Effect of nitric oxide on capillary hemodynamics and cell injury in the pancreas during Pseudomonas pneumonia-induced sepsis

Barbara Tribl,

Ryon M. Bateman,

Stephanie Milkovich,

William J. Sibbald,

Christopher G. Ellis

1Sunnybrook and Women’s College Health Sciences Centre, University of Toronto, Toronto M4N 3M5; and 2Lawson Health Research Institute and Department of Medical Biophysics, University of Western Ontario, London, Ontario, Canada N6A 5B8

Submitted 18 March 2003; accepted in final form 8 September 2003

A number of papers have proposed that sepsis or septic shock leads to a preferential redistribution of blood flow from the pancreas resulting in ischemic pancreatitis, which contributes to the progression of sepsis to multiple organ failure and ultimately death (5, 13, 20). Booke et al. (5) proposed that overproduction of nitric oxide (NO) by other splanchnic organs was the cause for a redistribution of blood flow from the pancreas. More recently, a potent vasoconstrictor, endothelin, has been implicated in the decrease in pancreatic blood flow (5, 286: H340–H345, 2004. First published September 11, 2003; 10.1152/ajpheart.00234.2003.—Sepsis-induced nitric oxide (NO) overproduction has been implicated in a redistribution of flow from the pancreas making it vulnerable to ischemic injury in septic shock. To test this hypothesis in a remote injury model of normotensive sepsis, we induced Pseudomonas pneumonia in the rat and used intravital video microscopy (IVVM) of the pancreas to measure functional capillary density, capillary hemodynamics (red blood cell (RBC) velocity, lineal density, and supply rate), and lethal cellular damage (propidium iodine staining) at 6 and 24 h after the induction of pneumonia. With pneumonia, plasma nitrite/nitrate [NO\(_2\)/NO\(_3\)] levels were doubled by 21 h (P < 0.05). To assess the effect of NO overproduction on microvascular perfusion, NO\(_3\)-(1-iminoethyl)-L-lysine (L-NIL) was administered to maintain NO\(_3\) levels at baseline. Pneumonia did cause a decrease in RBC velocity of 23% by 6 h, but by 24 h RBC velocity and supply rate had increased relative to sham (P < 0.05). L-NIL treatment demonstrated that this increase was due to NO overproduction. With pneumonia, there was no change in functional capillary density and only modest increases in cellular damage. We conclude that, in this normotensive pneumonia model of sepsis, NO overproduction was protective of microvascular perfusion in the pancreas.

A NUMBER OF PAPERS HAVE PROPOSED that sepsis or septic shock leads to a preferential redistribution of blood flow from the pancreas resulting in ischemic pancreatitis, which contributes to the progression of sepsis to multiple organ failure and ultimately death (5, 13, 20). Booke et al. (5) proposed that overproduction of nitric oxide (NO) by other splanchnic organs was the cause for a redistribution of blood flow from the pancreas. More recently, a potent vasoconstrictor, endothelin, has been implicated in the decrease in pancreatic blood flow associated with a peritonitis model of septic shock in pigs (14). All of these reports appear to confirm the earlier findings of more severe pancreatic injury in sepsis than in other organs (6, 11, 12).

However, recent clinical studies of mechanically ventilated intensive care patients with sepsis and septic shock have presented a very different picture (25, 26). The septic shock patients demonstrated severe exocrine pancreatic dysfunction without histological evidence of necrosis or biochemical evidence of tissue injury as indicated by elevated levels of amylase or lipase (25). Although no blood flow measurements were made, it is unlikely that these patients experienced pancreatic ischemia as predicted by the experimental models. The difference between the experimental studies and this clinical report may be the experimental models employed in these studies. Endotoxin models have been criticized as not adequately mimicking the clinical situation (9). Peritonitis models, which more closely model the progression of sepsis in patients, may cause direct inflammatory injury to the pancreas and its microvasculature resulting in pancreatitis. However, in the clinical study, only a small number of patients had an abdominal focus of inflammation. Sepsis arising from a focus of injury outside of the abdominal cavity and causing a remote injury to the pancreas may have very different characteristics. Studies of remote injury models of sepsis have shown microvascular dysfunction without extensive areas of ischemia (3, 10). A focus of infection in the gut caused a significant increase in stopped flow capillaries in rat skeletal muscle (from <10% in sham to >25% in sepsis), but the loss of perfused capillaries was very heterogeneous with both stopped flow and perfused capillaries observed in the same microscopic field of view (3, 10). Inhibition of NO overproduction with aminoguanidine prevented this loss of perfused capillaries. The goals of the present study were to investigate whether remote inflammatory injury to the pancreas would cause a similar pattern of microvascular dysfunction and whether inhibition of NO overproduction would prevent the remote injury. We chose to study remote injury in the pancreas with intravital video microscopy using a nonlethal Pseudomonas pneumonia model of sepsis in the rat.

METHODS

Marine model of Pseudomonas pneumonia-induced sepsis. The following experimental protocols were approved by the University of Western Ontario Council on Animal Care.

Male Sprague-Dawley rats were anesthetized with halothane (1–2%) and oxygen (100%). Animal temperature (36.5–37.5°C) was maintained by heat lamp and monitored by a rectal probe (Simpson 383). The right carotid artery was cannulated [polyethylene (PE)-10/PE-50, Clay Adams] to enable monitoring of mean arterial pressure (MAP; Digi-Med pressure analyzer and Baxter pressure transducers). The left jugular vein was cannulated (Bio-Sil tubing, Silmed) for intravenous fluid resuscitation. In pneumonia rats, a 2-mm transverse incision was made in the anterior trachea, through which a 20-gauge Angiocath catheter (Becton Dickinson) was inserted; 40 μl/100 g body wt of broth of 2 × 10⁸ colony-forming units/ml Pseudomonas aeruginosa were instilled through the catheter (prepared by the Department of Microbiology, Victoria Campus, London Health Sciences Centre). The tracheotomy was covered with a 5 × 5-mm piece of gauze (26). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of Gelfoam, and the wound was closed. The control procedure consisted of cutting the trachea and placing the Gelfoam. Dextrose (5%) and sodium chloride (0.9%), 1 ml-100 g body wt\(^{-1}\) h\(^{-1}\), was administered during surgery and in the postoperative period to prevent hypotension and support the emergence of the hyperdynamic circulatory state (29). Fentanyl was added to the infusion at a rate of 0.5 mg-100 g body wt\(^{-1}\) h\(^{-1}\) for analgesia, and 0.5 U-100 g body wt\(^{-1}\) h\(^{-1}\) heparin was added to maintain catheter patency. Catheters were tunneled subcutaneously to the interscapular region where they exited and were connected to a swivel-harness device. Rats were maintained in cages with free access to water and standard rat chow.

Pancreas preparation. At 6 and 24 h after \(P.\) aeruginosa instillation, rats were reanesthetized by an intravenous injection of pento-barbital (with temperature monitored and maintained as before). The pancreas was exteriorized through a left lateral abdominal incision by pulling the spleen. After the animal was transferred onto the microscopic stage, the pancreas was placed at the center of the microscopic stage, kept moist with physiological saline solution, covered with a thin, transparent plastic wrap (to prevent tissue drying), and slightly compressed by a coverslip. The microvascular preparation was allowed to stabilize for 30 min. To avoid any bias introduced by the preparation technique, pancreas preparation was done by a single person throughout the study. Animals in all groups were exposed to 30% oxygen via a mask.

Intravital video microscopy of the pancreas. The pancreas was transilluminated by a 100-W xenon lamp, and its surface microcirculation was observed using a Nikon Diaphot 300 microscope at \(\times 20\) × 0.4, with an effective magnification on the monitor of \(\times 1,740\). High-contrast images were achieved with a 440-nm band-pass filter (Nikon DF440-DF30). Video images of the microcirculation were obtained with a charge-coupled device camera (Dage-MTI-CCD272), viewed on a monitor (Panasonic WV-BM1400) via a closed-circuit video system, and recorded (Panasonic AG 7350 VCR) on S-VHS videotape for off-line analysis. Time code (Telekom Research) was placed on the audio track. After the stabilization period, 3-min video recordings were made of 10 random fields/experiment.

Analysis of capillary hemodynamics. A Silicon Graphics O2 Image Analysis workstation was used to capture and store 45 s of video recording (1,350 frames) for each field of view to be analyzed for capillary hemodynamics. Each video sequence was processed to show the location of perfused capillaries within the field of view. This “functional” image was used for locating each in-focus capillary segment to be analyzed for hemodynamic data. From the functional image, the user selected each capillary segment and the analysis software automatically processed the light intensity data for each capillary from the stored frame-by-frame video data. Red blood cell (RBC) velocity (in mm/s) was calculated using a spatial correlation technique that determines how far RBCs move from frame to frame. The number of RBCs in the capillary segment in each frame was determined from the RBC optical density \(\text{OOD} = \log (I/I_0)\), where \(I_0\) is the plasma light intensity and \(I\) is the RBC light intensity. The total OD in the segment in each frame was divided by the OD for a single RBC to yield the number of RBCs in that length of capillary and hence the number of RBCs/mm, i.e., RBC lineal density. The RBC supply rate (in RBCs/s) was calculated from the product of RBC velocity and RBC lineal density. Each field of view yielded from 1 to 10 capillary segments with the hemodynamic data for each expressed as mean ± SD of the 45-s sample.

Functional capillary density analysis. Functional capillary density (FCD; in cm\(^{-1}\)), defined as the length of all erythrocyte-perfused capillaries per observation area, was assessed in each of 10 randomly selected areas in accordance with the method of Schmid-Schoenbein et al. (23). A grid system with a grid width representing 50 \(\mu\)m was superimposed on the video monitor. By counting the number of intersections between the grid and the capillaries \((N)\), the FCD for each observation area was calculated in accordance with the following equation: \(\text{FCD} = n/2 \times N/L\) (cm\(^{-1}\)), where \(L\) represents the total length of the grid system.

Fluorescence microscopy of lethal cell injury. Severely injured cells were labeled in vivo with an intra-arterial bolus of the fluorescent vital dye propidium iodine (PI; 0.05 mg/100 g body wt, Sigma-Aldrich) just before the end of each experiment. Nuclei labeled with PI (excitation wavelengths, 500-545 nm; emission barrier filter, >600 nm, Omega Optics XF 103–2) were used to identify irreversibly damaged cells (15–17) and are expressed as the number of PI-labeled nuclei per field.

Drug dose and delivery. \(N^\beta-(1\text{-iminoethyl})\text{-L-lysine (}-\text{L-NIL;}\text{ Sigma-Aldrich; St. Louis, MO}\) was administered intravenously at 0.3 mg/kg \(\text{h}^{-1}\) starting at 10 h to maintain plasma nitrite/nitrate \((\text{NO}_x)\) levels at ≥20% baseline throughout the experiment.

Measurement of plasma levels of NO metabolites. Plasma \text{NO}_x measurements were made using an optimized \text{NO} chemiluminescent system (2). In brief, whole blood was collected isovolumically from the arterial catheter at 1, 9, 15, and 21 h and at the end of the experiment and immediately centrifuged \((3,000 \times g\) for 5 min at \(4^\circ\text{C}\)). Plasma was stored at \(-20^\circ\text{C}\) before analysis. \text{NO}_3 species (\text{NO}_3; and \text{NO}_2; were reduced to \text{NO} \((0.05 \text{ M} \text{ V(III) in 1 M HCl at } 90^\circ\text{C}}\) and quantified by chemiluminescent reaction with ozone using a Sievers 270B NO analyzer. The system was calibrated against known concentrations of \text{NO}_3;.

Statistical analysis. All values are expressed as means ± SD unless otherwise stated. For all tests of significance, \(P\) values of \(< 0.05\) were considered statistically significant. A \(2 \times 2\) ANOVA model was used for statistical analysis of physiological and microcirculation data. Accordingly, the injury effect, the time effect, and the interaction between injury and time were analyzed as described previously (3). A one-way ANOVA was used to assess differences in physiological and microcirculation parameters between 24-h sham and 24-h pneumonia animals with and without \(\text{-NIL}\) treatment. Tukey’s multiple-comparison tests for all pairwise differences were performed to identify

Table 1. Physiological parameters at 6 and 24 h after the induction of sepsis

<table>
<thead>
<tr>
<th></th>
<th>6 h</th>
<th>24 h</th>
<th>(P) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>106±11</td>
<td>101±11</td>
<td>NS</td>
</tr>
<tr>
<td>WBCs, G/l</td>
<td>4,640±1,262</td>
<td>3,283±964</td>
<td>NS</td>
</tr>
<tr>
<td>Erythrocytes, T/l</td>
<td>4.82±0.14</td>
<td>5.50±0.19</td>
<td>NS</td>
</tr>
<tr>
<td>Platelets, G/l</td>
<td>636±354</td>
<td>677±130</td>
<td>NS</td>
</tr>
<tr>
<td>Lactate, mg/dl</td>
<td>2.3±0.34</td>
<td>2.35±0.22</td>
<td>NS</td>
</tr>
<tr>
<td>Lipase, u/l</td>
<td>17±2</td>
<td>16±4</td>
<td>NS</td>
</tr>
<tr>
<td>Anylase, u/l</td>
<td>1,359±195</td>
<td>1,215±181</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 1. Physiological parameters at 6 and 24 h after the induction of sepsis

MAP, mmHg: mean arterial pressure; WBCs, white blood cells; NS, not significant. \(P\) values were determined by two-way ANOVA unless otherwise noted. *\(P < 0.05\), 6-h pneumonia vs. 24-h pneumonia (post hoc analysis with Tukey’s test); \(\dagger P < 0.05\), 6-h sham vs. 24-h sham (post hoc analysis with Tukey’s test); \(\ddagger P < 0.05\), 6-h sham vs. 6-h pneumonia (post hoc analysis with Tukey’s test); \(\S P < 0.05\), 24-h sham vs. 24-h pneumonia (post hoc analysis with Tukey’s test).
reflected only small differences between groups. The white
fimonia in septic animals.
109 mmHg in the L-NIL group. Analysis of the MAP data
from a low of 98 mmHg in the 24-h sham group to a high of
Erythrocyte count increased slightly over time in the sham
compared with the untreated pneumonia group (Table 2).
Animals received L-NIL after the induction of pneumonia at
microvascular parameters at 6 and 24 h after the induction of sepsis
Table 3. Microcirculatory parameters at 6 and 24 h after the induction of sepsis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>Pneumonia</th>
<th>Pneumonia with L-NIL</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC velocity, μm/s</td>
<td>104±84</td>
<td>80±71†</td>
<td>114±76</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lineal density, RBCs/mm</td>
<td>80±34</td>
<td>83±35</td>
<td>80±28</td>
<td>NS</td>
</tr>
<tr>
<td>Supply rate, RBCs/s</td>
<td>7.2±5.9</td>
<td>6.7±6.4</td>
<td>7.9±6.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FCD, cm⁻¹</td>
<td>169±23</td>
<td>180±31</td>
<td>167±42</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Value are means ± SD. RBCs, red blood cells; FCD, functional capillary density. P values were determined by two-way ANOVA unless otherwise noted. †Significance relative to sham and pneumonia with L-NIL (post hoc analysis with Tukey’s test). NS: not significant.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
rat was chosen as the focus of inflammation for the remote injury to the pancreas. This model has been demonstrated to be hyperdynamic, to be associated with gut mucosal and microvascular injury, and to lead to the development of gut barrier dysfunction with bacterial translocation as early as 24 h (29). We observed that rats with pneumonia developed severe dyspnoea, tachypnoea, piloerection, and a significant drop in WBCs by 24 h. There was a statistically significant increase in plasma NO\textsuperscript{x} levels to twice baseline, which is another indicator of systemic inflammation (3). Although there was a statistically significant difference in MAP between the 24-h sham and 24-h pneumonia animals, it was not physiologically relevant, indicating that our fluid resuscitation protocol maintained MAP. All of these signs demonstrate that our pneumonia animals developed normotensive sepsis by 24 h.

Choice of l-NIL. l-NIL was chosen as a selective inhibitor of inducible NO synthase (iNOS), which is responsible for the increased production of NO in sepsis (1). Aminoguanidine, another selective iNOS inhibitor, was not chosen because it has antioxidant properties that prevent one from drawing mechanistic conclusions concerning the role of NO overproduction in the pathogenesis of sepsis (28). Nonselective inhibitors, such as N-monomethyl-l-arginine, used by Booke et al. (5), have adverse effects on organ perfusion because they also inhibit eNOS, the constitutive form of NOS, which plays a key role in regulating microvascular blood flow.

Time course of NO overproduction. As shown in Fig. 1, NO\textsuperscript{x} levels were not significantly increased until 21 h after the induction of pneumonia, which is rather late compared with several lipopolysaccharide (LPS) and cecal ligation and perforation models with a more rapid increase in NO\textsuperscript{x} (19, 22, 24). Because NO\textsuperscript{x} levels began to increase between 9 and 15 h after the induction of pneumonia, l-NIL infusion was not started until 10 h.

Alterations in pancreatic perfusion with pneumonia. The results of the intravital video microscopy study of microvascular perfusion of the pancreas after the induction of pneumonia were unexpected. Although velocities in capillaries at 6 h after the induction of pneumonia were significantly decreased by 23% relative to sham animals, this occurred without a loss of FCD or a decrease in the RBC supply rate. By 24 h, when sepsis was clearly established, the expected progressive deterioration of microvascular perfusion of the pancreas had not occurred. Surprisingly, there was no change in FCD, but there was a 22% increase in RBC velocity and a 38% increase in the RBC supply rate above the 24-h sham values. Relative to the 6-h pneumonia group, the increases in RBC velocity and RBC supply rate were 74% and 62%, respectively, indicating a substantial rebound and increase in microvascular blood supply in these capillaries. This substantial increase in capillary blood flow with the onset of normotensive sepsis has not been previously reported in the pancreas.
Mechanisms responsible for changes in pancreatic perfusion. The decrease in velocity at 6 h cannot be attributed to NO overproduction because plasma NO\textsuperscript{-} levels did not begin to increase until sometime after 9 h. A possible cause for the decrease in velocity could be increased levels of endothelin. Krejci et al. (14) reported that an endothelin antagonist, bosentan, restored blood flow in the pancreas in a peritonitis septic shock model in pigs, thus indicating that endothelin was responsible for the decreased pancreatic blood flow in their model. If the decrease in velocity we observed was due to endothelin, then endothelin levels have increased in the pancreas before the upregulation of iNOS and overproduction of NO.

Although we had not expected the increase in velocity and supply rate at 24 h, the 1-NIL data does allow us to address the mechanism responsible for this increase. When 1-NIL was administered to maintain plasma NO\textsuperscript{-} levels at baseline, mean RBC velocity fell relative to the 24-h pneumonia group (significantly different, Table 4) to the level of the 24-h sham group (not significantly different). Because 1-NIL is a highly selective inhibitor of iNOS and only sufficient 1-NIL was administered to inhibit the overproduction of NO [NO\textsubscript{x} levels remained at baseline (Fig. 1) with no significant change in MAP (Table 2)], we can state that the mechanism responsible for the increase in RBC velocity with pneumonia at 24 h was NO overproduction in the pancreas.

1-NIL treatment also resulted in a significant decrease in mean RBC lineal density (relative to both the 24-h sham and 24-h pneumonia groups, Table 4). RBC lineal density is a measure of capillary hematocrit. Because the decrease in lineal density occurred without a decrease in systemic RBC count (Table 2), there must have been a redistribution of RBC flow away from the capillaries being observed at the surface to other regions deeper within the pancreas (capillaries in these regions would have higher lineal densities). These data demonstrate that without the overproduction of NO in sepsis, there would have been a maldistribution of RBC flow within the pancreas.

The decrease in RBC velocity and lineal density with 1-NIL treatment resulted in a substantial decrease in RBC supply rate, from 10.9 ± 8.8 RBCs/s in the untreated pneumonia animals to 4.2 ± 4.2 RBCs/s (Table 4). Without NO overproduction, the RBC supply would have been seriously impaired, a situation that would be consistent with the decrease in flow observed in LPS and peritonitis sepsis models. These results suggest that the difference between our study and these models likely reflects whether vasodilation (NO) or vasoconstriction (endothelin) dominated the inflammatory response (4).

Pancreatic injury in this pneumonia model. Unlike the endotoxin models of sepsis, which did demonstrate evidence of pancreatitis and pancreatic injury (21, 27), there was no significant increase in amylase or lipase levels indicative of substantial pancreatic damage in this model of sepsis. This is consistent with the microcirculatory data, which showed no evidence of pancreatic ischemia. Amylase did increase significantly from 6 to 24 h, but this occurred in both sham and pneumonia groups. Although PI staining did demonstrate a statistically significant fourfold increase in lethal cell injury in the 24-h pneumonia group (Fig. 2), this injury was not sufficient to be reflected in increased amylase or lipase levels. We speculate that the PI staining data reflect an underlying impairment of pancreatic function as found in the clinical study of septic shock patients, but clearly, additional studies are required.

The mechanism responsible for the increased lethal cell injury is not apparent from the present study. 1-NIL treatment had no effect on amylase and lipase results or on PI staining data, indicating that elevated levels of NO were not responsible for the cell injury. It is unlikely that WBC accumulation in the pancreas was responsible because 1-NIL treatment prevented the fall in WBC counts at 24 h (Table 2), which is consistent with several other animal models of inflammation where iNOS inhibitors suppressed the infiltration of inflammatory cells (7, 8, 18).

In conclusion, pneumonia-induced sepsis caused a reduction in pancreatic capillary perfusion at 6 h and an unexpected rebound and increase in RBC velocity and supply rate at 24 h, without a loss in perfused capillary density. NO overproduction was beneficial to pancreatic capillary perfusion and not responsible for cell injury. Further studies are needed to establish whether this normotensive pneumonia model of sepsis induces pancreatic cellular dysfunction similar to what was demonstrated in the previous clinical study with septic shock patients (25). Clearly, the time course of upregulation of mediators involved in the sepsis-induced changes of microvascular perfusion and of cell injury and pancreatic dysfunction deserve further investigation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the advice of Dr. Pei Yu and Dr. Richard Potter.

GRANTS

This research was supported by Heart and Stroke Foundation of Ontario Grant T4476 (to W. J. Sibbald) and Canadian Institutes of Health Research Grant MOP-49416 (to C. G. Ellis). B. Tribl was supported by a Charlotte-Bühler stipend from the Fonds zur Förderung der Wissenschaftlichen Forschung (Austria).

REFERENCES

16. Liu S, Adcock IM, Old RW, Barnes BJ, and Evans TW.
17. Maddock HL, Mocanu MM, and Yellon DM.
20. Raper RF, Sibbald WJ, Hobson J, and Rutledge FS.
27. Wray GM, Millar CM, Hinds CJ, and Thiemermann C.
28. Yu P and Martin CM.