Hypertonic saline reduces neutrophil-epithelial interactions in vitro and gut tissue damage in a mouse model of colitis

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ALTHOUGH INFLAMMATORY DISEASES of the intestine are etiologically diverse, many of these diseases express similar histological features. Accumulation of neutrophils [polymorphonuclear neutrophils (PMN)] in the lamina propria is a major hallmark of the acute inflammatory response in disorders ranging from inflammatory bowel disease (IBD) to shock-induced gut dysfunction. In IBD, PMN also transmigrate from the lamina propria in the gut lumen. By measuring fecal excretion of leukocytes labeled with indium-111, migration of PMN from the circulation in the diseased intestine has been estimated to be increased by at least 10-fold in IBD patients (23). PMN are rapidly recruited from the bloodstream by cell adhesion to the vascular endothelium, transendothelial migration, and chemotaxis to the local inflammatory site. After their recruitment to such sites, PMN exert cytotoxic effects that can damage gut tissues via the release of cytotoxic mediators. A complex assortment of these cytotoxic agents, including reactive oxygen metabolites and proteolytic enzymes, is released during this inflammatory process and causes host tissue damage. In addition, PMN can further modulate such inflammatory processes by immunoregulatory functions they exert via the secretion of several important cytokines, including interleukin (IL)-1, IL-1 receptor antagonist, IL-6, IL-8, tumor necrosis factor-α, granulocyte colony-stimulating factor, and granulocyte macrophage colony-stimulating factor (15).

The transepithelial migration of PMN in response to chemotactic stimuli depends on expression of integrins such as CD11b/18 and results in a disruption of epithelial barriers as demonstrated by transient decreases in transepithelial resistance in various in vitro epithelial models (17, 20). Several studies have shown that this decrease in epithelial barrier function is directly related to the number of transmigrating PMN. The integrity of the intestinal epithelium is crucial for preventing translocation of luminal pathogens in the lamina propria. Dysfunction of the epithelial barrier, therefore, could initiate an uncontrolled mucosal immune response. Thus the transepithelial migration of PMN, associated with a decrease in epithelial resistance, is likely an important step in the inflammatory cascade associated with IBD. Similar processes may be responsible for gut barrier dysfunction in hemorrhagic shock where hypoxemia causes the impairment of epithelial barrier function that is followed by translocation of luminal contents and stimulation of subepithelial immunocytes (9). Recent evidence suggests that cytotoxic mediators from the gut carried in the mesenteric lymphatics mediate secondary tissue injury (e.g., in the lungs) in hemorrhagic shock conditions (16).

Different strategies have been developed to inhibit intestinal inflammatory responses in attempts to treat IBD. These strategies range from the use of anti-inflammatory drugs to immune modulating substances. However, the efficacy of these treatments is limited, and some are associated with considerable side effects. Hence, additional treatment strategies for IBD are needed. Recently, there has been renewed interest in the use of hyperosmolar resuscitation fluids in shock therapy. Infusion of hypertonic saline (HS) results in a rapidly increased intravascular blood volume due to osmotically induced fluid shifts from the extravascular compartments to the vascular bed (28).
Hypertonic fluids have been shown to benefit patients with hypovolemic shock and head injury (2, 25, 29). Moreover, HS also modulates PMN function (reviewed in Ref. 26). Treatment of isolated PMN with HS attenuates several cytotoxic responses, including the release of proteolytic enzymes and reactive oxygen metabolites or upregulated \( \beta_2 \)-integrin expression induced by \( N \)-formyl-methionyl-leucyl-phenylalanine (fMLP) (6, 12, 27). In a dorsal skinfold chamber model, postischemic leukocyte rolling and sticking were significantly reduced when animals were treated with HS (18). Recent studies have shown that the underlying mechanisms involve the release of cellular ATP, formation of extracellular adenosine, and the stimulation of A2a adenosine receptors that increase intracellular cAMP and thereby block PMN activation (4, 5, 19).

This work suggests that HS-treatment may reduce PMN-associated tissue damage in IBD. We therefore sought to determine if treatment of isolated PMN with HS could inhibit disruption of epithelial barrier function and gut tissue damage in a mouse model of colitis.

**MATERIALS AND METHODS**

**Isolation of PMN.** Heparinized blood was obtained from healthy donors. After sedimentation in 5% dextran T500, red blood cells were discarded, and PMN were isolated from the remaining fraction using gradient centrifugation with 55 and 73% Percoll. The resulting PMN preparation was washed two times with Hanks’ balanced salt solution (HBSS). Cell viability was >98%, as determined by trypan blue dye exclusion. After isolation, cells were resuspended in HBSS and adjusted to a concentration of 10⁷ cells/ml. For transmigration studies, PMN were incubated with different concentrations of HS (ranging from 5 to 40 mM). After 20 min, cells were washed and resuspended in HBSS.

**T84 cell monolayers.** T84 human colonic epithelial cells (passages 23–28) were seeded on 0.6-cm² Millipore polycarbonate filters and grown in Dulbecco’s modified Eagle’s medium-F-12 medium supplemented with 5% newborn calf serum until they formed confluent monolayers with resistances >1,500 \( \Omega \cdot \text{cm}^2 \), using methods that have been described previously (1).

**Electrophysiological studies of PMN transepithelial migration.** Plates containing confluent T₈₄ monolayers on filter supports were placed horizontally on a heating block at 37°C, and spontaneous potential differences generated across the cell monolayers were negated by the application of short-circuit current via agar bridges. Isolated PMN (400 \( \mu \)l of a cell suspension containing \( 1 \times 10^7 \) cells/ml) were carefully placed on the apical side of the monolayer. To establish a chemotactic gradient, fMLP (10⁻⁶ M) was added to the basolateral compartment. Changes in monolayer conductance (the inverse measure of transepithelial resistance) associated with PMN transmigration were then monitored for 70 min.

**Fluorescence-activated cell sorter analysis.** CD11b expression was analyzed using flow cytometry and single color staining. Briefly, PMN (0.5 × 10⁶ cells) were incubated with human serum (10 \( \mu \)l/100 \( \mu \)l cell suspension) to block nonspecific binding. The cells were then incubated with a monoclonal mouse anti-CD11b antibody (Serotec, Oxford, UK). After two washings, PMN were incubated with a phycocerythrin-labeled rabbit anti-mouse IgG antibody (Accurate, Westbury, NY). Cells were washed, and fluorescence was analyzed and reported as the mean channel fluorescence using a FACScalibur (BD Biosciences, San Jose, CA). An isotype-matched mouse IgG antibody (Accurate) served as the negative control. All steps were performed at 4°C.

**Induction of colitis.** Male Balb/c mice weighing 18–20 g were used for induction of colitis. All studies received approval from the University of California San Diego Committee on Investigations Involving Animal Subjects. Animals were housed in a room maintained at...
22°C and kept on standard laboratory pellet food. Mice were treated ad libitum with 5% dextran sulfate sodium (DSS), molecular weight 40,000 (ICN Biomedicals, Aurora, OH), in the drinking water for 7 days. The treatment groups received 4 or 8 ml/kg of 7.5% saline intraperitoneally two times daily. One control group was injected with comparable volumes of normal saline (NS), whereas another control group received no intraperitoneal treatment. Disease was determined according to a disease activity index (DAI), which reflects weight loss, stool consistency, and positive hemoccult or rectal bleeding (7). The DAI was calculated daily. After 7 days, the mice were killed, and colonic tissue was obtained for histological grading. Colonic tissue samples were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histological grading was performed by an experienced pathologist (Wolf) who was blinded to the treatment modalities. A score from 0 to 4 was used, reflecting epithelial architecture and subepithelial leukocyte infiltration (Table 1).

Statistical analyses. All data are expressed as means ± SE for a series of n experiments. Student’s t-test or ANOVA with post hoc analysis (least-significant difference test) was used to compare mean values as appropriate; P values <0.05 were considered to be significant.

RESULTS

Treatment of PMN with HS inhibits the disruption of T84 intestinal epithelial cell monolayers. Figure 1 shows representative micrographs of T84 cell monolayers before and after PMN transmigration, resulting in a disruption of the continuity of the epithelial cell lining by interposed PMN. As shown in Fig. 2, the conductance of T84 monolayers essentially doubled 3 min after adding PMN (4 × 10⁶) apically and fMLP (10⁻⁶ M) basolaterally. The conductance continued to increase over 70 min, suggesting ongoing damage to the epithelial monolayer by transmigrating PMN. However, pretreatment of PMN with HS (to increase the extracellular tonicity by 40 mM) for 20 min before addition of the PMN to T84 monolayers significantly inhibited damage to the T84 epithelial cell monolayers. This effect of HS was concentration dependent over a range of 5–40 mM (Fig. 3), which represents clinically feasible levels of hypertonicity. These data combined with previous reports that HS can block PMN chemotaxis (12) suggest that decreased PMN transmigration is responsible for the decrease in T84 cell damage.

Fig. 3. The inhibitory effect of HS on PMN transmigration is dose dependent. Neutrophils were incubated with different doses of HS (5, 10, 20, and 40 mM beyond isotonicity) or HBSS (PMN alone). *Time points where the responses were significantly different among the groups. *P < 0.05 vs. PMN alone by ANOVA, means for n = 3–5. Error bars have been omitted for clarity.

Fig. 4. The ability of HS to inhibit responses associated with PMN transmigration is not caused by downregulation of CD11b. Isolated PMN were incubated in HS (40 mM hypertonicity) or HBSS and stimulated with fMLP (10⁻⁶ M) for 20 min. CD11b expression on unstimulated cells (PMN) was compared with that on fMLP-stimulated PMN (fMLP) or PMN coincubated with fMLP and HS (fMLP + HS), as determined by fluorescence-activated cell sorter (FACS) analysis. MCF, mean channel fluorescence. *Significant differences between both fMLP-treated groups. **P < 0.01 by ANOVA, means ± SE, n = 5.
Inhibition of PMN transmigration by HS is not caused by downregulation of CD11b. To investigate whether the inhibitory effect of HS on PMN transepithelial migration was due to downregulation of the cell-surface expression of CD11b, we stimulated PMN with fMLP at the same concentration used in transmigration studies (i.e., $10^{-6}$ M) in the absence or presence of HS treatment and determined CD11b expression by fluorescence-activated cell sorter analysis. fMLP stimulation resulted in a significant increase in CD11b expression compared with unstimulated PMN. This effect, however, was not attenuated by either simultaneous treatment of PMN with fMLP and HS (Fig. 4), or by pretreatment of PMN with 40 mM HS before

![Fig. 5. Effect of HS or normal saline (NS) treatment on macroscopic and histological appearance of dextran sulfate sodium (DSS)-induced colitis in mice. Treatment with 7.5% HS (4 and 8 ml/kg), bid ip, but not with NS resulted in a marked reduction of inflammatory tissue damage in DSS mice. Healthy mice served as controls. As shown in A, after 7 days of DSS treatment, the colon was hyperemic with gross blood in the cecum and a lack of formed stool pellets. In HS-treated DSS mice, only mild hyperemia and no gross bleeding was observed. Stool pellets were seen in the colonic lumen of HS-treated mice and controls. Colons from mice treated with DSS + NS, on the other hand, still showed evidence of inflammation and poorly formed stools. B: corresponding histological appearances. In colonic sections of mice receiving DSS alone, severe epithelial damage (block arrow) and dense PMN infiltration was seen (note infiltrating PMN in the submucosa). A similar appearance was seen in NS-treated mice. In HS-treated mice, only slight inflammation was seen (hematoxylin and eosin staining).]
addition of fMLP (data not shown). These data suggest that HS inhibits epithelial transmigration by affecting PMN responses other than CD11b expression. The results also indicate that the effect of HS on PMN function documented above is likely not a reflection of nonspecific cytotoxicity.

**HS treatment improves the clinical and histological activity of DSS colitis in Balb/c mice.** To determine whether the effects of HS on PMN-epithelial interactions observed in vitro were paralleled in an in vivo setting, we studied the effect of HS treatment in a well-established murine colitis model. As expected, administration of DSS to mice for 7 days caused severe colitis (Fig. 5) accompanied by an increase in the DAI (Fig. 6).

In keeping with our findings with HS in vitro, treatment with 7.5% HS at a dose of 4 or 8 ml/kg resulted in a significant reduction in intestinal tissue damage. This protective effect was evident macroscopically (Fig. 5A). Histological grading of tissue injury revealed that, in animals receiving DSS in combination with HS or NS, only HS had a significant beneficial effect at both concentrations (Fig. 7). These data suggest that the ability of HS to diminish PMN transmigration and perhaps other PMN functions may ameliorate their deleterious effect on epithelial function. Similarly, while treatment with 7.5% HS at a dose of 4 ml/kg resulted in a significant reduction in DAI, such effects were not seen with administration of 4 ml/kg NS. However, when higher volumes (8 ml/kg) of HS or NS were administered, the clinical benefit of NS was superior to that of HS. This implies that at least a part of the overall ability to reduce clinical signs of disease may relate to improved hydration of the animals and/or that toxic side effects may occur at the higher dose of HS.

**DISCUSSION**

Intestinal inflammation underlies a diverse group of digestive diseases that are often accompanied by migration of neutrophils in the intestinal lumen. HS, on the other hand, has recently emerged as a simple, inexpensive, and largely non-toxic means to modulate PMN function. In the current study, we examined whether HS could alter the interactions of PMN with intestinal epithelial cells and whether this might have beneficial consequences in the setting of colitis. In fact, in addition to other inhibitory effects of HS on PMN function described previously, we could demonstrate a HS-mediated inhibition of PMN transmigration across epithelial monolayers in vitro. Rosengren et al. (21) demonstrated in a Boyden chamber system that hyperosmolar shrinking decreases PMN migration toward fMLP (21). Similarly, Junger et al. (12) showed that HS reduces PMN chemotaxis induced by zymosan-activated serum. However, and significantly, pretreatment with HS was also able to abrogate inflammatory responses and tissue injury in a mouse model of colitis.

Osmotic stress is associated with a rearrangement of various components of the cytoskeleton. DiCiano et al. provided evidence for a hypertonicity-induced accumulation of F-actin, cortactin, and increased myosin light chain phosphorylation (10, 11). Newly polymerized F-actin localizes predominantly under the cell membrane in the form of a thick ring that persists as long as hypertonicity is maintained. The authors speculate that this mechanism could offer mechanical protection against the deleterious effects of cell shrinkage. On the other hand, this response to HS results in “immobilization” and impaired migratory functioning of PMN. The ability of HS to modulate transepithelial migration of PMN could also be due to altered expression of cell-surface adhesion molecules. However, in contrast to the findings of Thiel et al. (27), CD11b expression was not inhibited by HS in our in vitro setting. The apparent discrepancy between our findings and the prior work is possibly attributable to the relatively high concentration of the chemotactant (i.e., fMLP 10^-6 M) that was used here. Nevertheless, the findings underscore the fact that HS likely

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Fig. 6. Effect of HS and NS on disease activity index (DAI) in mice with DSS-induced colitis. Clinical activity in DSS colitis was improved by treatment with 4 ml/kg HS (HS4, n = 6) and 8 ml/kg HS (HS8, n = 10), but also by fluid replacement therapy with 8 ml/kg normal saline (NS8, n = 10), compared with DSS mice without further treatment (DSS, n = 12). Statistical analysis was done by ANOVA. *P < 0.05 and **P < 0.01 compared with DSS alone. Values are means ± SE.

Fig. 7. Effect of HS and NS on histological scores in mice with DSS-induced colitis. The histological score in DSS colitis was improved by treatment with 4 ml/kg HS (HS4, n = 6) and 8 ml/kg HS (HS8, n = 10), but not with 4 ml/kg NS (NS4, n = 6) or 8 ml/kg NS (NS8, n = 10). Statistical analysis was done by ANOVA. *P < 0.05 and **P < 0.0001 vs. DSS mice without further treatment (DSS, n = 12). Values represent means ± SE.
has multifaceted effects on PMN that impair their ability to participate in inflammatory responses.

Because transmigration of PMN is considered as a crucial event in the pathophysiology of IBD, the inhibition of PMN transmigration suggested that HS might also exert beneficial effects in the setting of intestinal inflammation. Accordingly, we were able to demonstrate, in an in vivo model of murine colitis, that HS pretreatment indeed results in a significant reduction of various indexes of gut tissue damage. Our findings demonstrate that HS exerts potent anti-inflammatory effects, resulting in decreased infiltration of PMN in the intestinal mucosa. At the lower dose, these effects of HS were paralleled by clinical signs of disease improvements.

In clinical trials investigating the efficacy of HS as a resuscitation fluid for the treatment of hemorrhagic shock, HS is typically administered intravenously as a single dose of 4 ml/kg of 7.5% NaCl solutions. In an attempt to obtain maximal anti-inflammatory effects, we tested this and a higher dose of HS in the studies presented here. Repeated peritoneal administration of HS at a dose of 4 ml/kg proved to be both clinically and histologically superior to an equivalent volume of NS. NS administered at this dose had no significant effects on either DAI or the histological score used to assess gut tissue inflammation and damage. However, at a dose of 8 ml/kg (a dose comparable to 1,120 ml/day in a 70-kg adult), the effect of HS on DAI was similar to that of NS, even though HS but not NS at this dose clearly prevented histological signs of inflammation and gut tissue injury. This may suggest that, if the osmotic load exceeds a certain amount, adverse effects may occur that counterbalance the protection provided by the ability of HS to prevent PMN infiltration and damage of gut tissues. Such adverse effects may be related to metabolic side effects like hyperchloremic acidosis. As shown by Scheingraber et al. (24), infusion of 30 ml·kg⁻¹·h⁻¹ (total amount 70 ml/kg) of 0.9% saline leads to metabolic acidosis, associated with hyperchloremia. This dose is comparable to 8.4 ml/kg of HS and therefore approximates the dose administered in our high-dose group. Hyperosmolarity could also lead to an increased uptake of DSS and thereby aggravated colitis. Because most cell membranes are freely permeable to water, an increase in extracellular osmolarity may result in a shift of water from the intracellular to the extracellular compartment, leading to activation of the osmoregulatory system. Changes in osmolarity as small as 2–3% increase water uptake in adults (8). Likewise, it was demonstrated that subcutaneous injections of HS result in robust water drinking by mice (22). In addition, increased diuresis was reported after the administration of hypertonic solutions (28). Therefore, in the HS group, a higher consumption of DSS provided through the drinking water may have occurred. Conversely, fluid replacement by NS may have reduced the intake of DSS-water. Thus a potential methodical drawback of the DSS colitis model used in our studies may have led to greater amounts of DSS ingested in the HS groups. Despite this, however, we found reduced PMN infiltration and tissue damage in the HS groups compared with all other groups. These findings along with the considerations described above suggest that grading of the clinical signs of disease with DAI may not fully correspond with that actual gut tissue injury inflicted by PMN.

In clinical studies addressing hypovolemic conditions, HS has been administered intravenously (14). In this study, we chose the intraperitoneal route for administration of HS or NS for practical reasons. It has been shown that intraperitoneal HS infusion causes an increase in plasma osmolarity comparable to that obtained through the intravenous route (3). Similarly, in emergency settings when rapid intravenous access cannot be obtained (e.g., in combat casualties) the intravenous route has been suggested, and entry of HS in the vascular space at a rate comparable to that achieved with HS administration via the intravenous route has been demonstrated (13).

It is likely that HS administration may affect the functions of other cell populations, including endothelial and epithelial cells. Interestingly, hyperosmolar activation of the Na⁺/K⁺-2Cl⁻ cotransporter (NKCC) was demonstrated in vascular endothelial cells (25). We observed similar effects in T₈₄ epithelial cells (data not shown). Because chloride secretion is markedly downregulated in the setting of colonic inflammation, and may contribute to a loss of barrier function, an upregulation of NKCC activity by HS could help to restore epithelial secretory function (23, 41).

In summary, the data presented here demonstrate for the first time inhibitory effects of HS on PMN transmigration across model epithelia in vitro and on PMN-induced gut tissue inflammation in vivo. The underlying mechanisms may be related to suppressive effects of HS on PMN chemotaxis or other PMN functions, including superoxide generation and release of granule contents (5, 6, 17, 26). Our findings support the idea that hypertonic fluids exert inhibitory effects on PMN function that may be beneficial in inflammatory conditions of the intestine, such as IBD, or in patients whose intestinal integrity has been compromised by shock/trauma. Additional work will be required to evaluate if HS can be useful as an adjunctive therapy with conventional pharmacological agents such as steroids to control IBD flares.

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