IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice

Runkuan Yang, Xiaonan Han, Takashi Uchiyama, Simon K. Watkins, Arino Yaguchi, Russell L. Delude, and Mitchell P. Fink. IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice. Am J Physiol Gastrointest Liver Physiol 285: G621–G629, 2003. First published May 28, 2003; 10.1152/ajpgi.00177.2003.—We sought to determine the role of IL-6 as a mediator of the alterations in gut barrier function that occur after hemorrhagic shock and resuscitation (HS/R). C57Bl/6 wild-type (WT) and IL-6 knockout (KO) mice on a C57Bl/6 background were subjected to either a sham procedure or HS/R. Organ and tissue samples were obtained 4 h after resuscitation. In WT mice, HS/R significantly increased ileal mucosal permeability to fluorescein isothiocyanate-labeled dextran (average molecular mass, 4 kDa) and bacterial translocation to mesenteric lymph nodes. These alterations in gut barrier function were not observed in IL-6 KO animals. HS/R increased ileal steady-state mRNA levels for IL-6, TNF, and IL-10 in WT but not in IL-6 KO mice. Ileal mucosal expression of the tight junction protein, ZO-1, decreased after HS/R in WT but not IL-6 KO mice. Collectively, these data support the view that expression of IL-6 is essential for the development of gut barrier dysfunction after HS/R.

mesenteric lymph nodes; mucosal permeability; inducible nitric oxide synthase

USING VARIOUS RODENT MODELS, we (42, 47–50) and others (6, 39) have shown that both mucosal permeability to hydrophilic solutes and bacterial translocation to mesenteric lymph nodes (MLNs) increases after resuscitation of rodents from hemorrhagic shock. These findings may have clinical implications, because increased intestinal permeability has been shown to be associated with an increased risk of complications, multiple organ dysfunction syndrome (MODS), or even mortality in critically ill patients (3, 13, 15, 33). It is conceivable that the development of intestinal epithelial hyperpermeability after hemorrhage directly promotes the development of MODS, perhaps by permitting systemic contamination with gut-derived microbes or microbial products. Alternatively, gut mucosal hyperpermeability might simply be a regional manifestation of a more generalized acquired defect in epithelial barrier function that also affects other organs, such as the lungs, liver, and kidneys.

The basis for the development of gut barrier dysfunction after hemorrhage is undoubtedly complex and multifactorial (17). Nevertheless, two lines of evidence suggest that increased production of the pluripotent cytokine IL-6 may be an important factor that contributes to the development of gut barrier dysfunction after hemorrhagic shock. First, IL-6 has been implicated as being at least partially responsible for increased gut mucosal permeability in mice with a condition that is associated with systemic inflammation, namely polymicrobial peritonitis induced by cecal ligation and perforation (44). Our laboratory and others (5, 31, 49, 50) have shown that hemorrhagic shock and resuscitation (HS/R) is also associated with increased expression of proinflammatory mediators in the mucosa of the gut as well as in other tissues. Thus the pathophysiological mechanisms responsible for organ dysfunction after HS/R might bear at least some similarities to those pertinent to sepsis. Second, Wang et al. (45) reported that HS/R in rats is associated with increased systemic arterial and portal venous plasma levels of IL-6, and we (49, 50) recently showed that HS/R in mice is associated with marked upregulation of IL-6 mRNA expression in samples of ileal mucosa. Prompted by this reasoning, we hypothesized that IL-6 knockout mice with targeted genetic disruption of IL-6 expression would be protected from gut mucosal barrier dysfunction induced by HS/R. The studies reported herein were carried out to test this hypothesis.

MATERIALS AND METHODS

This research protocol complied with the regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh Medical School. Male C57BL/6 wild-type and IL-6 knockout mice, age 4–8 wk, were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were maintained at 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 the experiments. All

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were performed: tobarbital sodium (90 mg/kg), and the following procedures: plus intra-arterial injection of 280 mmHg by the administration of all remaining shed blood/H11350. The animals were resuscitated to an initial MAP of 30 mmHg. MAP was maintained at 30 mmHg for 2.0 h with continuous monitoring of blood pressure and withdrawal and return of blood as needed. Cannulas, syringes, and tubing were flushed with heparin sodium (1,000 U/ml) before all procedures. The animals were resuscitated to an initial MAP of 80 mmHg by the administration of all remaining shed blood plus intra-arterial injection of 2× shed blood volume of RLS. Four hours after resuscitation (or sham shock), surviving mice were reanesthetized with intramuscular pento- tobarbital sodium (90 mg/kg), and the following procedures were performed: 1) a segment of ileum was harvested for determination of mucosal permeability; 2) the MLN complex was removed, weighed, and placed in a grinding tube containing 0.5 ml of ice-cold PBS. The MLN were homogenized with glass grinders, and a 250-μl aliquot of the homogenate was plated onto brain-heart infusion and MacConkey’s agar (Becton Dickinson, Franklin Lakes, NJ). The plates were examined 24 h later after being aerobically incubated at 37°C. The colonies were counted, and results were expressed as colony-forming units per gram of tissue.

**Serum ALT measurement.** Blood (200 μl) was obtained by cardiac puncture and placed in a 0.5-ml centrifugation tube on ice. The samples were then centrifuged at 5,000 g for 3 min. The serum was collected and assayed for ALT by using an automated assay system.

**Determination of IL-10 protein concentrations.** IL-10 levels were determined in serum samples by ELISA by using a commercially available kit from Pharmingen (San Diego, CA). The lower limit of detection as described by the manufacturer was <1 pg/ml.

**Immunoprecipitation and Western blot analysis.** An 8- to 9-cm segment of ileum was gently scraped twice with a glass microscope slide to obtain the mucosal tissue. The scrapings were homogenized on ice with a Polytron tissue homogenizer in 1 ml of cold Nonidet P-40 (NP-40) isoinsoluble buffer (in mM: 25 HEPES, 1,4,5-triphosphate buffer (in mM: 25 HEPES, 150 NaCl, 4 EDTA, 25 NaF, 1 Na3VO4, 1 4-amidinophenylmethanesulfonyl fluoride, and 1% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4). The samples were centrifuged at 12,000 g for 30 min at 4°C, and the pellets (membrane fraction) were resuspended in SDS-dissolving buffer (in mM: 25 HEPES, 4 EDTA, 25 NaF, 1 Na3VO4, pH 7.5, and 1% SDS) by using five strokes with a Dounce homogenizer (pestle B) followed by sonication with a microtip on power setting 3. Sonication was continued until the precipitates were completely dissolved.

One hundred micrograms of the NP-40-insoluble fraction was immunoprecipitated with either rabbit anti-occludin or anti-ZO-1 polyclonal antibodies (Zymed Laboratories, South San Francisco, CA). The lysate was precleared by adding 0.25 μg of normal mouse IgG, together with 20 μl of suspended protein A/G agarose (Santa Cruz Biotech, Santa Cruz, CA). After incubation at 4°C for 30 min, the beads were collected by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube, 3 μg of anti-occludin or anti-ZO-1 antibody were added, and the tube was incubated on a rocker platform for 2 h at 4°C. Resuspended agarose AV (20 μl) was added to the tube, and the incubation was continued overnight at 4°C with gentle shaking. The agarose beads were washed five times with 1 ml of NP-40 lysis buffer. Proteins were eluted by boiling in 1× Laemmli buffer (10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, 0.1 M Tris-HCl, pH 6.8, and 0.2% bromphenol blue) for 10 min.

Equal volumes of samples were electrophoresed on 7.5% precast SDS-PAGE (Bio-Rad, Hercules, CA). The proteins were electroblotted onto Hybond-P polyvinylidene difluoride membrane (APBiotech, Leicester, England) and blocked with Blotto (1% BSA, 5% milk, 0.05% Tween-20, and 0.2% NaN3) for 60 min. The filter was incubated at room temperature for 1 h with anti-ZO-1 or anti-occludin antibody at a 1:4,000 dilution in PBST (PBS and 0.02% Tween-20). After being washed three times in PBST, immunoblots were exposed for 1 h to a 1:20,000 dilution of anti-rabbit horseradish peroxidase antibody. After washing three times, the membranes were incubated in an enhanced chemiluminescence Western blotting reagent (Amersham, Arlington Heights, IL) for 5 min at room temperature. The membranes were then exposed to x-ray film for 1 to 2 days at −80°C.
ish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). After three washes in PBST and two washes in PBS, the membrane was impregnated with the enhanced chemiluminescence substrate (Amersham Pharmacia Biotech) and used to expose X-ray film. Autoradiographs were captured by using a ScanJet 6300s (Hewlett Packard, Palo Alto, CA). Band intensities were quantified by densitometry and expressed as mean area density by using GelExpert version 3.5 software (Nucleotech, San Mateo, CA).

**RESULTS**

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**Intestinal barrier dysfunction.** Consistent with previously published results (49, 50), subjecting WT mice to HS/R significantly increased ileal mucosal permeability to FD-4 (Fig. 1A). In contrast, when IL-6 KO mice were subjected to HS/R, ileal mucosal permeability was not increased. Interestingly, intestinal permeability in sham-treated IL-6 KO mice was somewhat higher than in sham-treated WT controls. Although this difference did not quite achieve statistical significance, the observation suggests that basal IL-6 production may be necessary for formation of a completely normal mucosal barrier. As expected, bacterial translocation to MLN was minimal in sham-hemorrhaged animals, irrespective of whether they carried the IL-6<sup>+/+</sup> or IL-6<sup>−/−</sup> genotype (Fig. 1B). Translocation increased significantly in conventional mice subjected to HS/R, but not in IL-6 KO mice subjected to the same insult.

**Hepatocellular damage.** The mean plasma ALT concentration increased significantly after HS/R in both conventional and KO mice (Fig. 2). The circulating levels of ALT were significantly lower after HS/R in IL-6 KO compared with WT animals.

**Circulating IL-10 concentrations.** We were prompted to measure circulating levels of IL-10 because of find-
proteins, such as ZO-1 and occludin, involved in the changes in the expression and localization of various epithelial permeability are associated with marked inflammation-induced changes in intestinal epithelium in vitro (22) and in vivo (10) studies support the view that in septic animals. In sham-treated WT and KO animals. After HS/R, however, large gaps in ZO-1 staining were apparent in WT mice. In the WT-HS/R group, ~50% of the villi showed marked abnormalities in ZO-1 immunofluorescent staining. However, the pattern of ZO-1 immunofluorescence was minimally affected after HS/R in KO mice. Among sham-treated animals, ileal mucosal expression of occludin was greater in IL-6−/− compared with IL-6+/+ mice (Fig. 8). After HS/R, occludin expression decreased in both WT and KO groups. However, after HS/R, occludin almost vanished in samples from WT mice, whereas high levels of occludin expression were still apparent in KO mice.

DISCUSSION

IL-6 is a 26-kDa pluripotent cytokine that is produced by many different cell types including activated monocytes and macrophages (1), endothelial cells (27), adipocytes (32), T cells (25) and enterocytes (29). The biological effects of IL-6 are quite diverse and range from stimulation of hepatocyte proliferation to suppression of the pituitary-thyroid axis (34). With regard to the innate immune response, IL-6 has been shown to have both pro- (7, 24, 31, 35) and anti-inflammatory effects (2, 12) depending, at least in part, on whether the cytokine is acting in a paracrine or endocrine manner (16).

A number of previous studies have examined the role of IL-6 as a mediator of organ system injury or dysfunction in experimental models of HS/R. Based on results from these investigations, we know that circulating levels of IL-6 are increased in mice (40) and rats formation of tight junctions. Accordingly, we sought to assess the effects of HS/R in WT and IL-6 KO mice on ZO-1 and occludin expression. In WT mice, ileal mucosal ZO-1 expression, as assessed by immunoprecipitation and Western blotting, markedly decreased after HS/R (Fig. 6). In contrast, ileal mucosal ZO-1 expression was not affected by HS/R in IL-6 KO mice. These findings were confirmed when we performed immunofluorescence with the use of an anti-ZO-1 antibody. As shown in Fig. 7, staining for ZO-1 was apparent as a continuous band along the villous epithelium in sham-treated WT and KO animals. After HS/R, however, large gaps in ZO-1 staining were apparent in WT mice. In the WT-HS/R group, ~50% of the villi showed marked abnormalities in ZO-1 immunofluorescent staining. However, the pattern of ZO-1 immunofluorescence was minimally affected after HS/R in KO mice. Among sham-treated animals, ileal mucosal expression of occludin was greater in IL-6−/− compared with IL-6+/+ mice (Fig. 8). After HS/R, occludin expression decreased in both WT and KO groups. However, after HS/R, occludin almost vanished in samples from WT mice, whereas high levels of occludin expression were still apparent in KO mice.

Data from prior in vitro (22) and in vivo (10) studies support the view that inflammation-induced changes in intestinal epithelial permeability are associated with marked changes in the expression and localization of various proteins, such as ZO-1 and occludin, involved in the

Expression of proinflammatory and anti-inflammatory genes. We used semiquantitative RT-PCR to estimate steady-state mRNA levels for several proinflammatory cytokines as well as the anti-inflammatory cytokine IL-10. 18S RNA was used as an internal control to document equal loading of RNA. Consistent with earlier results from our laboratory (49), HS/R was associated with marked upregulation of IL-6 expression in liver (Fig. 4) and ileal mucosa (Fig. 5) in WT mice. As expected, IL-6 mRNA was undetectable in KO mice. In the liver, HS/R was associated with greater upregulation of COX-2 expression in WT compared with KO mice (Fig. 4). However, hepatic upregulation of TNF and IL-10 mRNA induced by HS/R tended to be greater in IL-6−/− compared with IL-6+/+ animals. In ileal mucosa, mice in both the KO-SHAM and KO-HS/R groups had relatively low levels of COX-2 and IL-10 mRNA expression compared with mice in the WT-SHAM and WT-HS/R groups (Fig. 5). Whereas iNOS, IL-10, and TNF mRNA expression in ileal mucosa tended to increase after HS/R in WT mice, expression of these genes was minimally changed or even decreased after HS/R in KO mice.

Expression of tight-junction proteins. Data from prior in vitro (22) and in vivo (10) studies support the view that inflammation-induced changes in intestinal epithelial permeability are associated with marked changes in the expression and localization of various proteins, such as ZO-1 and occludin, involved in the

![Fig. 2. Effect of HS/R on circulating alanine aminotransferase (ALT) concentration assessed 4 h after the end of shock (or sham shock) in WT and KO mice. Sample sizes were as follows: WT-SHAM, n = 18; KO-SHAM, n = 6; WT-HS/R, n = 22; KO-HS/R, n = 6 mice. Results are means ± SE. Groups and symbols are the same as in Fig. 1.](http://ajpgi.physiology.org/)

![Fig. 3. Plasma IL-10 concentration assessed 4 h after the end of shock (or sham shock) in WT and IL-6 KO mice. Data are means ± SE (n = 6 mice/condition). Groups and symbols are the same as in Fig. 1.](http://ajpgi.physiology.org/)
(21) after resuscitation from hemorrhage. Expression of IL-6 mRNA and protein is upregulated in the lungs, liver, and intestinal tracts of rodents subjected to HS/R (23, 24, 49, 50). Furthermore, based on studies carried out with the use of IL-6−/− mice, IL-6 seems to be essential for the development of inflammation in the lung and liver after HS/R (31). Herein, we extended these observations by showing that gut mucosal barrier dysfunction was attenuated in KO compared with WT mice.

Our results are largely consistent with some earlier findings regarding the role of IL-6 as a mediator of intestinal mucosal injury in various conditions associated with transient mesenteric hypoperfusion and subsequent inflammation. For example, Cuzzocrea et al. (8) reported that gut mucosal inflammation and histological damage were markedly attenuated in IL-6−/− compared with IL-6+/+ mice subjected to splanchnic artery occlusion and reperfusion. In another study (9), the same laboratory showed that intestinal inflammation induced by intraperitoneal administration of zymosan was decreased in IL-6−/− compared with IL-6+/+ mice. More recently, Wang et al. (44) compared changes in intestinal permeability to FD-4 in WT and KO mice 16 h after the induction of sepsis by cecal ligation and perforation. Whereas sepsis was associated with a marked increase in mucosal permeability in IL-6+/+ mice, intestinal permeability was essentially unchanged after the induction of sepsis in IL-6−/− mice. Also, in another study (4), blocking the IL-6-dependent signaling pathway by administering a neutralizing antibody against the IL-6 receptor decreased mucosal inflammation in several murine models of inflammatory bowel disease.
Despite the similarity of our results with the findings from some of these earlier studies, one key observation was quite different. In the study cited above by Wang et al. (44), plasma levels of the counterregulatory cytokine IL-10 were 20-fold higher in septic IL-6−/− compared with septic IL-6+/+ mice. Furthermore, mucosal concentrations of IL-10 protein were substantially higher in both sham-operated and septic IL-6−/− mice compared with sham-treated or septic IL-6+/+ mice (44). Our findings in mice subjected to HS/R were quite different. Circulating levels of IL-10 were more than five times higher in hemorrhaged compared with sham-treated mice, regardless of whether the genotype was IL-6+/+ or IL-6−/−. Furthermore, IL-10 mRNA expression in ileal mucosa increased dramatically after HS/R in WT mice, but there was little or no increase in IL-10 mRNA expression after HS/R in mucosal samples from IL-6 KO mice. At first glance, it is hard to rationalize the marked differences in our findings from those reported by Wang et al. (44) with respect to local and systemic expression of IL-10. However, the key difference between the two studies was the nature of the inciting proinflammatory stimulus, i.e., polymicrobial peritonitis in the earlier report vs. HS/R in the present one. In the study by Wang et al. (44), in addition to IL-10, circulating levels of IL-1β were also much higher in KO compared with WT mice, suggesting that the absence of circulating IL-6 may have exaggerated the proinflammatory systemic response to bacterial contamination of the peritoneum. Because the amount of IL-10 released by stimulated macrophages is very dependent on the presence of IL-1β and TNF (18), the increased release of IL-10 observed in the study by Wang et al. (44) may have reflected

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**Fig. 5.** Ileal mucosal expression of IL-6, IL-10, iNOS, COX-2, and TNF mRNA in WT or IL-6 KO mice subjected to HS/R or the sham procedure. Results were obtained by using semiquantitative RT-PCR as described in MATERIALS AND METHODS. Data in bar graphs are means ± SE (n = 3 or 4 mice/condition). Representative gels are depicted. 18S RNA was measured to verify equal loading of RNA. Lanes 1, 2, 3, and 4 represent WT-SHAM, WT-HS/R, KO-SHAM, and KO-HS/R, respectively.
disinhibition of the inflammatory response to sepsis due to the absence of circulating IL-6. In contrast to the findings in the cecal ligation and perforation model, targeted deletion of IL-6 expression in the HS/R model appeared to result in global downregulation of the inflammatory response to this insult in the ileal mucosa.

Although our findings support the view that excessive IL-6 secretion is essential for gut mucosal barrier dysfunction after HS/R, and these findings are consistent with the apparently essential role of IL-6 as a mediator of inflammation in other organs after hemorrhage (31), the results presented here seem to conflict with a series of papers showing that enteral administration of recombinant IL-6 protects rodents from mucosal damage caused by mesenteric ischemia and reperfusion or HS/R (36–38). Our data also might be
construed as conflicting with data obtained by Meng et al. (30), who showed that intravenous administration of exogenous IL-6 downregulates post-HS/R inflammation in the lung and liver of rats. The apparent discordance between the pharmacological effects of exogenous IL-6 compared with the pathophysiological effects of endogenous IL-6 on post-HS/R inflammation and/or organ dysfunction is not easy to explain. However, as already noted, IL-6 defies simple categorization as a proinflammatory or anti-inflammatory mediator (16, 28, 43), and the qualitative effects of this cytokine seem to be concentration dependent.

Normal epithelial permeability is maintained and regulated by the tight junctions between adjacent cells (11, 41). The formation of tight junctions requires the assembly of several proteins anchored directly or indirectly to the actin-based cytoskeleton. Integral membrane proteins involved in tight junction formation include occludin and members of a large class of proteins called claudins. Recent results (26) suggest that phosphorylation of occludin may be important in the regulation of tight junction permeability in response to histamine and lysophosphatidic acid. Cosegregation assays of tight junction proteins suggest that there is a strong interaction between occludin and ZO-1, which is another protein associated with tight junction formation (14). ZO-1 has been shown to interact with the cytoplasmic tails of occludin and the claudins. The integrity of tight junctions is maintained by these (and probably other proteins), and thus the normal control of paracellular permeability is highly dependent on their proper expression and localization. In the present study, we used immunoprecipitation and Western blotting and immunofluorescence to show that HS/R in WT mice was associated with altered localization and decreased expression of ZO-1 and occludin in intestinal epithelium. To our knowledge, these findings have not been reported previously. Moreover, we showed that the alterations in expression of ZO-1 and occludin were much less apparent in KO mice, suggesting that IL-6 was required for both the functional and structural abnormalities in the gut epithelial barrier after HS/R.

In summary, we used inbred mice with a genetic deficiency of IL-6 to show that production of this cytokine is essential for the development of gut barrier dysfunction after HS/R. It is noteworthy that previous studies (19, 20) showed that the administration of an anti-IL-6 antibody decreases bacterial translocation in burn injured rodents. Taken together, these findings support the view that therapeutic strategies targeting IL-6, such as neutralizing antibodies directed against IL-6 or its receptor, might prove beneficial in myriad conditions associated with abnormalities in gut barrier function. Further investigative efforts along these lines are warranted.

DISCLOSURES

This study was supported by National Institute of General Medical Sciences Grants GM-53789, GM-37631, and GM-58484.

REFERENCES