Cellular mechanisms of heparinase III protection in rat traumatic shock

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Hayward, Reid, Rosario Scalia, Bruce Hopper, James Z. Appel III, and Allan M. Lefer. Cellular mechanisms of heparinase III protection in rat traumatic shock. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H23–H30, 1998.—Pentobarbital-anesthetized rats subjected to traumatic shock developed a shock state characterized by marked hypotension to 65–70 mm Hg, a survival time of 88 ± 13 min, significant increases in ileal myeloperoxidase activity (P < 0.01), and severe endothelial dysfunction as evidenced by a significant (P < 0.01) decrease in vasorelaxation to endothelium-dependent dilators. Treatment with heparinase III (45–67 µg·kg⁻¹·min⁻¹) 10 min posttrauma prolonged survival time to 223 ± 19 min (P < 0.001), significantly attenuated ileal myeloperoxidase activity (P < 0.01), and significantly preserved endothelial function (P < 0.05). Intravital microscopy of the rat mesentery showed that infusion of heparinase III (45–67 µg·kg⁻¹·min⁻¹) significantly (P < 0.01) attenuated both leukocyte rolling and adherence in the rat mesenteric microvasculature in response to N⁶-nitro-L-arginine methyl ester stimulation. Immunohistochemical localization of surface-expressed P-selectin on mesenteric venules showed that heparinase III infusion at 45–67 µg·kg⁻¹·min⁻¹ significantly (P < 0.05) attenuated the increase in surface P-selectin expression. The beneficial effects of heparinase III are mediated at least in part by attenuating leukocyte-endothelial cell interactions via a P-selectin-dependent mechanism.

endothelial dysfunction; myeloperoxidase activity; leukocyte rolling; P-selectin; intravital microscopy

TRAUMATIC SHOCK induced by Noble-Collip drum rotation is characterized by severe hypotension, marked visceral tissue injury, and a high mortality rate (8, 23, 31). This has been primarily attributed to an inflammatory response in which platelets and leukocytes as well as endothelial cells are activated, releasing humoral mediators, and to the activation of the complement cascade (15, 29, 31). The inflammatory response is triggered by an early endothelial dysfunction characterized by a decreased release of nitric oxide (NO) from the endothelium (16). This loss of endothelial-derived NO occurs within 15 min following traumatic shock and increases endothelial adheresiveness for polymorphonuclear neutrophils (PMN), which results in transendothelial migration of many of the adherent PMN, leading to subsequent tissue injury (29).

The migration of PMN from the circulation to the target tissue has been described as a three-step process (5). First, the vascular endothelium must recognize circulating leukocytes, resulting in the characteristic rolling along the endothelial surface. This is followed by a strengthening of adhesive forces and a firm adherence of PMN to the endothelium. Finally, many of the adherent PMN subsequently extravasate through the endothelium and come in close proximity to the target tissue, where they are able to produce their injurious effects. Early leukocyte-endothelium interactions are dependent on the actions of the selectin family of adhesion molecules, identified as P-, E-, and L-selectin. On stimulation, P-selectin is rapidly translocated to the surface of the endothelial cell, where it can bind to carbohydrate structures expressed on the surface of PMN (19). E-selectin, which is also expressed by endothelial cells, requires de novo protein synthesis and can be upregulated by cytokines and bacterial endotoxin over a period of 4–6 h (2, 27). In contrast to P- and E-selectin, L-selectin is constitutively expressed on the cell surface of leukocytes.

Selectins have been shown to bind both carbohydrate and glycolipid structures. A number of studies have demonstrated the capability of selectins to bind sialylated fucosylated structures as well as heparin and heparin-like molecules (2, 25). Moreover, a sialyl Lewisx (SLe⁰) oligosaccharide has been shown to attenuate reperfusion injury in myocardial ischemic cats (4). Recent attention has focused on heparan sulfate proteoglycans (HSPG), whose glucosamine residues are enriched with unsubstituted amino groups (24), as a major ligand for the selectin adhesion molecules. This is consistent with experiments showing that the administration of heparin can inhibit leukocyte rolling along the vascular endothelium (17).

Heparinase are a family of enzymes capable of degrading both heparin and heparan sulfate. Heparinase I primarily cleaves heparin, heparinase II cleaves both heparin and heparan sulfate, and heparinase III selectively cleaves heparan sulfate (18, 33). Previous work in our laboratory (22) has indicated a significant protective effect of heparinase III in a feline model of myocardial ischemia-reperfusion in which leukocyte-mediated injury was significantly attenuated. However, the cellular mechanisms responsible for the protective effects of heparinase III have not been determined. Because murine traumatic shock has many of the pathophysiological sequelae in common with myocardial ischemia-reperfusion (i.e., a neutrophil-mediated inflammatory sequence initiated by endothelial dysfunction), we investigated the effects of heparinase III in this model of trauma. Thus the purpose of this study was to determine the overall effects of heparinase III in a well-established model of murine traumatic shock and to investigate the cellular mechanisms of leukocyte-endothelium interactions in the rat mesentery by employing intravital microscopy techniques.
HEPARINASE III IN TRAUMATIC SHOCK

MATERIALS AND METHODS

Experimental protocol. Male Sprague-Dawley rats weighing 175–225 g were anesthetized with pentobarbital sodium (40 mg/kg ip). Traumatic shock was induced in anesthetized rats by whole body trauma utilizing a Noble-Collip drum apparatus (23). All animal protocols have been approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Traumatized rats were subjected to a total of 525 revolutions at 60 revolutions/min. Immediately after the induction of trauma, the trachea was cannulated with PE-240 polyethylene tubing (Becton-Dickinson, Parsippany, NJ) to maintain a patent airway. Polyethylene catheters (PE-50) filled with heparinized 0.9% NaCl solution were inserted into the right common carotid artery for recording mean arterial blood pressure (MABP) and into the left external jugular vein for administration of either heparinase III or its vehicle (PBS). The total time required to complete all surgical procedures was <10 min. MABP was continuously recorded and tabulated every 30 min over the entire 5-h observation period using a Grass model 7 oscillographic recorder (Grass Instruments, Quincy, MA) and Statham P23 pressure transducers (Gould, Cleveland, OH). Rats were randomly assigned to one of five experimental groups: 1) sham-trauma rats receiving vehicle (PBS; n = 6), 2) sham-trauma rats receiving 45 µg·kg⁻¹·min⁻¹ heparinase III (n = 6), 3) traumatized rats receiving inactive heparinase III (n = 14), 4) traumatized rats receiving 4.5 µg·kg⁻¹·min⁻¹ heparinase III (n = 7), or 5) traumatized rats receiving 45 µg·kg⁻¹·min⁻¹ heparinase III (n = 11).

Heparinase III, inactive heparinase III, or vehicle was administered as an intravenous infusion at a rate of 0.1 ml/h over the entire 5-h observation period. Sham-trauma rats were anesthetized and subjected to all of the same surgical procedures as the traumatized rats except that they did not undergo drum trauma. Additional pentobarbital was given intraperitoneally throughout the observation period to maintain a surgical plane of anesthesia.

Five hours posttrauma, or when MABP fell below 45 mmHg, the experiments were terminated. Samples of rat small intestine were obtained for measurement of myeloperoxidase (MPO) activity. Survival time was defined as that interval between removal from the drum to the end of the experiment (i.e., MABP <45 mmHg). All rats were autopsied to confirm the presence of gross evidence of traumatic injury to the splanchnic viscera (i.e., bowel ischemia, serosanguineous ascites, splancnic vascular engorgement). Rats were excluded from the study if these findings were not observed to a significant degree or if the rat died sooner than 30 min posttrauma. Fewer than 15% (i.e., 5 of 35 rats) of the rats studied were discarded from the study for such reasons, and they were randomly distributed among all trauma groups.

Determination of tissue MPO. Small intestinal activity of MPO, an enzyme occurring virtually exclusively in PMN, was determined using the method of Bradley et al. (3) as modified by Mullane et al. (20). A hemorrhage-free area of the small intestine was obtained at 5 h or at the time MABP fell below 45 mmHg. The segment of ileum was dissected and carefully rinsed in 0.9% NaCl before homogenization in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical, St. Louis, MO; dissolved in 50 mM KH₂PO₄ buffer at pH 6.0) using a Polytron (PCU-2) homogenizer (Kinematika, Lucerne, Switzerland). Homogenates were centrifuged at 12,500 g at 4°C for 30 min. The supernatants were then collected and reacted with 0.167 mg/ml of o-dianisidine dihydrochloride (Sigma Chemical) and 0.0005% H₂O₂ in 50 mM phosphate buffer at pH 6.0. The resultant change in absorbance was determined spectrophotometrically at 460 nm. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol H₂O₂/min at 25°C.

Isolated mesenteric artery ring studies. At the end of the experiment, the superior mesenteric artery (SMA) was rapidly removed from animals and placed into warmed Krebs-Henseleit (KH) buffer consisting of (in mmol/l): 118 NaCl, 4.75 KCl, 2.54 CaCl₂, 2H₂O, 1.19 KH₂PO₄, 1.19 MgSO₄·7H₂O, 12.5 NaHCO₃, and 10.0 glucose. Isolated vessels were carefully freed of connective tissue and cut into rings 2–3 mm in length. The rings were then mounted on stainless steel hooks, suspended in a 10-ml tissue bath, and connected to FT03 force displacement transducers (Grass Instruments) to record changes in force on a Grass model 7 oscillographic recorder. The baths were filled with KH buffer and aerated at 37°C with 95% O₂-5% CO₂. A resting force of 0.5 g was applied to SMA rings, and then they were equilibrated for 60–90 min, during which time the buffer in the tissue bath was replaced every 20 min and the resting force of the vascular rings was adjusted until 0.5 g of preload was maintained. This resting force was selected because it does not injure the endothelium or interfere with the release of NO in response to endothelium-dependent vasodilators. After equilibration, the rings were exposed to 100 nM U-46619 (9,11-epoxy methanoprostaglandin H₂; Biomol Research Laboratories, Plymouth Meeting, PA), a thromboxane A₂ mimetic, to generate ~0.5 g of developed force. Once a stable contraction was obtained, ACh, an endothelium-dependent vasodilator, was added to the bath in cumulative concentrations of 0.1, 1, 10, and 100 nM. At the cumulative response stabilized, the rings were washed and allowed to equilibrate to baseline once more. The procedure was repeated with an endothelium-independent vasodilator, acidified NaNO₃ (0.1, 1, 10, and 100 µM). NaNO₃ was prepared by dissolving the compound in 0.1 N HCl and titrating it to pH 2.0. Equal volumes of pH 2.0 solution had no vasoactive effect on rat SMA rings. One hundred percent relaxation was defined as the return to precontraction force following U-46619-induced contraction.

Intravital microscopy. Male Sprague-Dawley rats, weighing 250–270 g, were anesthetized with pentobarbital sodium (40 mg/kg) injected intraperitoneally. A tracheotomy was performed to maintain a patent airway throughout the experiment. A polyethylene catheter was inserted in the left carotid artery to monitor MABP. In addition, a jugular vein was cannulated for administration of supplemental pentobarbital sodium as needed to maintain a surgical plane of anesthesia throughout the experiment. Blood pressure was recorded on a Grass model 7 oscillographic recorder using a Statham P23 AC pressure transducer. The abdominal cavity was opened via a midline laparotomy.

A loop of ileal mesentery was exteriorized through the midline incision and placed in a temperature-controlled, fluid-filled Plexiglas chamber for observation of the mesenteric microcirculation via intravital microscopy. The mesentery was placed over a Plexiglas pedestal in the superfusion chamber, and the ileum was secured with fine-gauge stainless steel pins on a cork board for stabilization of the viewing field. The ileum and mesentery were superfused throughout the experiment with KH solution warmed to 37°C and bubbled with 95% N₂-5% CO₂.

A Nikon Microphot microscope (Nikon, Tokyo, J apan) with a ×40 objective lens and a ×10 ocular was used to visualize the mesenteric microcirculation. The image was projected by a high-resolution video camera (model XC77, Hamamatsu, Hamamatsu, J apan) onto a Sony high-resolution video monitor, and the image was recorded using a videocassette recorder. Red blood cell velocity was determined on-line with
the use of an optical Doppler velocimeter obtained from the Microcirculation Research Institute (College Station, TX). This method gives an average red blood cell velocity that allows for the calculation of vascular wall shear rates.

The rats were allowed to stabilize for 30 min following surgery. After stabilization, a 30- to 50-µm-diameter postcapillary venule was chosen for observation. A baseline recording was made to establish basal values for leukocyte rolling and adherence. The mesentery was then superfused with 50 µM Nω-nitro-L-nitro-L-methyl ester (L-NAME) dissolved in KH solution for 60 min. Video recordings were made at 0, 15, 30, 45, and 60 min after initiation of superfusion for quantification of leukocyte rolling and adherence. Rats were randomly divided into one of five groups: 1) KH-superfused rats infused with heparinase III at 67 µg·kg⁻¹·min⁻¹ (n = 7), 2) 50 µM L-NAME-superfused rats (n = 9), 3) 50 µM L-NAME-superfused rats infused with inactive heparinase III at 67 µg·kg⁻¹·min⁻¹ (n = 6), 4) 50 µM L-NAME-superfused rats infused with heparinase III at 6.7 µg·kg⁻¹·min⁻¹ (n = 7), and 5) 50 µM L-NAME-superfused rats infused with heparinase III at 67 µg·kg⁻¹·min⁻¹ (n = 9). In four additional rats, after L-NAME superfusion, heparinase III was infused at 45 µg·kg⁻¹·min⁻¹ for 2 h.

The numbers of rolling and adherent leukocytes were determined off-line by playback of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling is expressed as the number of cells moving past a designated point per minute (i.e., leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for >30 s. Adherence is expressed as the number of adherent leukocytes per 100 micrometers of vessel length. Red blood cell velocity (V) and venular diameter (D) were used to calculate venular wall shear rate (γ) employing the formula γ = 8(Vmean/D), where Vmean is average red blood cell velocity calculated as V/1.6.

Immunohistochemistry. Immunohistochemical localization of P-selectin was determined using monoclonal antibody (MAb) PB1.3, which only detects surface expression of P-selectin. After the trauma observation period or intravital microscopy procedures, both the SMA and superior mesenteric vein were rapidly cannulated for perfusion fixation of the small bowel. The ileum was first washed free of blood by perfusion with Krebs-Henseleit buffer warmed to 37°C, bubbled with 95% O₂-5% CO₂, and fixed in 4% paraformaldehyde for 90 min at 4°C. Immunohistochemical localization of P-selectin was accomplished with the use of the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent, Vector Laboratories, Burlingame, CA) as previously described by Weyrich et al. (32). Positive staining was defined as a venule displaying brown reaction product on >50% of the circumference of its endothelium. Fifty venules per tissue section were examined, with twenty sections analyzed per group, and the percentage of positive staining venules was tallied.

Heparinase III. Heparinase III (IBT 9302), purified from Flavobacterium heparinum, and chemically inactive heparinase III were provided by Drs. Achim Recktenwald and Paul Silver of IBEX Technologies (Montreal, Quebec, Canada).

Statistical analyses. All values for data listed in the text and Figs. 1–7 are presented as means ± SE of n independent experiments. Data were compared by ANOVA using post hoc analysis with Fishers corrected t-test. Survival times were compared utilizing Gehan’s generalized Wilcoxon test as described by Knapp and Wise (14). The survival rates were assessed by χ² analysis. P ≤ 0.05 was considered to be significant in all cases.

RESULTS

Effect of heparinase III on survival time and survival rate. Survival times for each experimental group are presented in Fig. 1. All sham-trauma rats survived the entire 5-h observation period regardless of whether heparinase III or its vehicle was administered. In contrast, traumatized rats receiving inactive heparinase III demonstrated a survival time of only 90 ± 14 min, a value significantly (P < 0.001) lower than that of sham-trauma rats and comparable to historic vehicle (i.e., 0.9% NaCl or PBS controls) in trauma rats. A significant prolongation of survival time was observed in traumatized rats receiving 45 µg·kg⁻¹·min⁻¹ heparinase III (P < 0.001), with values increasing ~2.5-fold. However, no significant difference was observed between trauma rats administered 4.5 µg·kg⁻¹·min⁻¹ heparinase III and trauma rats administered inactive heparinase III. Survival rate was also significantly (P < 0.01) increased in trauma rats administered 45 µg·kg⁻¹·min⁻¹ heparinase III compared with that in trauma rats administered inactive heparinase III (Fig. 2). No trauma rat administered 4.5 µg·kg⁻¹·min⁻¹ heparinase III or inactive heparinase III survived longer than 210 min, whereas 36% of trauma rats administered 45 µg·kg⁻¹·min⁻¹ heparinase III survived the entire 5 h. These results suggest that heparinase III at 45 µg·kg⁻¹·min⁻¹, but not at 4.5 µg·kg⁻¹·min⁻¹, significantly increases both survival time and survival rate in this model of traumatic shock.

Effect of heparinase III on MABP. Administration of heparinase III (45 µg·kg⁻¹·min⁻¹) or an equivalent volume of PBS (i.e., vehicle) had no significant effect on MABP over the 5-h observation period in sham-trauma rats. This finding indicates that the surgical procedures as well as the administration of heparinase III did not contribute to the hypotension observed in rats subjected to traumatic shock. The mean initial posttrauma MABP in trauma rats given heparinase III (4.5 or 45 µg·kg⁻¹·min⁻¹) or inactive heparinase III (45 µg·kg⁻¹·min⁻¹) ranged from 69 to 72 mmHg, and no significant differences were observed among these groups. No significant differences were observed be-

Fig. 1. Mean survival time of sham-trauma and traumatized rats. Columns represent means and bars indicate SE. Numbers in columns represent no. of rats in each group. NS, not significant.
between rats receiving 4.5 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III and rats receiving inactive heparinase III at any time interval, indicating that the inactive form of heparinase III has no positive effect.

**Effect of heparinase III on ileal MPO activity.** Ileal MPO activity was determined as an index of PMN infiltration into intestinal tissue, because it is exclusively present in PMN. All sham-trauma rats receiving either 45 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III or vehicle exhibited low MPO activities consistent with a low number of resident neutrophils (Fig. 3). In contrast, ileal MPO activity in the rats subjected to traumatic shock given inactive heparinase III (45 µg·kg\(^{-1}\)·min\(^{-1}\)) was significantly increased (\(P < 0.01\)) compared with that in sham-operated control rats. Moreover, treatment of traumatized rats with 45 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III significantly attenuated the increase in MPO activity (\(P < 0.01\)), thus indicating that heparinase III at this dose significantly retarded the accumulation of PMN in intestinal tissue following traumatic shock. Trauma rats treated with 4.5 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III exhibited no significant difference from trauma rats treated with inactive heparinase III (45 µg·kg\(^{-1}\)).

**Effect of heparinase III on SMA endothelial function.** Endothelial function was measured by comparing vasorelaxation of isolated SMA rings to the endothelium-dependent vasodilators ACh and A-23187, as well as to the endothelium-independent vasodilator NaNO\(_2\) (Fig. 4). Isolated SMA rings from sham-trauma rats, whether they received 45 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III or vehicle, exhibited full (>90%) relaxation to all three vasodilators. However, the endothelium-dependent vasorelaxant responses of SMA rings obtained from traumatized rats receiving either 4.5 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III or inactive heparinase III (45 µg·kg\(^{-1}\)·min\(^{-1}\)) were significantly reduced. Neither ACh nor A-23187 elicited a relaxation. However, these rings relaxed fully in response to NaNO\(_2\) (>95%). The degree of relaxation to ACh and A-23187 in rings isolated from traumatized rats treated with 45 µg·kg\(^{-1}\)·min\(^{-1}\) heparinase III was significantly preserved (\(P < 0.05\) and \(P < 0.01\), respectively), thus indicating that heparinase III at this dose significantly

**Fig. 2.** Comparison of survival rates from sham-trauma and traumatized rats. Ratio in each column indicates no. of survivors per total no. of rats in group. Statistical significance was assessed by \(\chi^2\) analysis.

**Fig. 3.** Intestinal myeloperoxidase (MPO) activity in sham-trauma and traumatized rats. Columns represent means and bars indicate SE. Numbers in columns represent no. of rats in each group.

**Fig. 4.** Summary of vasorelaxation responses of superior mesenteric artery rings from sham-trauma and traumatized rats to 100 nM ACh, 1 µM A-23187, and 100 µM NaNO\(_2\). Sham-trauma rats were given heparinase III (45 µg·kg\(^{-1}\)·min\(^{-1}\)), and traumatized rats were given either vehicle (PBS), heparinase III (45 µg·kg\(^{-1}\)·min\(^{-1}\)), or heparinase III (45 µg·kg\(^{-1}\)·min\(^{-1}\)). Columns represent means and bars indicate SE. Numbers in columns represent no. of segments studied. *\(P < 0.05\) compared with trauma + inactive heparinase III; **\(P < 0.01\) compared with trauma + inactive heparinase III; †† \(P < 0.01\) compared with sham + vehicle.
HePARINASE III IN TRAUMATIC SHOCK

Protected against endothelial dysfunction occurring during traumatic shock.

The preservation of endothelial function may be responsible for normalizing MABP values observed in heparinase III-treated rats. By preserving the integrity and function of the endothelium, vascular leakiness in the microcirculation is attenuated, which subsequently maintains blood volume and thus systemic blood pressure. Therefore, an improved relaxation of SMA rings to endothelium-dependent vasodilators demonstrates the maintenance of arterial blood pressure, resulting in a protective effect of heparinase III in trauma.

Effect of heparinase III on leukocyte rolling and adherence in mesenteric vasculature. Infusion of the rat mesentery with 50 µM L-NAME resulted in a time-dependent increase in leukocyte rolling and adherence in postcapillary venules of the rat mesenteric microvasculature (Figs. 5 and 6). Mean values for MABP ranged from 130 to 135 mmHg for all five groups of rats studied at 0 min (data not shown). No significant differences in MABP were noted among groups at any time interval, indicating that the adhesive interactions observed between leukocytes and endothelial cells were not due to changes in physical hydrodynamic forces or to spontaneous hemodynamic alterations brought about by the infusion of heparinase III.

Intravenous infusion of 67 µg·kg⁻¹·min⁻¹ heparinase III significantly attenuated L-NAME-induced leukocyte rolling. At 60 min, rolling was significantly (P < 0.01) reduced from 37 ± 5 cells/min in rats exposed to mesenteric superfusion of L-NAME (50 µM) to 10 ± 3 cells/min in rats given L-NAME (50 µM) and treated with 67 µg·kg⁻¹·min⁻¹ heparinase III. In a similar fashion, heparinase III (67 µg·kg⁻¹·min⁻¹) significantly attenuated leukocyte adherence to the microvascular endothelium. After 60 min of L-NAME (50 µM) superfusion, 15 ± 2 cells/100 µm were adherent to the microvasculature, whereas the superfusion of L-NAME (50 µM) in conjunction with the intravenous infusion of heparinase III resulted in only 3 ± 0.5 adherent cells/100 µm (P < 0.01). Infusion of heparinase III at 67 µg·kg⁻¹·min⁻¹ or inactive heparinase III (67 µg·kg⁻¹·min⁻¹) did not result in any significant attenuation in leukocyte rolling or adherence in L-NAME superfused rats. Additional intravital microscopy experiments were conducted using a 45 µg·kg⁻¹·min⁻¹ infusion rate of heparinase III (n = 6), and no significant differences in leukocyte rolling or adherence were observed between the 45 and 67 µg·kg⁻¹·min⁻¹ infusion rates. In terms of leukocyte adherence, both infusion rates attenuated leukocyte adherence comparably (2.5 ± 0.5 vs. 3 ± 0.5 adherent cells/100 µm at 45 vs. 67 µg·kg⁻¹·min⁻¹, respectively). Circulating white blood cells were counted in rats undergoing intravital microscopy. No significant differences were observed between L-NAME-superfused rats and L-NAME-superfused rats administered heparinase III (45 µg·kg⁻¹·min⁻¹) initially (12,400 ± 800 vs. 13,000 ± 500 cells/µl) or at 60 min (11,100 ± 600 vs. 12,000 ± 500 cells/µl). Therefore, in both protocols, there was no significant difference in circulating leukocyte count between the active agent and its control substance. Therefore, the attenuation of ileal MPO activity and the lower leukocyte rolling and adherence in heparinase III-infused rats were significantly different from each other and did not change significantly over the 60-min observation period for any group; final shear rates were 642 ± 28, 670 ± 34, 650 ± 25, 720 ± 28, and 700 ± 19 s⁻¹, respectively, for the five experimental groups. There was no significant difference in shear rates among the five groups, indicating that the adhesive interactions observed between leukocytes and endothelial cells were not due to changes in physical hydrodynamic forces or to spontaneous hemodynamic alterations brought about by the infusion of heparinase III.
adherence cannot be attributed to alterations in circulating leukocytes.

Effect of heparinase III on P-selectin expression. Immunolocalization of surface-expressed endothelial cell P-selectin was observed only on the venular endothelium in the rat ileum. Tissue sections obtained from sham-trauma rats showed faint, patchy cytoplasmic immunostaining primarily located at the endothelium in <5% of small intestine venules (Fig. 7). Conversely, in those rats administered inactive heparinase III, the surface expression of P-selectin was evident in intestinal venules of all rats following the induction of trauma. In the trauma + inactive heparinase III group, 72 ± 2% of venules exhibited positive staining for MAb PB1.3 (P < 0.01 vs. sham-operated controls). Administration of heparinase III (45 µg·kg⁻¹·min⁻¹) significantly attenuated surface expression of P-selectin, with only 48 ± 3% of venules staining positively for MAb PB1.3.

Likewise, immunohistochemical localization of P-selectin was also determined in rats utilized in intravitral microscopy experiments. The percentage of venules staining positively for P-selectin in ileal sections from control rats superfused with heparinase III (67 µg·kg⁻¹·min⁻¹) was consistently low. In contrast, superfusion with 50 µM l-NAME for 120 min resulted in a significant increase in P-selectin expression as quantified by the percentage of venules staining positively for P-selectin (P < 0.01). Intravenous infusion of heparinase III (67 µg·kg⁻¹·min⁻¹), in conjunction with superfusion of 50 µM l-NAME, significantly attenuated the number of venules staining positively for P-selectin (P < 0.05). In contrast, infusion of inactive heparinase III (67 µg·kg⁻¹·min⁻¹) did not attenuate the increase in P-selectin expression resulting from the superfusion of l-NAME. Thus heparinase III appears to suppress P-selectin expression on the surface of mesenteric endothelial cells following superfusion of l-NAME as well as traumatic shock, an effect that may account for much of the inhibition of leukocyte rolling and adherence.

**DISCUSSION**

Our data indicate that heparinase III exerts significant protective effects in a murine model of traumatic shock. Specifically, administration of heparinase III resulted in a significant prolongation of survival time and an attenuation of leukocyte infiltration as measured by lower intestinal MPO activity. Heparinase III also preserved endothelial function as evidenced by the maintained relaxation responses of SMA rings to the endothelium-dependent vasodilators ACh and A-23187. These findings are consistent with earlier work demonstrating that heparinase III plays a significant role in limiting myocardial ischemia-reperfusion injury in the cat and rabbit (12, 13).

Proteoglycans encompass a wide variety of molecules that serve numerous functions. One of the most extensively studied proteoglycans associated with endothelial cells is HSPG. Heparan sulfate is synthesized within endothelial cells and is subsequently incorporated into the more complex molecule HSPG, which is found within the extracellular matrix and on the cell surface. The HSPG located in extracellular matrix and expressed on the cell surface contributes to vascular permeability (28) as well as to the nonthrombogenic nature of blood vessels (9) and has been shown to serve as a ligand for a number of extracellular enzymes (1, 7).

Endothelial cell HSPG has been suggested to play a key role in the presentation of chemokines to circulating leukocytes. Chemokines are a group of heparin-binding cytokines produced by a number of cells on activation. These chemokines modulate leukocyte recruitment and extravasation by chemotaxis and by stimulating integrin-mediated leukocyte binding to endothelial cells (10).9 Proadhesive cytokines recruit leukocytes most effectively when the cytokines are immobilized on the endothelial surface. Therefore, to facilitate the effective recruitment of leukocytes to the site of inflammation, endothelial cell HSPG are believed to bind and therefore optimally localize high concentrations of these chemotactic cytokines.

In addition to their ability to localize cytokines, HSPG also serve as a ligand for specific adhesion molecules. The selectin adhesion molecules have been shown to bind several structures including SLEx, sulfosialylated molecules (11) and N-linked tetra-antennary difucosyl oligosaccharides (26) as well as noncarbohydrate phosphorylated or polysulfated molecules such as inositol polyphosphorylans (6). In addition, P- and L-selectin, but not E-selectin, have been shown to bind both heparin and heparin oligosaccharide structures (11, 22). Numerous studies have shown that competitors of specific selectin ligands can diminish the inflammatory response. Intravenous infusion of SLEx has been shown to significantly attenuate the inflammatory response to traumatic shock (30) and myocardial ischemia-reperfusion (4) as well as to cobra venom factor-induced lung injury (21). The role of HSPG as a ligand for specific selectin adhesion molecules suggests that the effects of heparinase III may be mediated by its ability to inhibit the initial PMN-endothelial cell inter-
actions (i.e., rolling) via a P-selectin-mediated mechanism.

Heparinase III selectively cleaves heparan sulfate at \(\alpha\text{-d-GlcNp2Ac(or 2S)6OH(1\rightarrow4)\beta\text{-d-GlcAp}}\), thus inactivating the ligand for L-selectin. The consequences of such an action would inhibit the initial leukocyte-endothelial cell interactions (i.e., rolling), thus arresting the progression of neutrophils from the vascular lumen to the site of inflammation. Secondary to the direct actions of heparinase III on HSPG, additional inhibition may be mediated by small saccharide fragments resulting from HSPG degradation. Norgard-Sumnicht and Varki (24) reported that after heparinase III digestion, fragments of approximately four saccharide units were still capable of binding to L-selectin. Likewise, Nelson et al. (22) demonstrated that heparin molecules containing four or more monosaccharide residues inhibited P- and L-selectin adhesive actions. Thus the degradation of HSPG by heparinase III may actually produce soluble P- and L-selectin ligands. In this regard, small saccharides produced by the heparinase III-induced degradation of HSPG may serve as competitive inhibitors to specific selectin adhesion molecules. The resulting soluble heparin-like saccharides could bind L-selectin on leukocytes as well as the P-selectin on platelets and on the vascular endothelium, thereby inhibiting selectin-mediated blood cell-endothelium interaction.

The intravenous administration of heparinase III attenuated mesenteric venule endothelial expression of P-selectin resulting from the superfusion of 50 \(\mu\text{M L-NAME}\) as well as that in rats subjected to trauma. These data support the hypothesis that heparinase III may not only degrade an endothelial ligand for L-selectin but also may attenuate the role of P-selectin in the mediation of leukocyte rolling. Our finding of lower positive staining of venules for P-selectin may be due to one of two processes. First, administration of heparinase III may cleave endothelial bound HSPG, releasing small saccharides that bind surface-expressed P-selectin competing for binding with the antibody utilized in the immunohistochemical staining process. Therefore, although P-selectin may be expressed on the endothelial cell surface, the binding of soluble saccharide fragments may prevent interaction between P-selectin and leukocytes. Second, effective doses of heparinase III may cleave endothelial bound HSPG, releasing small saccharide molecules that bind surface-expressed P-selectin, preventing initial interactions between endothelial cells and leukocytes (i.e., rolling). By limiting this initial cell-to-cell interaction, leukocytes release less proteases, cytokines, and oxygen-derived free radicals in high concentrations, which limits activation of the endothelium, resulting in a lower surface expression of P-selectin.

We observed a 45% decrease in P-selectin expression in contrast to a 73% reduction in leukocyte rolling and an 80% reduction in leukocyte adherence. Leukocyte rolling and, in particular, leukocyte adherence are importantly, but not exclusively, mediated by P-selectin. Similar results were obtained in rats subjected to trauma. In situations such as trauma, these processes are mediated by additional adhesion molecules including members of the immunoglobulin superfamily and the integrins. Heparinase III may provide protective effects by inhibiting the function of one or several of these adhesion molecules. The data presented here support the hypothesis that heparinase III exerts its protective effects via several mechanisms.

Heparinase III treatment attenuates the interaction between leukocytes and stimulated vascular endothelium, which preserves both endothelial cell integrity and function. We also show that heparinase III attenuates endothelial dysfunction normally associated with traumatic shock, as evidenced by the preservation of endothelium-dependent vasodilation. Endothelial dysfunction occurs rapidly following the induction of traumatic shock (16, 29) and is characterized by a marked impairment in NO release by the endothelium. NO mediates a variety of cellular processes, including vasodilation, inhibition of platelet aggregation, and attenuation of neutrophil adherence to the endothelium. Thus the loss of NO production in shock states promotes leukocyte adherence to the endothelium and the subsequent activation of neutrophils (16). Activated neutrophils are capable of mediating several cytotoxic processes via the release of oxygen-derived free radicals and elastase, which in turn can exacerbate endothelial dysfunction and tissue injury. This concept is supported by the observation that administration of either exogenous NO via an NO donor (8) or the NO synthase substrate L-arginine (unpublished observations) significantly preserves endothelial function following Noble-Collip drum trauma.

Endothelial cell surface HSPG appears to serve numerous functions, several of which are key to the pathophysiology of tissue inflammation. These functions include immobilizing cytokines at or near the site of tissue injury as well as serving as a ligand for specific selectin adhesion molecules. By compromising the integrity of these structures with heparinase III, the processes of leukocyte rolling, leukocyte adherence, and neutrophil extravasation into injured tissues are diminished. We now provide evidence in vivo that heparinase III exerts beneficial effects in murine traumatic shock that are mediated at least in part by attenuating leukocyte-endothelial cell interactions. These data taken together with previous investigations suggest the presence of at least two distinct categories of carbohydrate-containing selectin ligands (i.e., heparin- and SLe-like structures) that may serve complementary functions.

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