Regional Intestinal Blood Flow and Nitric Oxide Synthase Inhibition During Sepsis in the Rat

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Objective
Regional circulatory changes in intestinal mucosa were evaluated after the onset of septic shock and the effect of nitric oxide (NO) inhibition on mucosal blood flow was investigated at different locations along the intestine.

Summary Background Data
The response of intestinal blood flow to different physiologic and pharmacologic stimuli is known to vary along the intestine, but limited data are available on regional alterations in intestinal blood flow during septic shock. These regional variations in intestinal blood flow could become important because NO inhibition might restore the circulation of one segment of the gut or exacerbate ischemia that may be occurring concomitantly in another segment of the intestine.

Methods
Mucosal blood flow was studied with fluorescent microspheres in conscious unrestrained rats before and 2, 4, and 6 hours after lipopolysaccharide (LPS, 20 mg/kg intraperitoneally)-induced sepsis in the presence and absence of the nitric oxide synthase inhibitor N\textsuperscript{\textuuml;}-nitro-L-argininemethylester (L-NAME, 5 mg/kg subcutaneously).

Results
Control mucosal blood flow was significantly higher in the ileum than in the duodenum, jejunum, or colon. During LPS-induced sepsis, mucosal blood flow to the ileum decreased and perfusion to the remaining gut was preserved. This was accompanied by hypotension throughout the experiment. L-NAME administration during sepsis prevented hypotension and decreased mucosal blood flow to all segments of small intestine at 2 hours. In this group, mucosal blood flow to the proximal small intestine but not to the ileum returned to baseline levels at 4 and 6 hours. L-NAME alone decreased mucosal blood flow to the small intestine throughout the experiment.

Conclusions
This study indicates that mucosal blood flow alterations during septic shock vary along the intestine, with a significant change only in the ileum, suggesting that perfusion in the small intestine is dependent on physiologic NO production.
Septic shock is accompanied by alterations of blood flow to various tissues, including organs perfused by the splanchnic vasculature. Intestinal hypoperfusion or ischemia can lead to mucosal injury and a loss of the restrictive function of the luminal gut barrier. This loss is strongly associated with bacteria or bacterial wall products entering the circulation, thus initiating or aggravating existing sepsis. Current treatments for sepsis show minimal efficacy, underscoring the need for new approaches. However, it is now apparent that inhibition of NO can also be detrimental to vascular integrity, oxygen supply, organ perfusion, and survival in septic shock. The beneficial or detrimental effect of NO inhibition on intestinal integrity appears to be dependent on time and dose of administration.

The response of intestinal blood flow to different physiologic and pharmacologic stimuli is known to vary along the intestine, but limited data are available on regional alterations in intestinal blood flow during septic shock. These regional variations in intestinal blood flow could become important because NO inhibition might restore the circulation of one segment of the gut or exacerbate ischemia that may be occurring concomitantly in another segment.

The major objective of our study was to evaluate regional changes in intestinal mucosal perfusion after the onset of lipopolysaccharide (LPS)-induced septic shock in a rat. The effects of the administration of an NO inhibitor on segmental mucosal blood flow were also investigated, with and without LPS administration.

MATERIALS AND METHODS

Sixty-two male Harlan Sprague-Dawley rats (350–450 g), handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and with the approval of The University of Texas Medical School at Houston Animal Welfare committee, were housed at a constant temperature, exposed to a cycle of 12 hours of light and 12 hours of darkness, and given rat chow and water ad libitum during a 1-week acclimation period.

Animal Preparation

The rats were anesthetized with a mixture of Ketaset (80 mg/kg) and xylazine (6 mg/kg) given intraperitoneally. Vascular catheters were chronically implanted using sterile surgical techniques. A polyethylene 50 catheter was introduced into the left ventricle via the right carotid artery for microsphere injection. The position was confirmed by the typical left ventricular pressure curve and verified at autopsy. The second catheter (Silastic tubing 0.51 mm (0.02") I.D., 0.94 mm (0.037") O.D., with a polyethylene 50 tip) was placed in the left femoral artery for withdrawal of reference blood samples and evaluation of blood pressure and heart rate. Both catheters were tunneled to the back and exteriorized at the base of the skull. A metal harness secured the lines and allowed the rats free movement. The left ventricular line was flushed with 0.9% saline and occluded. The femoral line was continuously perfused with 0.9% saline at a rate of 0.5 mL/hr to prevent clotting. Rats were allowed to recover for 18 to 24 hours before blood flow measurements in individual cages with free access to water but not to food.

Blood Flow Measurements

All blood flow determinations were made in conscious unrestrained animals because anesthesia can alter blood flow to the intestine and may also affect the responsiveness of the vasculature to NO inhibition. The recently established fluorescence microsphere method was used to avoid manipulation of the intestine before measurement of mucosal blood flow. Blood flow was determined in each rat before and 2, 4, and 6 hours after treatment administration.

Experimental Groups

Rats were randomly assigned to one of four groups: 1) a control group consisted of untreated animals (n = 6) and those treated with saline (n = 8, 0.9% saline 1 mL/kg subcutaneously and 0.9% saline 1 mL/kg intraperitoneally); 2) an LPS group (n = 8) injected with LPS (20 mg/kg intraperitoneally) and 0.9% saline (1 mL/kg subcutaneously); 3) an LPS–N^G-nitro-L-argininemethylene (L-NAME) group (n = 8), injected with LPS (20 mg/kg intraperitoneally) and L-NAME (5 mg/kg subcutaneously); and 4) an L-NAME group (n = 8) injected with L-NAME (5 mg/kg subcutaneously) and 0.9% saline (1 mL/kg intraperitoneally). The untreated animals (n = 6) and those treated with 0.9% saline (n = 8) showed no differences and were pooled; they are referred to as the control group (n = 14).
Microsphere Injection

Four different fluorescent-labeled microspheres (blue, blue-green, yellow-green, and orange; FluorSpheres; Molecular Probes Inc., Eugene, OR) with a diameter of 15.5 ± 0.5 μm were randomly assigned to the different time points of blood flow measurement. After thoroughly vortexing, 0.2 mL of the microsphere solution (0.9% saline + 0.02% Tween 20), containing approximately 2 × 10^4 microspheres, was injected at a rate of 0.2 mL/min and flushed with 0.5 mL 0.9% saline at a rate of 0.3 mL/min. Withdrawal for reference blood samples started 10 seconds before microsphere injection and was continued for 60 seconds after the injection was finished at a rate of 0.22 mL/min. Micropumps (Instech Lab Inc., Plymouth Meeting, PA) were used for injection and withdrawal. Approximately 0.7 mL of blood withdrawn at each measurement, totaling 2.8 mL blood collected for each experiment, was replaced with 0.9% saline. The amount of microspheres injected was adjusted so that at least 400 microspheres were recovered in each organ and reference blood sample, the minimum required to reduce random variation.24

Blood pressure, heart rate, and hematocrit were evaluated at each of the four blood flow measurements. Animals were killed 10 minutes after the last microsphere injection by decapitation under methoxyflurane anesthesia. Mucosa and submucosa of the duodenum (complete), jejunum (a piece 15 cm long, beginning 10 cm distal to the ligament of Treitz), ileum (the final 15 cm measured from the ileocecal valve), and the entire colon were recovered for blood flow determination. The intestine was removed and opened along its mesentery. The mucosa was blotted dry and scraped from the submucosa with a glass slide. The muscularis was then stripped off the submucosa with a scalpel. Mucosa and submucosa were measured as one compartment because mucosal vasculature behaves as a bed serially connected to that of the submucosa. Proper separation of the layers was confirmed histologically. The muscular layer of the 15-cm intestinal segments did not contain sufficient microspheres to calculate blood flow accurately.24

Figure 1. Differences in mean arterial blood pressure (MAP) from baseline in controls (open circles), lipopolysaccharide (LPS)-induced sepsis (20 mg/kg intraperitoneally; open squares), N^3-nitro-L-argininemethyl ester (L-NAME; 5 mg/kg subcutaneously; black circles), and LPS-L-NAME groups (20 mg/kg intraperitoneally and 5 mg/kg subcutaneously; black squares). Actual baseline values for the different groups were (in mmHg) 98 ± 3.1, 102 ± 3.1, 95 ± 4.2, and 90 ± 3.5, respectively. Values are mean ± standard error of the mean of 8 to 14 rats/group. *p < 0.05; †p < 0.001 versus baseline values.

The number of injected microspheres was not increased due to concern for alterations in the microcirculation by excessive occlusion of capillaries. To ascertain equality of microsphere distribution, the blood flow from left and right kidneys was compared. Tissue samples were weighed (wet weight) and digested with 4 M KOH (blood samples with 16 M KOH) and filtered. Fluorescence dye was extracted with 2-(2-ethoxyethoxy)ethyl acetate (Aldrich Chemical Co., Milwaukee, WI), and the signal was determined with a spectrophotometer (Luminescence Spectrophotometer LS-50B, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK).

Organ blood flow per gram of tissue (V_O) was calculated as follows: V_O = V_R × S_O/S_R (mL/min/g), where V_R is the rate of reference blood sampling (0.22 mL/min), S_R is the fluorescent signal of the reference blood sample, and S_O is the fluorescent signal of the organ.

Statistical Analysis

Two-way mixed analysis of variance was used to compare the treatments in each organ or organ part. Two-way fully repeated analysis of variance was used to compare the different segments of intestine for each treatment. The Newman-Keuls post hoc test was used to calculate significance, which was accepted at p < 0.05. Simple regression analysis was used to compare left and right kidney blood flow. Results are expressed as mean ± standard error of the mean.

Drugs

We used LPS derived from Escherichia coli serotype 0111:B4 (Sigma Chemicals, St. Louis, MO), dissolved in 0.9% saline (20 mg/mL) and L-NAME (Sigma) dissolved in 0.9% saline to a concentration of 5 mg/mL.

RESULTS

Mean Arterial Pressure

Mean arterial pressure (MAP) in the control group remained stable over the duration of the experiment (Fig. 1).
In contrast, administration of LPS caused a 22% decline in MAP at 2 hours (from 102 ± 3.1 to 80 ± 5.5 mmHg) that was maintained throughout the experiment. Administration of L-NAME alone increased blood pressure above baseline level, although the difference between L-NAME and baseline at the 6-hour time interval was not significant. The administration of L-NAME during sepsis not only prevented LPS-induced hypotension, but also increased MAP significantly above baseline over the entire experiment.

**Mucosal Blood Flow—Control Group**

Figure 2 shows the baseline mucosal blood flow in each intestinal segment. Ileal mucosal blood flow (4.17 ± 0.25 mL/min/g) was significantly higher (p < 0.001) than that of the duodenum (2.54 ± 0.17 mL/min/g), jejunum (2.40 ± 0.46 mL/min/g), or colon (1.65 ± 0.30 mL/min/g). Colonic mucosal blood flow was lower than that of the rest of the gut (p < 0.05). This perfusion pattern persisted throughout the 6 hours of the experiment.

**Mucosal Blood Flow—Experimental Groups**

Figure 3 shows the mucosal blood flow over the course of sepsis development. Two hours after LPS administration, mucosal blood flow tended to increase in the duodenum and jejunum (25%–28% above baseline). In the ileum, mucosal blood flow declined by 43% from 3.91 ± 0.45 to 2.22 ± 0.39 mL/min/g (p < 0.005). These changes in mucosal blood flow continued for the duration of the experiment. Mucosal blood flow to the colon was not significantly altered in either direction by LPS administration.

Administration of L-NAME alone significantly decreased mucosal blood flow after 2 hours throughout the small intestine but not the colon (Fig. 4). Reductions of 37%, 30%, and 61%, respectively, occurred in the duodenum, jejunum, and ileum. In the latter, the baseline mucosal blood flow of 3.70 ± 0.27 mL/min/g dropped to 1.44 ± 0.21 mL/min/g. Of these, only duodenal mucosal blood flow returned to baseline levels by the end of the experiment.

Administration of L-NAME combined with LPS had an effect similar to that of L-NAME alone on small and large intestinal mucosal blood flow at the 2-hour time
Values are mean ± standard error of the mean of eight rats. *p < 0.05, **p < 0.001 versus baseline values.

Heart Rate and Hematocrit

Heart rate did not change significantly in any of the groups (Table 1). Hematocrits decreased significantly in the control and LPS groups throughout the experiment but remained stable in the L-NAME group. In the LPS–L-NAME group, the hematocrit was unchanged for the first 2 hours but then declined significantly (p < 0.001).

Kidneys

In the control group, blood flow increased insignificantly from 5.38 ± 0.18 to 5.85 ± 0.20 mL/min/g and returned to 5.22 ± 0.23 mL/min/g at the end of the experiment (Table 2). Blood flow declined steadily in the LPS group from 5.48 ± 0.24 to 2.78 ± 0.53 mL/min/g (p < 0.001). The LPS–L-NAME group showed the most dramatic fall in blood supply, from 5.54 ± 2.08 to 2.08 ± 0.31 mL/min/g (p < 0.001). Administration of L-NAME alone resulted in a decrease of blood flow from 5.22 ± 0.23 to 2.71 ± 0.16 mL/min/g at 2 hours, with a return toward baseline values at the end of the experiment. L-NAME administration in normal and septic rats resulted in an immediate (2 hours) decline rather than the steady decline in blood flow seen in septic rats alone.

Accuracy of Blood Flow Measurements

Blood flows to the left and right kidneys were compared, yielding the regression curve y = 0.98x + 0.063 with a regression coefficient of 0.937 (p < 0.001), indicating equal microsphere distribution during the measurements.

DISCUSSION

We performed this study with several purposes in mind. Due to the functional and structural heterogeneity of the intestine and an increasing awareness of the potential importance of the gut in sepsis, we wanted to determine whether regional differences in intestinal blood flow existed in an animal model of LPS-induced sepsis. The gut is assuming an ever-increasing role in the pathogenesis of multiple organ failure after sepsis. In view of the functional and structural heterogeneity of the gut, we em-

<table>
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<th>Heart rate (beats/min)</th>
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*p < 0.05, †p < 0.001, vs. baseline.
Values are mean ± SEM of eight to 14 rats/group.
barked on a series of experiments to assess regional differences in intestinal blood flow after LPS-induced sepsis in the rat. We wanted not only to determine normal patterns of flow and changes occurring during sepsis, but also to study the role of NO inhibition during these conditions. The fluorescent microspheres method\textsuperscript{23} was used to evaluate compartmental organ blood flow at multiple intervals. Comparison of the newly developed fluorescent microsphere method to the well-established radioactive microspheres method shows both to be equally well suited to measure blood flow. Due to the high sensitivity and good spectral separation of fluorescent microspheres, the amount of injected spheres can be as small as that used for radioactive microspheres to measure blood flow.\textsuperscript{27} The advantages over radioactive microspheres include avoidance of radiation hazards and cost savings in most experimental settings.\textsuperscript{27} Furthermore, fluorescent microspheres are superior to radioactive microspheres in chronic animal experiments because of better label stability and the avoidance of the need for special animal care facilities to confine radioactivity. The biochemical advantages of fluorescence over spectrophotometry (colored microspheres) and radioactivity were presented elsewhere.\textsuperscript{28} One problem with the fluorescent microsphere method is that the processing of samples is time-consuming and complex. Developments are under way to facilitate this procedure through automation (R. Koch, personnel communication).

Because the determination of relative blood flow with the microsphere method is dependent on the weight of the tissue being perfused,\textsuperscript{26,29} mucosal blood flow in this study was normalized to the mucosal and submucosal weight rather than to the weight of the full wall thickness. This approach more precisely represents the actual perfusion to the mucosa and submucosa because the relative contribution of the weight of these layers to total intestinal weight decreases from the duodenum to the ileum.\textsuperscript{30,31} If this is not taken into account, distal mucosal perfusion of the small intestine (in mL/min/g) would be underestimated. Another consequence of reporting mucosal blood flow relative to mucosal and submucosal weight rather than to full gut wall weight is that the calculated flow values are approximately twofold higher.\textsuperscript{26}

Before evaluating any blood flow alterations during sepsis, we first wanted to establish the perfusion pattern of the different segments along the gut in control animals. Previous studies expressing mucosal blood flow relative to whole gut wall weight reported a descending proximal-to-distal gradient in intestinal blood flow,\textsuperscript{30,31} although one group demonstrated equal distribution.\textsuperscript{32} When mucosal blood flow was reported relative to the weight of the mucosal compartment only, blood flow to the different small intestinal segments was found to be similar.\textsuperscript{29} In the present study, however, ileal mucosal blood flow, normalized to mucosal and submucosal weight, was found to be significantly higher than that of the rest of the intestine. We have no conclusive explanation for this difference in the present study, other than the possibility that the flow pattern in conscious, unrestrained rats may be different from that in anesthetized animals\textsuperscript{21} or those that are restrained.

In this study, LPS markedly reduced mucosal blood flow in the ileum but had no significant effect on perfusion of the remaining gut. Although duodenal and jejunal mucosal blood flows tended to be somewhat elevated throughout the development of sepsis, these changes were not significant. Using radioactive microspheres, Xu et al.\textsuperscript{20} also demonstrated a selective LPS-induced blood flow reduction to the ileal mucosa. This was only apparent, however, when they examined compartmental (mucosa vs. all intestinal layers) blood flow, normalized for the respective intestinal layer weight. We confirmed their findings not only for the very early phase, as in their study, but also over an extended period of time. In contrast, Lang et al.\textsuperscript{33} reported in septic animals that no change occurred in global small intestinal blood flow. Nevertheless, when segmental intestinal perfusion was examined, they found a selective increase to the jejunum. Using intravital microscopy, numerous studies have also reported decreased ileal blood flow during sepsis,\textsuperscript{34–36} usually with the assumption that this reflected the response of the entire intestine.
Our second goal was to investigate the effect of NO synthase inhibition on mucosal blood flow at different locations in the intestine.

After the administration of L-NAME alone, mucosal blood flow to all intestinal segments declined, accompanied by an increase in arterial pressure. The colonic mucosa also showed a decreased blood flow, but this finding was not significant. We concluded that a baseline production of NO is necessary to maintain physiologic intestinal blood flow. The colon seems to be an exception because no significant changes occurred during sepsis or NO synthase inhibition.

After the induction of septic shock and the simultaneous administration of L-NAME, mucosal blood flow to all regions in the small intestine was decreased at 2 hours. This initial decrease in organ blood flow was also demonstrated by Mulder et al. They demonstrated that complete or partial inhibition of NO production was deleterious in the first hour of endotoxic shock. In the LPS–L-NAME group, blood flow to the duodenum and jejunum returned to baseline values after the presumed stimulation of the inducible NO synthase (NOS-II) at 4 hours and remained normal until the termination of the experiment. In contrast, ileal blood flow remained depressed for the entire time interval. As mentioned above, colonic blood flow remained unchanged.

Upregulation of NOS-II has been shown to occur at about 3 hours after induction with LPS. In this model, changes in blood flow occurred to the ileum but not to the proximal gut 2 hours after the induction of sepsis. No further changes were observed at later time points, even though it is likely that NOS-II was upregulated and producing nitric oxide. Although we did not measure NOS-II upregulation in these experiments, this would suggest that NOS-II does not play a major role in the regulation of intestinal blood flow in sepsis. This observation needs further investigation, with more selective inhibitors for the different NOS isoforms and inhibition of cofactors essential for NOS-II activity (e.g., tetrahydrobiopterin) to analyze the role of NO in regulating intestinal blood flow during sepsis.

Taken together, these results confirm and expand the concept that normal structural and functional variations are present throughout the intestine. It is well known that there is a differential response in intestinal blood flow to physiologic stimuli, such as food intake, and pharmacologic substances. This study indicates that regional responses to pathologic perturbations also occur. Moreover, our results emphasize the danger of making generalizations about intestinal blood flow based on results from any one segment of the intestinal tract.

References


