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

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Yang, Runkuan, 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—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: surviving mice were reanesthetized with intramuscular pen-H11003
80 mmHg by the administration of all remaining shed blood procedures. The animals were resuscitated to an initial MAP return of blood as needed. Cannulas, syringes, and tubing were flush-mounted with digital readout (model 78342A; Hewlett Packard, Santa Clara, CA). The right artery was used for blood withdrawal and blood and fluid administration. After vascular cannulation, the mice were subjected to hemorrhagic shock by withdrawal of blood (2.25 ml/100 g body wt) over, above a mean arterial pressure (MAP) of 30 mmHg. MAP was maintained at 30 mmHg for 2 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 ≥80 mmHg by the administration of all remaining shed blood plus intra-arterial injection of 2× shed blood volume of RLS over 30 min. Four hours after resuscitation (or sham shock), surviving mice were reanesthetized with intramuscular pentobarbital 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 harvested to measure bacterial translocation; 3) blood was aspirated from the heart to measure the plasma concentration of alanine aminotransferase (ALT); and 4) portions of the liver and ileum were harvested for determination of the expression of several genes by the use of semiquantitative RT-PCR.

Intestinal mucosal permeability. Intestinal mucosal permeability to the fluorescent tracer, FITC dextran with a 4 kDa mol mass (FD-4), was determined by using an everted gut sac method, as previously described by Wattanasirichai-goon et al. (46) and modified by Yang et al. (49) for use with mice. Everted gut sacs were prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). One end of the gut segment was ligated with a 4-0 silk suture. The segment was then everted onto a thin plastic rod, and the resulting gut sac was secured with a 4-0 silk suture to the grooved tip of a 3-ml plastic syringe containing KHBB. The sac was gently distended by injecting 1.5 ml KHBB. The sac was suspended in a 50-ml beaker containing 40 ml KHBB plus FD-4 (40 mg/ml). The solution in the beaker was temperature-jacketed at 37°C and was continuously bubbled with a gas mixture containing 95% O2-5% CO2. A 1.0-ml sample was taken from the beaker before it was put in the gut sac to determine the initial external (i.e., mucosal surface) FD-4 concentration. The sac was incubated for 30 min in the KHBB solution containing FD-4. The length of the gut sac was measured. Fluid from the inside of the sac was aspirated for the determination of FD-4 concentration. The serosal and mucosal samples were centrifuged for 10 min at 1,000 g. Three hundred microliters of the supernatant were diluted with PBS (2.7 ml). Fluorescence was measured by using an LS-50 fluorescence spectrophotometer (Perkin-Elmer, Palo Alto, CA) at an excitation wavelength of 492 nm (slit width, 2.5 nm) and an emission wavelength of 515 nm (slit width, 10 nm). Permeability was expressed as the mucosal-to-serosal clearance of FD-4, as previously described (49).

Bacterial translocation. The skin was cleaned with 10% povidone-iodine. With the use of sterile technique, the abdominal cavity was opened and the viscera were exposed. 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) isositol-1,4,5-trisphosphate buffer (in mM: 25 HEPES, 150 NaCl, 4 EDTA, 25 NaF, 1 Na3VO4, 1 4-aminophenylmethanesulfonyl 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 0.1 W Fisher Scientific sonic dismembrator fitted 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 A/G (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 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.

After being washed three times in PBST, immunoblots were exposed for 1 h to a 1:20,000 dilution of anti-rabbit horserad-
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).

RT-PCR. Steady-state levels of TNF, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 mRNA were estimated by using semiquantitative RT-PCR and methods and primers that have been previously reported in detail by our group (49). The PCR conditions for amplifying cDNA for IL-10 and IL-6 were denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and polymerizing at 72°C for 45 s for 35 cycles. This number of PCR cycles was empirically determined to ensure that amplification was in the linear range. After the last cycle of amplification, the samples were incubated at 72°C for 10 min and then held at 4°C. The 5′- and 3′-primers for IL-10 were CCT GGT AGA AGT GAT GCC CCA G and GCA GTT GAT GAA GAT GTC AAA, respectively, and the expected product length was 237 bp. The 5′- and 3′-primers for IL-6 were TTC CAT CCA GTT GCC TTC TTG G and TTC TCA TTT CCA CGA TTT CCC AG, respectively, and the expected product length was 174 bp.

Imaging of ZO-1 using immunofluorescence. Frozen tissue sections (4 μm) were prefixed in cold acetone and then air dried. The sections were fixed with 4% paraformaldehyde and then washed three times with cold PBS. The sections were blocked with 1:10 donkey serum and then washed three times with cold PBS. Tissue sections were incubated with 1:200 dilutions of rabbit anti-ZO-1 polyclonal antibodies. After a 1-h incubation at room temperature, the sections were washed three times with PBS. Tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit secondary antibody was added and incubated for 45 min at room temperature. The sections were washed three times with PBS, and the nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes, Eugene, OR). Coverslips were mounted by using the Antifade Kit from Molecular Probes. Images were captured by using an Olympus Provis fluorescence microscope equipped with a cooled charge-coupled device camera (Magnafire; Olympus, Melville, NY) using the 400× oil immersion objective lens.

Statistical methods. In general, results are presented as means ± SE. Bacterial translocation data were analyzed by using the Mann-Whitney U-test. Other continuous data were analyzed by using Student’s t-test or ANOVA followed by Fisher’s least significant difference test, as appropriate. P values < 0.05 were considered significant. Summary statistics are presented for densitometry results from studies with the use of RT-PCR to estimate mRNA expression and Western blotting to estimate ZO-1 and occludin expression, but these results were not subjected to statistical analyses, because the methods employed were only semiquantitative and the sample sizes (n = 3–4) were small (49).

RESULTS

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 WT mice (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 epi-fl

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 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.

**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...
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 KO compared with septic IL-6 WT mice. Furthermore, mucosal concentrations of IL-10 protein were substantially higher in both sham-operated and septic IL-6 KO mice compared with sham-treated or septic IL-6 WT 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 KO or IL-6 WT. 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...
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. Cosedimentation 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

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