Relationship between plasma NOx and cardiac and vascular dysfunction after LPS injection in anesthetized dogs

Paul R. Forfia, Xioping Zhang, Francisca Ochoa, Manuel Ochoa, Xiobin Xu, Robert Bernstein, Pravin B. Sehgal, Nicholas R. Ferreri, and Thomas H. Hintze

Departments of Physiology, Anatomy and Cell Biology, and Pharmacology, New York Medical College, Valhalla, New York 10595

Forfia, Paul R., Xioping Zhang, Francisca Ochoa, Manuel Ochoa, Xiobin Xu, Robert Bernstein, Pravin B. Sehgal, Nicholas R. Ferreri, and Thomas H. Hintze. Relationship between plasma NOx and cardiac and vascular dysfunction after LPS injection in anesthetized dogs. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H193–H201, 1998.—The relationship between plasma nitrite, nitrate, and nitric oxide (NOx), cytokines, and cardiac and vascular dysfunction after lipopolysaccharide (LPS) was studied in chronically instrumented anesthetized dogs. LPS was administered (1 mg/kg iv), and hemodynamics were recorded at baseline, every 15 min for 1 h, and every hour for an additional 14 h. Dramatic reductions in mean arterial pressure (−48 ± 6%), cardiac output (−40 ± 8%), stroke volume (−42 ± 9%), and first derivative of left ventricular pressure (LV dP/dt, −38 ± 7%) were seen within 1 h after injection of endotoxin. Cardiac output was not different from control by 9 h, whereas mean arterial pressure (−19 ± 7%), stroke volume (−32 ± 8%), and LV dP/dt (−21 ± 10%) remained significantly depressed from control. Total peripheral resistance was not significantly different from control. Therefore, the hypotension appears to be due to a reduction in cardiac function and not to vasodilation. Levels of plasma NOx were not different from control until 4 h after LPS reached levels 597 ± 126% higher than control at 15 h. In vitro production of nitrite by coronary microvessels was also elevated, supporting our in vivo findings. In contrast, production of tumor necrosis factor-α and interleukin-6 occurred shortly after endotoxin injection, reaching peak levels at 45 and 150 min, respectively. Our data suggest that inducible nitric oxide synthase induction occurred after LPS injection. It is unlikely that nitric oxide contributed significantly to the hypotension and cardiac dysfunction early in our study, whereas cardiodepressive cytokines, particularly tumor necrosis factor-α, may be important. In contrast, the hemodynamic effects seen later after injection of endotoxin may be the result of an overproduction of nitric oxide, since there was a sixfold increase in plasma NOx levels at this time and a marked production of nitric oxide in isolated coronary microvessels in vitro.

plasma nitrate/nitrite; cytokines; tumor necrosis factor-α; interleukin-6; stroke volume; cardiac output; coronary microvessels; N-nitro-L-arginine methyl ester; aminoguanidine; S-methylisothiourea

Septic shock is the leading cause of death in intensive care units in the United States annually (22). Characterized by a complex metabolic and cardiovascular profile, sepsis is associated with hypotension and myocardial dysfunction that can often result in decreased organ perfusion and eventual multiple organ failure. Often the result of a gram-negative bacterial infection, the lipopolysaccharide (LPS) portion of the bacterial cell wall is thought to account for the toxicity of the infection (11). However, despite aggressive antibiotic treatment and the rapid clearance of LPS from the circulation, sepsis can progress long after the initial infection has been treated. On the basis of these findings, a cascade of mediators subsequent to the original infection have been implicated in the pathogenesis of septic shock.

Nitric oxide (NO) is one of the major mediators suspected in precipitating the cardiovascular collapse associated with septic shock. Under normal conditions, constitutively expressed isoforms of NO synthase (NOS) produce low levels of NO that, via the activation of soluble guanylate cyclase, participate in the regulation of vascular tone and platelet aggregation, in addition to a multiplicity of other functions (23). In contrast, during infection, LPS indirectly leads to the induction of an inducible isoform of NOS (iNOS) in a variety of tissues, including macrophages, vascular smooth muscle, cardiac myocytes, and endothelial cells (24). Originally described in 1985, Stuehr and Marletta (34) showed a sixfold increase in plasma nitrite/nitrate levels ~6 h after LPS injection in mice treated with a low dose of Escherichia coli LPS. In addition, macrophages from these mice incubated with LPS produced nitrite and nitrate. Studies by Szabo et al. (35) and Schulz et al. (31) confirmed the magnitude and time course of nitrite and nitrate production after LPS injection and also showed that the iNOS isoform was Ca2+-independent. Thus the Ca2+-independent isoform, which is not normally expressed in tissues, can, when induced, produce large amounts of NO over an extended period of time. Because high levels of NO are known to activate soluble guanylate cyclase, just as NO from a constitutive source does, it has been proposed that high levels of NO from iNOS may be partly responsible for the overt vasodilation and hypotension associated with septic shock. In addition, NO from this inducible isoform has been implicated as a potential negative inotropic agent (4, 36a) and, thus, a potential mediator of the cardiac dysfunction associated with experimental endotoxemia in animals and septic shock in humans. Using NOS inhibitors, Kilbourn et al. (17) and others (40) attempted to establish a role for NO in the hemodynamic alterations of endotoxemia. However, the induction of iNOS requires de novo protein synthesis of the enzyme, as well as the cofactor tetrahydrobiopterin. Therefore, a time delay of 4–6 h is required after injection of LPS to allow for induction of iNOS. For this reason, these studies (17, 40), none of which lasted >4 h, could not accurately evaluate the
role of NO from an inducible isoform of NOS in the development of cardiac and vascular dysfunction. Cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 have also been implicated in the pathogenesis of endotoxia and sepsis. More specifically, these signaling peptides, which are elevated almost immediately in LPS-treated animals as well as patients with sepsis (37), have been shown to be essential for the induction of iNOS (3, 36). In addition, there is substantial in vitro and in vivo evidence to suggest that TNF-α is a potent negative inotropic agent (7, 38). Thus cytokines such as TNF-α could mediate the early deleterious effects of endotoxin and provide the link between LPS and the expression of iNOS. Therefore, the goal of our study was to compare the time course for NO and cytokine expression and the time course of the cardiac and vascular dysfunction after a moderate dose of endotoxin in anesthetized dogs. Because of the time required for iNOS induction, a time course of 15 h was used to ensure an adequate time period for the induction and expression of NO by iNOS.

METHODS

Mongrel dogs of either sex (11 males and 2 females) weighing 24–28 kg were divided into two groups: an LPS-treated group (n = 7) and a non-LPS-treated (control) group (n = 6). The control group was used to ensure that the experimental preparation was stable over the 15-h time course and to rule out possible confounding effects of anesthesia on hemodynamic function. Both groups of animals were treated similarly with respect to surgical preparation, hemodynamic monitoring, blood gas analyses, and plasma assays. Pulmonary hemodynamics were monitored only in the LPS-treated animals.

Surgical Preparation

Dogs were sedated with acepromazine (0.3 mg/kg im; Ayerst Laboratories, New York, NY), anesthetized with pentobarbital sodium (25 mg/kg iv), then orotracheally intubated and ventilated with room air using a respirator (Harvard Apparatus, Boston, MA). With use of sterile surgical techniques, a thoracotomy was performed in the left fifth intercostal space. A Tygon catheter was placed in the descending thoracic aorta for the measurement of arterial pressure and for arterial blood sampling. A solid-state pressure gauge (model P6.5, Konigsberg Instruments, Pasadena, CA) was inserted into the left ventricle (LV) via an apical stab wound for the measurement of LV pressure, calculation of the first derivative of LV pressure (LV dp/dt), and measurement of heart rate. The catheter and wires were run subcutaneously and exited at the intrascapular region. The chest was closed in layers, and the pneumothorax was reduced. Antibiotics were given as needed while the dogs fully recovered from surgery (10–14 days). The protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals. Studies were performed in anesthetized animals to avoid the discomfort after LPS injection in recognition of the profound effects and limitations of the use of pentobarbital sodium.

On the day of the experiment an intravenous catheter was inserted in a peripheral vein for administration of drugs. The dogs were anesthetized with pentobarbital sodium (25 mg/kg iv), intubated, and allowed to breathe spontaneously. Supplemental doses of anesthesia were given as needed. An incision was made in the neck, and a 7-F Swan-Ganz thermocatheter was inserted through the left external jugular vein. The proximal and distal ports were positioned in the right atrium and pulmonary artery, respectively. Positions were confirmed by the presence of right atrial and pulmonary pressure waveforms on an oscilloscope. The Swan-Ganz catheter was used for the measurement of pulmonary arterial pressure, mixed venous blood sampling, and measurement of cardiac output.

Recording Technique

Aortic and pulmonary arterial pressures were measured by connecting the previously implanted catheters to strain gauge transducers (model P23 ID, Statham Instrument). Mean pressures were derived using a 2-Hz low-pass filter. LV pressure was measured from the solid-state pressure gauge, and maximal LV dp/dt was calculated using a microprocessor set as a differentiator with a frequency response flat to 700 Hz (model LM 324, National Semiconductor). Heart rate was derived from the LV pressure pulse interval using a cardiotachometer. All data were recorded on a direct-writing oscillograph (model 3800S, Gould). Cardiac output was determined by the thermodilution technique using the Swan-Ganz catheter and a computer (SP1435 cardiac index computer, Gould). Briefly, 10 ml of cold saline (6–8°C) were rapidly injected into the right atrium as an indicator, and cardiac output was calculated as the computed average of three injections with less than 10% variability between trials. We have used all these techniques previously (33, 41).

Experimental Protocols

Effects of LPS on hemodynamics. Baseline hemodynamics were recorded for ~60 min on the day of the experiment with the dogs anesthetized. For dogs in the LPS-treated group, E. coli LPS (serotype 026:B6, Sigma Chemical) was diluted in normal saline to a concentration of 10 mg/ml and administered as an intravenous bolus injection (1 mg/kg). The control group received an intravenous bolus injection of normal saline. Mean and phasic aortic and pulmonary (LPS group only) arterial pressure, LV pressure, LV dp/dt, heart rate, and cardiac output were recorded at 15-min intervals for the first 2 h, then every hour for an additional 14 h. Total peripheral resistance and pulmonary vascular resistance were calculated as mean arterial pressure divided by cardiac output and mean pulmonary arterial pressure divided by cardiac output, respectively.

Effects of LPS on blood gases, hemoglobin, and lactate. Arterial and venous blood samples were withdrawn at the same time points during which hemodynamics were measured. Arterial and venous pH, PO2, and PCO2 were measured with a blood gas analyzer (model 170, Corning, Medfield, MA). Arterial and venous blood total hemoglobin and percent O2 saturation were measured with a CO-oximeter system (model IL482, Instrumentation Laboratories, Lexington, MA). Arterial blood lactate was measured with a lactate analyzer (model 1500 Sport, Yellow Springs Instruments, Yellow Springs, OH). Arterial blood hematocrit was measured using a Sporicrit microhematocrit capillary tube reader (Mongrel Scientific, St. Louis, MO). Blood samples were transferred to 14-ml centrifuge tubes and spun at 3,000 g at 4°C for 15 min. The plasma from LPS-treated and control animals was then decanted, divided into three separate tubes (aliquots of 1 ml), and frozen for assays of plasma nitrite, nitrate, and NO (NOx), TNF-α, and IL-6.
Effects of LPS on plasma NOx. Methods for measuring plasma NOx have been previously described by our laboratory (41). Briefly, plasma samples from LPS-treated and control animals were thawed, vortexed, and centrifuged to remove any precipitated proteins. Supernatant (1 ml aliquots) was transferred to 5 ml polystyrene tubes. Plasma was then incubated with Aspergillus nitrate reductase (Boehringer Mannheim) to convert nitrate to nitrite. Tubes were capped with a rubber septum, and O2 in the headspace gas was displaced with argon. Nitrite was converted to NO by addition of HCl to bring pH below 2.0. Headspace gas was removed and injected into the NO analyzer (Sievers), where it mixed with ozone to form a chemiluminescent product. By addition of known concentrations of potassium nitrite and sodium nitrate to 1 ml of pooled plasma, a standard curve was constructed that related known concentrations of nitrate or nitrite to luminescence produced. Plasma NOx (µM) of dogs treated with endotoxin was calculated from the standard curve.

Effects of LPS on TNF-α and IL-6 production. Samples from both groups of animals were allowed to thaw at room temperature. Serum TNF-α levels were detected by enzyme-linked immunosorbent assay (Cytoscreen Immunobead Kit, Biosource International, Camarillo, CA). Briefly, standards consisting of recombinant TNF-α were used at concentrations of 0–1,000 pg/ml. Samples, including standards of known TNF-α concentration and unknowns, were pipetted into wells coated with antibody specific for TNF-α. A second biotinylated antibody, which binds to a second site on the TNF-α antigen, was then added. Samples were incubated for 1.5 h at room temperature and then aspirated to remove any excess or unbound biotinylated antibody. The enzyme streptavidin-peroxidase, which binds to the TNF-α-bound biotinylated antibody, was added. After a second incubation and washing to remove any unbound enzyme, substrate solution was added. This solution acts on bound enzyme to produce color. Absorbance of colored product was measured by spectrophotometric analysis at a wavelength of 450 nm. The absorbance of standards was plotted, and serum TNF-α concentrations were calculated (pg/ml) on the basis of the standard curve.

IL-6 concentrations were determined by bioactivity assay. Briefly, all plasma samples were allowed to thaw and then heat-inactivated at 56°C for 30 min. Bioactivity was measured by monitoring their ability to induce proliferation of murine B9 hybridoma cells using standard procedures described previously (1, 30). The World Health Organization reference standard 89/548 was included in every assay. IL-6 concentrations were calculated (pg/ml) on the basis of the standard curve.

Elective effects of LPS on NOx release from coronary microvessels in vitro. At the end of the experiment, the dogs were killed with an overdose of pentobarbital sodium (25 mg/kg iv). Hearts were immediately excised and kept in ice-cold phosphate-buffered saline (PBS) at pH 7.4. As described previously, large coronary arteries were freed from the epicardial surface and discarded (32). Coronary microvessels were obtained from the LV free wall using the methods of Gerritsen and Printz (10). Briefly, the myocardiun was cut into small pieces, minced with a tissue chopper (Mcllwain), and suspended in ice-cold PBS. The suspension was homogenized and then poured over a nylon mesh sieve. The material that adhered to the nylon was collected and transferred to a tissue bath containing PBS. The bath was oxygenated with 95% O2-5% CO2 for 30 min. Tissue samples (20 mg) were placed in 5 ml plastic tubes and incubated with 500 µl of PBS as control or 450 µl of PBS and 50 µl of drugs. The drugs were used to stimulate or inhibit nitrite release from the coronary microvessels. Coronary microvessels from a separate group of non-LPS-treated dogs were used as the control in this series of experiments. Microvessels from these normal dogs (n = 11) were prepared using the methods described above.

To measure stimulated nitrite release, tissue samples from normal and LPS-treated dogs were incubated in PBS alone and in the presence of increasing concentrations of bradykinin (10^-2-10^-7 M), Nitro-L-arginine methyl ester (L-NAME), aminoguanidine (AG), and 5-methylisothiouracil (SMT) were used to inhibit nitrite release from the coronary microvessels. Tissues from LPS-treated dogs were incubated in PBS alone and in the presence of increasing concentrations of the inhibitors (10^-5-10^-2 M). Nitrite release was measured using the Griess reaction. A standard curve was prepared using known concentrations of sodium nitrite. Absorbance was measured at 540 nm with a spectrophotometer (model 930, Uvikon). Absorbance of standards was plotted, and nitrite production (pmol/mg) was calculated from the standard curve.

Chemicals

L-NAME, AG, SMT, bradykinin, and LPS were purchased from Sigma Chemical.

Statistical Analysis

Values are means ± SE. Maximal dP/dt was used as an index of cardiac contractile state and is referred to as dP/dt. Comparison between groups of samples was performed with a one-way repeated-measures analysis of variance using Dunnett’s method. P < 0.05 was considered statistically different. Figures were produced with Slide Write Plus 3.0 for Windows on a personal computer (AST 486 DX).

RESULTS

Although the data were sampled 19 times during the experiment, we focus on data at control and 30, 360, and 900 min after injection of LPS.

Effects of LPS on Hemodynamics

Table 1 summarizes the hemodynamic data for LPS-treated and control dogs. In the LPS-treated group, LV systolic pressure, LV dP/dt, mean arterial pressure, and calculated stroke volume were significantly reduced from baseline at 30, 360, and 900 min after LPS injection. Heart rate was significantly elevated from control in the LPS-treated animals at 360 min (from 160 ± 9.7 to 195 ± 12 beats/min) and remained at this level for the next 8 h before returning to baseline by 900 min. In contrast, control dogs showed no significant changes from baseline for mean arterial pressure, cardiac output, stroke volume, and heart rate (Table 1). The only significant changes reported were small increases in LV systolic pressure at 360 and 900 min and LV dP/dt at 900 min. Total peripheral resistance remained essentially constant in both groups of animals, with a transient increase reported in the LPS-treated group at 240 min (to 41.0 ± 5.1 from 28.0 ± 1.6 mmHg·l^-1·min^-1). Figure 1 illustrates the dramatic and maintained reductions in mean arterial pressure and LV dP/dt in the LPS-treated animals. In marked contrast, mean arterial pressure and LV dP/dt remained relatively stable over the full time course in the control group. In fact, increases in mean arterial pressure and LV dP/dt were reported in these animals.
Figure 2 shows the changes in cardiac output and stroke volume after injection of endotoxin. Stroke volume remained significantly reduced from control throughout the experiment, whereas cardiac output, although seemingly depressed, was not different from control from 540 to 900 min. There were no significant changes from baseline detected for either of these parameters in control animals.

Increases from baseline in mean pulmonary arterial pressure (to $15.0 \pm 1.5$ from $9.7 \pm 0.47$ mmHg) and pulmonary vascular resistance (to $5.0 \pm 1.0$ from $2.7 \pm 0.32$ mmHg·l$^{-1}$·min$^{-1}$) were detected at 900 min in endotoxin-treated animals.

**Effects of LPS on Blood Gases, Hemoglobin, and Lactate**

Table 2 summarizes the values for blood gases, hemoglobin, and lactate concentrations in LPS-treated and control dogs. Arterial PO$_2$ was significantly reduced from control at 30, 360, and 900 min after LPS adminis-

---

### Table 1. Hemodynamics of anesthetized dogs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>30 min</th>
<th>360 min</th>
<th>900 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV systolic pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>130 ± 10</td>
<td>130 ± 11</td>
<td>139 ± 7*</td>
<td>142 ± 7*</td>
</tr>
<tr>
<td>+LPS</td>
<td>134 ± 10</td>
<td>94 ± 10*</td>
<td>104 ± 10*</td>
<td>107 ± 5*</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>2.365 ± 410</td>
<td>2.413 ± 400</td>
<td>2.718 ± 513</td>
<td>2.849 ± 507*</td>
</tr>
<tr>
<td>+LPS</td>
<td>2.459 ± 359</td>
<td>1.582 ± 252*</td>
<td>1.666 ± 274*</td>
<td>1.496 ± 115*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>111 ± 7</td>
<td>109 ± 10</td>
<td>122 ± 4</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>+LPS</td>
<td>109 ± 5</td>
<td>73 ± 7*</td>
<td>87 ± 6*</td>
<td>91 ± 4*</td>
</tr>
<tr>
<td>Cardiac output, l/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>3.56 ± 0.30</td>
<td>3.58 ± 0.25</td>
<td>3.55 ± 0.31</td>
<td>3.84 ± 0.40</td>
</tr>
<tr>
<td>+LPS</td>
<td>3.75 ± 0.36</td>
<td>2.43 ± 0.22*</td>
<td>2.85 ± 0.36*</td>
<td>3.29 ± 0.43</td>
</tr>
<tr>
<td>Stroke volume, ml/beat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>27 ± 2.0</td>
<td>25 ± 1.7</td>
<td>22 ± 2.4</td>
<td>25 ± 3.1</td>
</tr>
<tr>
<td>+LPS</td>
<td>24 ± 1.8</td>
<td>16 ± 1.7*</td>
<td>15 ± 1.2*</td>
<td>20 ± 2.7*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>135 ± 8</td>
<td>142 ± 5</td>
<td>159 ± 8</td>
<td>155 ± 10</td>
</tr>
<tr>
<td>+LPS</td>
<td>161 ± 10</td>
<td>156 ± 8</td>
<td>196 ± 12*</td>
<td>170 ± 5</td>
</tr>
<tr>
<td>Total peripheral resistance, mmHg·l$^{-1}$·min$^{-1}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LPS</td>
<td>32 ± 2.0</td>
<td>32 ± 2.1</td>
<td>35 ± 2.2</td>
<td>32 ± 2.8</td>
</tr>
<tr>
<td>+LPS</td>
<td>28 ± 1.6</td>
<td>30 ± 2.1</td>
<td>33 ± 4.4</td>
<td>32 ± 8.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. +LPS and -LPS, lipopolysaccharide-treated and -untreated animals; LV, left ventricular; dP/dt, 1st derivative of pressure. *P < 0.05 compared with respective control.
small but significant decreases in venous PO2 and venous blood pH remained essentially constant throughout the experiment. Arterial PCO2 was significantly reduced from baseline early, at 360 min, and at 900 min. Venous PCO2 did not change early but was significantly reduced at 360 and 900 min. Despite the fall in venous PCO2, arterial PCO2 difference at 30 and 360 min. Arterial and venous blood pH remained essentially constant throughout the experiment. In the control animals, excluding small but significant decreases in venous PO2 and arterial PCO2 reported at 900 min, blood gases, pH, and hematocrit levels remained essentially constant.

Arterial hematocrit was significantly elevated from control at all time points after LPS administration. This hemococoncentration was also reflected by significant increases in arterial and venous total hemoglobin. These effects were not seen in the control animals.

Blood lactate concentrations were statistically different from control at 30 min after LPS, showing increases of 150 ± 25% In contrast, blood lactate concentrations did not change early in the control animals, with significant decreases reported late in the experiment.

Effects of LPS on Plasma NOx

The effects of endotoxin on arterial plasma NOx concentration are illustrated in Fig. 3. Plasma NOx concentration was not different from control until 240 min after LPS injection. From 360 to 900 min, there was a 108 ± 34% increase in plasma NOx, from 9.8 ± 1.7 to 16 ± 0.9 µM. Overall, plasma NOx increased approximately sixfold from control (from 3.7 ± 0.9 to 16 ± 0.9 µM). Administration of normal saline to dogs had no effect on arterial plasma NOx concentration.

Effects of LPS on IL-6 and TNF-α Production

Figure 4 shows the changes in plasma concentrations of TNF-α and IL-6 after endotoxin administration. TNF-α levels were significantly elevated from control between 30 and 60 min, with peak concentrations at 45 min (from 365 ± 153 to 1,460 ± 436 pg/ml). IL-6 plasma concentrations were significantly increased from baseline at 120, 180, and 240 min after endotoxin. From their respective peak concentrations in plasma, TNF-α and IL-6 levels rapidly returned to baseline values and were not different from control at 360 and 900 min. Plasma levels of TNF-α and IL-6 remained at baseline values in animals treated with normal saline.

Effects of LPS on Nitrite Release from Coronary Microvessels

The effects of LPS on baseline and stimulated nitrite release from the microvessels of normal dogs are shown in Fig. 5. Baseline, or unstimulated, nitrite release was approximately fourfold higher in microvessels from LPS-treated than in microvessels from normal dogs (292 ± 5 vs. 69 ± 6 pmol/mg). There was a dose-dependent increase in nitrite release from the microvessels of normal dogs (n = 11) with bradykinin, showing a 149 ± 20% increase from control at 10^{-5} M. In contrast, bradykinin...
at any dose (10^{-7}–10^{-5} M) did not cause an increase in nitrite release from the microvessels of LPS-treated dogs (n = 6).

Figure 6 illustrates the effects of three known NOS inhibitors on baseline nitrite release from the coronary microvessels of LPS-treated dogs. L-NAME was used as a nonselective NOS inhibitor; AG and SMT were used as more selective inhibitors of iNOS. Dose-dependent reductions in nitrite release were demonstrated by L-NAME, AG, and SMT, showing reductions of 59.6\% , 56.3\% , and 60.2\% , respectively, at the highest dose (10^{-2} M). Percent reductions in nitrite release by the three NOS inhibitors were not statistically different from each other.

DISCUSSION

The most striking finding of our study was that the early hypotension and cardiac dysfunction in dogs were not associated with an increase in plasma NOx production, whereas the late cardiovascular effects of endotoxin were associated with a sixfold increase in plasma NOx concentration. Therefore, the early effects of endotoxin could not be explained by an increase in NO production, but NO may be partly responsible for the late effects of endotoxin. Alternatively, the early effects of endotoxin could be explained by the release of cardiodepressive cytokines, particularly TNF-α.

In the dog a moderate dose of endotoxin (1 mg/kg) resulted in immediate and sustained reductions in mean arterial pressure, cardiac output, stroke volume, and LV dP/dt. These effects were not demonstrated in control animals, thus ruling out the possibility that the hemodynamic alterations reported in the LPS-treated group were due to anesthesia and/or the long time course used in this study. Plasma NOx was significantly increased from control at 4 h after LPS injection, with concentrations increasing steadily thereafter, reaching levels sixfold higher than control at 15 h. In vitro production of NO2 by coronary microvessels was also elevated in these dogs, supporting our in vivo findings. Additionally, the Ca^{2+}-dependent agonist bradykinin caused a dose-dependent increase in NO2 release from the microvessels of normal dogs but had no effect on nitrite release in the microvessels from LPS-treated animals. Cytokine production occurred shortly after endotoxin injection, with peak TNF-α and IL-6 levels at 45 and 120 min, respectively. In addition, a pronounced increase in lactate production was seen during the first 300 min after LPS, whereas arterial PO2 and PCO2 were reduced throughout the experiment. Control animals did not show significant alterations in blood gases, lactate, or cytokine production, suggesting that these effects were due to LPS and not to anesthesia.

In experimental sepsis the cardiovascular profiles characteristic of each model tend to be quite variable. This variability can be partly accounted for by the numerous serotypes of endotoxin used, as well as differences in dosage and method of administration (6,
17). Furthermore, species-specific responses to endotoxin have been demonstrated (5, 15). In our study an immediate hypotension occurred after LPS injection. Cardiac output and stroke volume were acutely depressed, whereas total peripheral resistance did not change. Wei et al. (39) and others (16) suggested that the immediate hypotensive phase is due to an increase in hepatic vascular resistance, resulting in an acute fall in venous return. Although the underlying mechanism is unclear, it has been postulated that the immediate venoconstriction is mediated through histamine or via a direct effect of endotoxin (14). In addition, a substantial reduction in LV dP/dt occurred immediately after endotoxin injection. However, the apparent acute cardiac dysfunction (within 5 min) after LPS could be a function of dramatic alterations in cardiac preload and afterload (21, 27). From 2 to 4 h after endotoxin injection, mean arterial pressure recovered from −40 to −15% from control. Interestingly, during this increase in afterload, LV dP/dt remained depressed. This finding contrasts with those of Hinshaw et al. (13) and suggests that an inherent cardiac dysfunction persists during this phase of endotoxemia and that the cardiac dysfunction cannot be solely attributed to alterations in the peripheral circulation and loading conditions. The recovery in blood pressure was associated with a 44% increase in total peripheral resistance and no change in cardiac output. The mechanism responsible for the transient increase in total peripheral resistance is unclear. Although cardiac output did appear to remain depressed late after endotoxin, values were determined to be not statistically different from control from 540 through 900 min. This is most likely due to the relatively large variability inherent in cardiac output measurement by thermodilution and explains how mean arterial pressure was significantly depressed at this time in the face of normal total peripheral resistance. Stroke volume and LV dP/dt remained substantially reduced until the end of the experiment. The recovery in cardiac output was associated with significant increases in heart rate, which compensated for, and may have partially contributed to, the reduced stroke volume. However, because of the positive inotropic effect of high heart rate on LV dP/dt, i.e., the Treppe effect (27), it is likely that the cardiac depression we observed late in our dogs is more pronounced than our data would suggest.

The large increase in plasma NOx reported in our dogs after LPS is consistent with previous studies in vivo (6) and in vitro (34). In our study an overall sixfold increase in plasma nitrite/nitrate was reported at 15 h after LPS injection. Additionally, a statistically significant increase in plasma NOx was not detected until 4 h after endotoxin administration. These findings were used to document the induction and time course for expression of iNOS in our studies. The fact that plasma NOx levels did not change in the control animals strongly suggests that the increase in NO production in the LPS-treated group was indeed due to LPS.

In previous studies, NOS inhibitors have reversed LPS-induced hypotension within 140 min of administration (17, 40), thus establishing a role for NO early after endotoxin administration. The results of those studies might be due to increased activity of the constitutively expressed endothelial isoform of NOS (28) or increased circulating levels of histamine (14) or kinins (25). Alternatively, because the inhibitors used in these studies were nonselective for isoforms of NOS, the reversal of the hypotension reported in these studies could simply be the result of the blockade of the constitutively expressed endothelial isoform that is maintaining basal vascular tone. Nevertheless, because of the short time course (180 min) used in these studies, determination of a role for NO from the inducible isoform of the enzyme is unclear. The hypotension that occurs early in our dogs is not associated with increases in plasma NOx or a fall in vascular resistance. Therefore, NO does not appear to be a major determinant of vascular resistance or blood pressure early after LPS treatment. In contrast, the persistent hypotension that occurred late (after 240 min) was associated with a dramatic increase in plasma NOx. Interestingly, the increase in plasma NOx during this phase did not worsen the preexisting hypotension or cause a precipitous fall in total peripheral resistance. This suggests that the amount of NO produced late in our model of endotoxemia may not have been sufficient to override the vasoconstrictive effects of other shock-related substances such as catecholamines, serotonin, various prostaglandins, and angiotensin II (12, 29). Alternatively, NO production may not have been sufficient to directly dilate peripheral blood vessels. This most likely relates to the moderate dose of endotoxin used in our study. This was done by design so that we could study the progression of endotoxemia over an extended period of time and not precipitate a premature cardiovascular collapse and death (before 10 h), which occur in dogs after high doses of LPS (6). Alternatively, because NOx are cleared primarily by the kidney and have a half-life in plasma of ~4 h (17), the increase in plasma NOx may be explained by a reduction in NOx clearance and not to an increase in production. However, the fourfold increase in nitrite release from the coronary microvessels in vitro suggests that the increase in plasma NOx in our dogs was due to an increase in NO production and not to a reduced clearance.

In addition to the hemodynamic changes, various metabolic derangements have been reported. In a porcine model of endotoxemia (9), injection of endotoxin caused a reduction in arterial Po2, a finding we observed in our studies as well. This may be the result of an increase in the diffusion distance for O2, inasmuch as studies have shown an increase in extravascular lung water in pigs treated with endotoxin (9). Additionally, we observed a profound increase in blood lactate concentrations during the first 300 min after LPS. This most likely resulted from the marked decrease in O2 delivery (depressed cardiac output and arterial Po2) seen during this time. Although a significant reduction in arterial pH was not detected, a concurrent decrease in arterial PCO2 suggests that a peripheral chemorecep-
tor-mediated respiratory compensation (hyperventilation) did occur. Furthermore, a significant reduction in venous Pco₂ was observed from 240 min after LPS until the end of the experiment. This decreased CO₂ production, indicative of a reduction in tissue metabolism, occurred over the same time course as the increase in plasma NOx. Recent evidence from our laboratory (33) and others (18) has demonstrated that NO has an inhibitory effect on tissue O₂ consumption. Thus it is possible that the reduction in CO₂ production seen during the late phase is partly mediated by NO.

TNF-α and IL-6 are believed to be important mediators of host defense against infection. However, they, along with IL-1, IL-2, and interferon-γ, have also been implicated in the pathogenesis of septic shock (11, 22). As shown by Natanson et al. (26), many of the effects of endotoxin can be mimicked by administration of these cytokines, providing a partial explanation for the continued progression of sepsis long after the causative bacteria have been cleared from the circulation. After LPS, TNF-α and IL-6 plasma concentrations reached peak levels at 45 and 150 min, respectively, with levels returning to control values shortly thereafter. This time course has been demonstrated previously (37) and supports the finding that TNF-α plays a pivotal role in the release of IL-6 and other cytokines (8). Thus it is logical that a lack of TNF-α production in control dogs was coupled with a lack of IL-6 production. Moreover, this cascade of cytokines is thought to be essential for the induction of iNOS. Liu et al. (19) showed that, in rats, pretreatment with dexamethasone, an inhibitor of TNF-α production (3), prevented the induction of iNOS after LPS injection. Thus it has been proposed that the protective effects of glucocorticoid pretreatment in experimental sepsis and endotoxemia are due to a suppression of cytokine cascades and subsequent prevention of iNOS induction. Additionally, a potential role for TNF-α as a negative inotropic agent has been shown on papillary muscle in vitro (7) and in an in vivo study that showed a marked reduction in the end-systolic pressure-volume relationship after TNF-α infusion in dogs (38). Therefore, it is possible that the high plasma levels of TNF-α detected are partially responsible for the marked cardiac dysfunction seen early in our model of endotoxemia.

In addition to the increase in plasma NOx in our study, baseline nitrite release from the coronary microvessels of the LPS-treated dogs (in vitro) was fourfold higher than from the coronary microvessels of normal dogs and could not be stimulated by bradykinin (10⁻⁷–10⁻³ M). Nitrite release was significantly attenuated by the nonspecific NOS inhibitor l-NAME, as well as by the putative selective iNOS inhibitors AG and SMT. Thus a role for the l-arginine pathway in the production of nitrite was established; because the level of nitrite inhibition from each inhibitor was not different, however, the isoform of NOS responsible for NO production could not be determined. However, the markedly elevated nitrite release, coupled with the inability of the Ca²⁺ agonist bradykinin to stimulate it further, strongly suggests that an induction of the Ca²⁺-independent iNOS occurred in the coronary microvessels from our dogs. Although the presence of iNOS in endothelial cells, vascular smooth muscle, and cardiac myocytes has been demonstrated (24), our study is the first to show direct evidence of iNOS activity in the coronary microvasculature. Because of the short diffusion distance between the microvasculature and cardiac myocyte, one would predict that a significant proportion of the excess NO produced in the microvessels during sepsis could reach the cardiac myocyte. As shown by Brady et al. (4) and others (36a), NO via an unknown mechanism could be partly responsible for mediating the cardiac dysfunction associated with sepsis. Interestingly, in a recent study from our laboratory, it was shown that blockade of endogenous NO production in vivo led to an increase in myocardial O₂ consumption, suggesting that NO has an inhibitory action on cellular metabolism in the working heart (2). Therefore, the possibility exists that the excessive levels of NO produced during sepsis and endotoxemia may lead to pathological inhibition of myocardial O₂ consumption, leading to reductions in ATP production and subsequent myocardial dysfunction.

In summary, intravenous injection of a moderate dose of E. coli endotoxin resulted in marked hemodynamic and metabolic derangements characterized by hypotension, cardiac depression, and increased blood lactate levels. These changes were accompanied by a sharp and immediate rise in plasma TNF-α and IL-6, with levels of both cytokines returning to control values within 120 min. In addition, an overall sixfold increase in plasma NOx was reported, with levels reaching statistical significance 4 h after endotoxin. None of these changes could be mimicked in anesthetized animals treated with normal saline. Moreover, in vitro production of NO₂ in sieved coronary microvessels was fourfold higher that in normal dogs and could not be stimulated by bradykinin. However, because NO production did not occur until 4 h after LPS injection, it is unlikely that NO contributed to the hypotension and cardiac dysfunction early in our study. These effects could be explained by the release of cardiodepressive cytokines, particularly TNF-α. In contrast, the hemodynamic effects late after injection of LPS may be the result of an overproduction of NO, since there was a sixfold increase in plasma NOx levels at this time and a marked production of NO in isolated coronary microvessels in vitro.

This study was supported by National Heart, Lung, and Blood Institute Grants PO-1HL-43023, HL-53053, and HL-50442 (T. H. Hintze), New York State Affiliate of the American Heart Association Grant-in-Aid AI-16262 (N. R. Ferreri), and a contract from the National Foundation for Cancer Research (P. B. Sehgal). X. Zhang was supported by a Fellowship from the New York State Affiliate of the American Heart Association.

This work was presented by P. R. Forfia at Experimental Biology '96 and was used in partial fulfillment for a master of science degree (1997).

Address for reprint requests: T. H. Hintze, Dept. of Physiology, New York Medical College, Valhalla, NY 10595.

Received 18 July 1997; accepted in final form 5 September 1997.

REFERENCES
