was prevented when mice were treated with sRAGE, the development of mucosal hyperpermeability at the earlier time point was not. Collectively, these observations support the view that mechanism(s) underlying the earlier phase of mucosal hyperpermeability are different from those responsible for the later phase. In the earlier phase, factors such as ATP depletion are likely important (47), whereas the proinflammatory effects of RAGE-dependent signaling appear to be important in the later phase.

Our laboratory previously reported that incubating Caco-2 monolayers with HMGB1 increases the permeability of these model epithelia (39). This effect can be partially inhibited by the addition of an anti-RAGE antibody (39), suggesting that at least some of the increase in permeability induced by HMGB1 is due to the interaction of this protein with RAGE. This notion is plausible since it is known that Caco-2 cells express RAGE (58, 59). In the present study, we showed that incubating Caco-2 monolayers with another potential ligand for RAGE, namely S100b, also increased epithelial permeability, further supporting the idea that RAGE-dependent signaling can lead to derangements in tight junction function in this enterocyte-like cell line. As was the case in our previously reported studies of Caco-2 cells incubated with HMGB1 (39), a longer incubation with the mediator (16 vs. 8 h) was associated with a greater increase in the permeability of the monolayers. Presumably, the alterations in tight junction formation that are induced by these mediators (i.e., HMGB1 and S100b) become progressively more apparent over time.

Although hemorrhagic shock is associated with a myriad of physiological and biochemical changes, one of the more prominent features is mesenteric hypoperfusion and, hence, gut mucosal hypoxia (56). Accordingly, it is noteworthy that previous studies using Caco-2 or T84 enterocyte-like monolayers have shown that hypoxia alone (46) or hypoxia followed by reoxygenation (7, 51) increases permeation of the epithelium by various hydrophilic probes. Herein, we confirmed these earlier observations and, in addition, showed that hypoxia/reoxygenation promoted the release of HMGB1 into the culture medium, a finding that is consistent with our recent report showing that immunostimulated enterocytes secrete HMGB1 (26). Herein, we also showed that adding either sRAGE or anti-RAGE antibody to the culture medium largely prevented the development of hyperpermeability induced by hypoxia/reoxygenation. In this reductionist model system, the protective effect of sRAGE was presumably due, at least in part, to neutralization of HMGB1 that was released into the medium as a result of hypoxia/reoxygenation, although neutralization of other (unmeasured) RAGE ligands also might have been important. The anti-RAGE antibody presumably interfered with the interaction of HMGB1 (or other unmeasured RAGE ligands) with RAGE on the surface of Caco-2 cells and thereby provided protection against hypoxia/reoxygenation-induced epithelial hyperpermeability. In addition to promoting the release of HMGB1 or other RAGE ligands, hypoxia/reoxygenation might also upregulate RAGE expression.

The notion that gut mucosal hypoxia or hypoxia/reoxygenation leads to increased expression of RAGE ligands is supported by the immunohistochemical and biochemical findings

**Fig. 7.** Effect of hypoxia/reoxygenation on HMGB1 release by Caco-2 monolayers growing in Transwell chambers. Hypoxia was maintained for a total of 16 h, and then normoxia was re-established for an additional 8 h. HMGB1 levels in apical supernatants were determined by Western blotting. A representative gel is depicted, and the densitometry represents results from 4 replicates per condition.
from the present study. In particular, we showed that HS/R increased expression of S100b and HMGB1 in ileal specimens. After HS/R in wild-type C57Bl/6 mice, focal areas of S100b expression were apparent, being localized for the most part to the muscularis propria. Previous studies (55) have shown that some postnatal myenteric neurons express S100b, so it is possible that these cells increase their expression of this protein after HS/R. It seems more likely, however, that increased S100b expression in the muscularis propria reflects infiltration by inflammatory cells. Previous studies by Kalff et al. (19) and Hierholzer et al. (14) showed that there is marked infiltration by neutrophils and macrophages into the muscularis propria of mice subjected to either the sham procedure or HMGB1 in mucosal scrapings. Additionally, treatment with sRAGE and macrophage markers, however, suggest that S100b was present in these cells types, previously shown to infiltrate the muscularis propria after HS/R (14, 19).

In mice treated with MSA, HS/R increased the amount of HMGB1 in mucosal scrapings. Additionally, treatment with sRAGE in mice subjected to either the sham procedure or HS/R decreased HMGB1 expression. Since ileal mucosal HMGB1 levels (as assessed by Western blotting) were not much different in MSA-treated sham hemorrhaged animals compared with completely normal (unmanipulated) mice (data not shown), our findings suggest that expression of this protein in the mucosa is constitutively upregulated by a mechanism that can be interrupted by treatment with sRAGE. It is tempting to speculate that the proinflammatory effects of the normal intestinal microflora contribute to this process (13), but further work will be needed to explore this idea.

In summary, we used both a pharmacological (treatment with sRAGE) and a genetic strategy (comparison of responses in rage +/+ vs. rage −/− mice) to demonstrate that RAGE-dependent signaling is important in the pathogenesis of gut barrier dysfunction in mice subjected to HS/R. Our findings, while emphasizing the importance of RAGE and its ligands in the pathogenesis of organ dysfunction after severe hemorrhage, should not be interpreted as indicating that other inflammatory pathways, such as signaling through Toll-like receptor 4 (35) or activation of the complement cascade (18), are not important as well.

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GRANTS
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