Selective Inhibition of Endothelium-Dependent Vasodilator Capacity by *Escherichia coli* Endotoxemia

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Increased release of endothelium-derived relaxing factor/nitric oxide has been proposed as the final common pathway for vasodilator responses to gram-negative lipopolysaccharide (endotoxin). To test this hypothesis, we examined endothelium-dependent and endothelium-independent vasodilator agents in vascular smooth muscle isolated from guinea pigs 16 hours after injection of saline (control group) or induction of *Escherichia coli* endotoxemia; aortic rings (≈1 mm in diameter) were studied with standard isometric tension techniques. Endotoxemia resulted in a significant loss of vasodilator responses to the endothelium-dependent receptor agonists acetylcholine (10^{-9}–10^{-5} M) and ADP (10^{-6}–10^{-5} M). In contrast, endotoxemia did not affect vasodilator responses to either the endothelium-dependent receptor agonist substance P (10^{-11}–10^{-7} M), the endothelium-dependent and receptor-independent agonist A23187 (10^{-5}–10^{-4} M), or the endothelium-independent agonist nitroprusside (10^{-6}–10^{-4} M). The nitric oxide synthase inhibitor N^g^{0}-nitro-L-arginine methyl ester (L-NAME) inhibited the vasodilator response to acetylcholine more in vessels from lipopolysaccharide-injected than control guinea pigs. Unexpectedly, L-NAME converted the endothelium-dependent vasodilator action of ADP to an endothelium-dependent vasoconstrictor response that was blocked individually by the cyclooxygenase inhibitor indomethacin, the thromboxane synthase inhibitor dazoxiben, and the thromboxane A2 receptor antagonist SQ29548. We conclude that in vivo endotoxia inhibits the constitutive isoform of nitric oxide synthase in endothelial cells by selectively disrupting receptor-coupled activation mechanisms shared by acetylcholine and ADP. Furthermore, since L-NAME unmask a thromboxane A2-mediated vasoconstrictor action of the endogenous purinoreceptor agonist ADP, drugs that inhibit nitric oxide synthase could exacerbate sepsis-induced vasoconstriction and ischemia by synergizing with lipopolysaccharide-induced inhibition of endothelial nitric oxide synthase. (*Circulation Research* 1993;72:539–551)

**KEY WORDS** • lipopolysaccharide • nitric oxide synthase • vascular smooth muscle

Systemic hypotension associated with the circulatory dysfunction produced by gram-negative lipopolysaccharide (LPS [endotoxin]) is an important determinant of the morbidity and mortality incurred during the hypodynamic phase of gram-negative sepsis. Endotoxin-induced changes in blood vessel function are expressed as a heterogeneous pattern of vasodilatation and vasoconstriction in different organs, culminating in a fall in total peripheral vascular resistance concomitant with regional maldistribution of blood flow and tissue ischemia. The mechanisms responsible for LPS-induced disorganization of vascular function remain obscure; however, recent studies have introduced a new hypothesis about this issue. Evidence in the last two years has indicated that LPS modulates the activity of a key endogenous regulator of vascular smooth muscle tone—endothelium-derived relaxing factor (EDRF).

EDRF has been identified as either nitric oxide (NO), a closely related nitrosothiol, or both. NO migrates from the endothelium and activates cytosolic guanylyl cyclase of nearby vascular smooth muscle (VSM); this activation accelerates conversion of GTP to cGMP, with cGMP leading in turn to VSM relaxation and its accompanying vasodilatation. It has now been proposed that LPS and its endogenous biological response modifiers promote the release of NO-like factors, with the latter bearing responsibility for expressing the hypertensive actions of the former. This hypothesis is based on different lines of evidence, including observations that pharmacological inhibition of the NO synthase (NOS) enzyme 1 improved blood pressure in patients with septicemic shock and 2) antagonized the hypertensive response to LPS and human recombinant tumor necrosis factor-α in laboratory animals.
The proposal that LPS and its mediators promote the release of EDRF/NO in intact subjects seems inconsistent with some other data suggesting just the opposite in isolated tissues; i.e., LPS and tumor necrosis factor-α seem to interfere with the release of EDRF/NO in different vascular and endothelial preparations.\textsuperscript{16–20} Disparate interpretations about interactions between endotoxin and EDRF/NO based on in vivo versus in vitro experiments partially reflect inherent complexities associated with the study of intact subjects and their multifaceted circulatory responses to LPS and its many endogenous vasoactive mediators. Another confounding influence is that there are different isoforms of NOS, and LPS can modulate NO synthesis in tissues other than blood vessels.\textsuperscript{21} Resolution of this complex problem is important because drugs that inhibit NOS have been proposed as novel therapeutic strategies for treating septic shock,\textsuperscript{10,14,15} and these drugs are now entering clinical trials in patients critically ill with sepsis.\textsuperscript{14,22} In the present study, we focused on LPS–endothelium interrelations; blood vessels were isolated from guinea pigs subjected to a standardized model of Escherichia coli endotoxemia, and endothelium-dependent and endothelium-independent vasoactive agents were compared in an ex vivo environment free from the metabolic and circulatory complexities operative in the intact host.

Materials and Methods

Methods used for inducing endotoxemia in vivo in guinea pigs were reported previously,\textsuperscript{23–25} as were in vitro techniques for assessing intrinsic contraction–relaxation properties of isolated blood vessels\textsuperscript{16}; these procedures are summarized below.

Endotoxemia Model

Hartley-strain male albino guinea pigs, weighing between 250 and 400 g, were used throughout the study. Animal room temperature was maintained between 22° and 24°C; a 12-hour light–dark cycle was maintained by artificial illumination; and experiments were timed so that blood vessel isolation began between 8:30 and 9:00 AM. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missouri, Columbia.

Gram-negative endotoxemia was produced by intraperitoneal injection of 4 mg/kg purified E. coli endotoxin (lipopolysaccharide W, 0127:B8; Difco Laboratories, Detroit, Mich.). Hemodynamic instrumentation was not done in this study; however, we have shown previously that this procedure consistently results in a state of circulatory shock characterized by central nervous system depression, hypothermia, \textasciitilde20% mortality, early compensatory hyperpnea, minimal changes in arterial blood gases, and marked decreases in systolic and diastolic blood pressures by 16–18 hours after injection of endotoxin.\textsuperscript{23,24} In the present study, blood vessels were isolated 16–18 hours after the injection of either endotoxin or an equivalent volume of sterile isotonic saline solution (control vessels). A control and endotoxemic guinea pig (and respective vessel preparations) were usually examined concurrently to pair environmental influences and nonspecific variables as much as possible.

Vessel Collection and Preparation

Aortic rings. Guinea pigs were decapitated, and the abdominal aorta from the diaphragm to the iliac bifurcation was rapidly removed and placed in ice-cold aerated bicarbonate-buffered medium. The aorta was cleaned of fat and connective tissue and cut transversely into separate rings; care was taken to collect rings from sections of aorta that did not contain major branches, such as the mesenteric and renal arteries. Each abdominal aorta yielded three or four separate rings with axial lengths of 3–4 mm; care was taken to avoid damage to the intimal surface. In designated experiments, a small forceps was inserted and rotated gently in the vessel lumen to remove the endothelium.

The aortic rings were mounted on two stainless-steel wires (Rocky Mountain Orthodontics) passed through the vessel lumen. One wire was attached to a force transducer (model FTO3c, Grass Instrument Co., Quincy, Mass.), and the other was attached to a micrometer (Stoelting/Prior microdrive, Stoelting Co., Wood Dale, Ill.) mounted on the transducer. This system allowed the vascular rings to be stretched by known increments while isometric force was measured. The stainless-steel wires with the vessels attached were lowered into individual tissue baths (Harvard Apparatus, South Natick, Mass.).

After in vitro stabilization, each aortic vessel was systematically stretched to the optimum of its length–active tension relation. A known amount of stretch was placed on the vessel, and a contraction was induced with 30 mM KCl Krebs’ solution. This procedure was repeated at progressive levels of stretch until the increase in active tension was \textless5% of that at the previous stretch. Vessel stretch was recorded as the calculated internal circumference (L\textsubscript{max}) that yielded maximal active contractile response to 30 mM KCl. We have found 30 mM KCl to be near the E\textsubscript{C50} for KCl contraction and to yield L\textsubscript{max} values at the asymptote of the length–active tension relations generated by higher KCl concentrations. L was calculated using the formula

\[ L = 2f + 4d/2 + 2(\pi d/2) \]

or

\[ L = 2f + d(2 + \pi) \]

where d is wire diameter and f is distance between wires as measured by the micrometer. All subsequent pharmacological responsiveness studies were performed with vessels at L\textsubscript{max}.

Coronary rings. In some experiments the heart was removed and placed in ice-cold medium for excision of the left circumflex coronary artery. Coronary rings were prepared and mounted in an isometric microvessel myograph system (Living Systems Instrumentation, Burlington, Vt.) as previously described.\textsuperscript{26} This system allows direct determination of vessel wall force while internal circumference is controlled.\textsuperscript{26} Briefly, the microvessel was threaded carefully onto two tungsten wires (20-µm diameter). The wires were attached to a force transducer (Kulite Semiconductor Products Inc., Ridgefield, N.J.) and a digitalized micrometer, respectively. The vessels were superfused with oxygenated bicarbonate-buffered medium (7–8 ml/min) maintained at 37±0.5°C and allowed to equilibrate at least 45
minutes. During equilibration, the vessel internal diameter was set (using the micrometer) to yield a resting tension of ~0.1–0.2 mN/mm. The resting tension–internal circumference relation was then determined, with progressive vessel stretches and measurements of passive tension and internal vessel circumference L at each level of stretch. The vessels were set at an internal circumference of L_o, where L_o = 0.8 L_max and L_100 is the estimated internal circumference of the vessel under transmural pressure of 100 mm Hg. We have determined that L_o is at or near (within 10%) the optimal degree of passive stretch (i.e., L_max) yielding maximal active tension to KCl in guinea pig coronary arteries. A Zeiss 57 stereomicroscope was used during vessel preparation, with a Filar micrometer eyepiece for measurements of vessel diameter and axial length and distance between the tungsten wires. After equilibration, the coronary vessels were contracted three times with 100 mM KCl, rinsed, and reequilibrated before drug experiments.

**Solutions and Drugs**

The Krebs' bicarbonate solution was similar to the one used in previous studies with isolated muscletext25,27,28; it contained (mM) NaCl 131.5, KCl 5.0, NaH2PO4 1.2, MgCl2 1.2, CaCl2 2.5, NaHCO3 23.5, and glucose 11.2. This solution was aerated with 95% O2–5% CO2 (pH 7.4) and was maintained at 37±0.5°C. The KCl concentration was increased in designated solutions. All solutions contained 3 μM propranolol and 0.025 mM EDTA.

Unless indicated otherwise, the following drugs were purchased from Sigma Chemical Co., St. Louis, Mo.: prostaglandin F2α (PGF2α, Upjohn Co., Kalamazoo, Mich.), propranolol, EDTA, sodium nitroprusside, ADP, A23187, L-arginine, N⁶-monomethyl-L-arginine (L-NMMA, Calbiochem Corp., La Jolla, Calif.), N⁶-nitro-L-arginine methyl ester (L-NAME), norepinephrine (NE), indomethacin (INDO), substance P (Bachem, California, Torrance, Calif.), dazoxiben (Pfizer Laboratories, New York), and acetylcholine (Ach). SQ29548 was a gift from E.R. Squibb & Sons, Inc., Princeton, N.J. Concentrated stock solutions were prepared with deionized water, 30% ethanol (SQ29548), or 1% dimethyl sulfoxide (A23187); subsequent dilutions of SQ29548 and A23187 were made with deionized water. Endotoxin was purchased as lipopolysaccharide W, 0127:B8 (Difco) and was prepared for administration in sterile isotonic saline solution.

**Pharmacological Responsiveness**

For aortic rings, concentration–response curves to vasoactive agents were obtained by cumulative additions of small aliquots of concentrated stock solutions directly to the tissue bath; drug concentrations were increased when the response to the preceding concentration was maximal. Some rings were incubated continuously with enzyme substrate, enzyme inhibitor, or receptor antagonist beginning at least 30 minutes before determination of agonist activity. The total volume of drug solution added to the tissue bath was <3.0% of the bath volume, and drug vehicles in the concentrations used did not discernibly affect vascular function. For coronary rings, concentration–response curves were obtained by cumulative additions of small aliquots of concentrated drug solutions to the microvessel perfusate.

**Vascular Protocol and Data Analyses**

After in vitro setup, vascular tissues were allowed to equilibrate for 45–60 minutes before the study. Contractile responses of aortic rings were presented as absolute values in grams developed tension (force) and also normalized to percentage of maximal response. Active wall tension in coronary rings was expressed as the increase in wall force (in millinewtons) divided by twice the axial length of the vessel segment, yielding millinewtons per millimeter. The IC₅₀ values were calculated using nonlinear regression analysis of the concentration response data. The IC₅₀ values for vasodilator agents were derived similarly and indicated inhibition of vasoconstrictor responses to KCl, PGF₂α, or NE. IC₅₀ and EC₅₀ values determined in this manner were similar to those calculated by a log–linear interpolation method. Drug concentration–response curves were compared using repeated-measures analysis of variance (ANOVA) and the Bonferroni correction for multiple comparisons. A one-way ANOVA was used for comparing respective IC₅₀ and EC₅₀ values for vessels from control and LPS groups, and Student’s unpaired t test was used to compare intergroup differences in vessel dimensions. A value of p < 0.05 was considered significant, n values reflect the number of animals, and data are presented as mean±SEM.

**Results**

**Vascular Dimensions and L_max**

Table 1 presents average values for outer and luminal diameters, vessel wall thickness, axial length, L_max, and resting tension at L_max for LPS and control blood vessels. All dimensions of aortic and coronary rings isolated for evaluation were similar between control and respective LPS groups (p > 0.05).

Aortic vessels were progressively stretched by calculated percentages of the initial resting outer diameter, and contractile responses to 30 mM KCl were determined at each level of stretch until maximal active contractile responses to KCl were obtained. Vessel stretch was recorded as the absolute internal circumferential length at L_max. Length-dependent active tension responses were essentially identical in control and LPS aortas (p > 0.05). Likewise, L_max and resting tension at L_max in the LPS group were equivalent to corresponding values for control aorta (Table 1). Neither L-NAME (100 or 300 μM) nor L-arginine (1 or 3 mM) affected passive (resting) tension in either LPS (n = 8) or control (n = 9) aortas (data not shown).

Analogous to findings in aortic rings, values for circumferential length at L_max in coronary arteries were not significantly different in LPS and control groups. Resting tension at L_max was somewhat lower in coronaries isolated from the LPS animals (Table 1). All subsequent drug response experiments were conducted at optimal resting length for individual coronary and aortic vessels.

**Vasoconstrictor Agonists**

Aortic rings isolated from LPS-injected guinea pigs exhibited ~40% reduction in the maximal vasoconstrictor responses to NE; the maximal contractile tension and corresponding EC₅₀ values for NE are summarized in Table 2 for control and LPS aortas. In coronary arterial rings from LPS-injected guinea pigs, vasocon-
strictor responses to PGF$_{2\alpha}$ were reduced (Table 2). In contrast to the LPS-induced inhibition of responses to the receptor-dependent agonists NE and PGF$_{2\alpha}$, the maximal and EC$_{50}$ values for the vasoconstrictor response to K$^+$ depolarization were not inhibited in either aortas or coronary vessels of the LPS group (Table 2). In fact, the maximal K$^+$ response was somewhat greater in the LPS group of coronary arteries (Table 2).

**Vasodilator Agonists**

*General.* Because of the LPS-induced inhibition of vasoconstrictor responses to receptor-dependent agonists (Table 2), it was necessary to use either K$^+$ (not inhibited by LPS) or different agonist concentrations to achieve equivalent absolute levels of vasoconstrictor tone in control and LPS vessels. NE concentrations near 3 x 10$^{-3}$ and 10$^{-2}$ M were used in control and LPS vessels, respectively, to gain equivalent vasocostricter tone of 2–3 g in both groups of aortic rings. This level of NE precontraction was equivalent to the contractions produced by EC$_{50}$ of K$^+$ in both groups. Since NE produces little or no vasowstriction in guinea pig 100–300-μm coronary arteries (authors' unpublished observations), these vessels were precontracted with either PGF$_{2\alpha}$ (from 10$^{-6}$ to 3 x 10$^{-5}$ M) or K$^+$ (30 mM). After vasoconstrictor responses to NE, K$^+$, or PGF$_{2\alpha}$ achieved equivalent stabilized precontractions, vasodilator responses were determined.

Concentration-dependent vasodilation consistently was produced by ACh, ADP, substance P, and the Ca$^{2+}$ ionophore A23187; data from control and LPS aortas are contrasted in Figure 1. Denuding of endothelium inhibited vasodilator responses to ACh, ADP, substance P, and A23187 by 70–95% in both groups of vessels.

**Acetylcholine.** The vasodilator response to ACh was consistently reduced in aortas from the endothelium group (Figure 1A). Excess L-arginine (1 mM) failed to affect vasodilator responses to ACh alone in either control or LPS populations (Figure 2). However, L-NAME (100 or 300 μM) consistently inhibited vasodilator responses to ACh in LPS and control vessels, substantially reducing the maximal ACh response by 80–90% (Figure 2). Incubation with L-NMMA (100 μM) similarly inhibited the ACh response by 65–80%. The IC$_{50}$ for ACh was increased by L-NAME significantly more in LPS than in control vessels (Figure 3); however, incubation with INDO (5 μM) did not change vasodilator responses to ACh in either group. The complete ACh–vasodilator curves in the presence and absence of L-arginine and L-NAME are included in Figure 2, and the IC$_{50}$ values for ACh in the presence and absence of INDO and L-NAME are shown in Figure 3 for LPS and control aortas.

To determine if reduced vasodilator responses in LPS vessels were restricted to NE-induced precontractions, additional experiments were conducted with control

### Table 1. Dimensional Characteristics of Abdominal Aortic Rings and Left Circumflex Coronary Artery Rings From Control and Lipopolysaccharide-Injected Guinea Pigs

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Group</th>
<th>Outer diameter (μm)</th>
<th>Lumen diameter (μm)</th>
<th>Wall thickness (μm)</th>
<th>Axial length (μm)</th>
<th>L (μm)</th>
<th>RTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Control</td>
<td>1,075±17</td>
<td>746±18</td>
<td>127±3</td>
<td>3,904±33</td>
<td>4,676±49</td>
<td>2.15±0.08</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>1,130±17</td>
<td>774±18</td>
<td>133±3</td>
<td>3,957±24</td>
<td>4,516±67</td>
<td>2.09±0.09</td>
</tr>
<tr>
<td>LCCA</td>
<td>Control</td>
<td>254±14</td>
<td>166±11</td>
<td>34±2</td>
<td>1,610±40</td>
<td>915±91</td>
<td>0.53±0.06</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td>240±16</td>
<td>152±13</td>
<td>34±1</td>
<td>1,620±20</td>
<td>845±78</td>
<td>0.33±0.05*</td>
</tr>
</tbody>
</table>

L, calculated luminal circumferential length that yielded maximal active contractile response to 30 mM KCl (aorta) or calculated luminal circumferential length at L80, where L80=0.8 Loo and Loo is the estimated circumference of the vessel under transmural pressure of 100 mm Hg (left circumflex coronary artery [LCCA]); RTL, resting (passive) tension at L; LPS, lipopolysaccharide. RTL is expressed in grams for aorta and millinewtons per millimeter for LCCA. Values for LPS and control groups are mean±SEM of data from 10–12 LCCA rings and from 31 or 32 aortic rings from 10–12 guinea pigs. LPS data were not significantly different from corresponding control values (p>0.05), except RTL of LCCA.

*p<0.05 vs. RTL of control LCCA.

### Table 2. Vasoconstrictor Responses to Norepinephrine, Prostaglandin F$_{2\alpha}$, and K$^+$ in Abdominal Aortic Rings and Left Circumflex Coronary Artery Rings From Control and Lipopolysaccharide-Injected Guinea Pigs

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Maximal contractile response</th>
<th>EC$_{50}$ (–log M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LPS</td>
</tr>
<tr>
<td>Aorta</td>
<td>4.60±0.21</td>
<td>2.54±0.04*</td>
</tr>
<tr>
<td>NE</td>
<td>5.07±0.41</td>
<td>4.51±0.28</td>
</tr>
<tr>
<td>K$^+$</td>
<td>6.05±0.01</td>
<td>1.31±0.01</td>
</tr>
<tr>
<td>LCCA</td>
<td>0.99±0.16</td>
<td>0.33±0.14*</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>2.78±0.48</td>
<td>4.90±0.57*</td>
</tr>
</tbody>
</table>

LPS, lipopolysaccharide; NE, norepinephrine; LCCA, left circumflex coronary artery; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$. Each value is the mean±SEM of data from 6–10 (aorta) or 14–16 (LCCA) vascular rings. Maximum contractile response is expressed in grams for aorta and millinewtons per millimeter for LCCA. Responses to NE and PGF$_{2\alpha}$ were reduced in the LPS aorta and LCCA, respectively; however, K$^+$ responses were unaffected by LPS in aorta (p>0.05) and potentiated by LPS in LCCA.

*p<0.05 vs. corresponding control value.
and LPS aortas precontracted with K⁺ (40 mM). As with NE contractions, K⁺-contracted vessels from endotoxic subjects consistently showed inhibited vasorelaxant responses to ACh. Maximal vasodilation to ACh (10⁻⁵ M) in control (n=10) and LPS (n=10) rings was 61±7% and 34±11% (p<0.01), respectively.

The ACh-vasodilator response in coronary arteries precontracted by either K⁺ or PGF₂α was significantly inhibited in the LPS groups (p<0.05 and p<0.001, respectively), with the concentration–response curves shifted to the right and IC₅₀ values of the LPS vessels increased twofold to fourfold over respective control values (Figure 4).

ADP. The vasodilator response to ADP was significantly inhibited in aortas from the endotoxin group (Figure 1B). As with the ACh response (Figure 2), incubation with 1.0 mM l-arginine did not reverse the LPS-induced inhibition of the vasodilator response to ADP (Figure 5).

Preincubation with either dazoxiben or INDO potentiated the vasodilator response to ADP in control and LPS vessels (Figure 6), suggesting that ADP exerted a cyclooxygenase and thromboxane synthase–dependent vasoconstrictor action that was masked by simultaneous release of NO. Indeed, L-NAME not only inhibited the endothelium-dependent vasodilator response to ADP but also converted it to an endothelium-dependent vasoconstrictor response in both control and LPS vessels, as shown in Figure 7. The vasoconstrictor response to ADP expressed in L-NAME–treated vessels was inhibited in both control and LPS groups by 5 μM INDO (Figures 7B and 7F), by 10 μM dazoxiben (Figures 7C and 7G), and by 10 μM SQ29548 (Figures 7D and 7H).

Substance P. In contrast to LPS-induced inhibition of vasodilator responses to ACh and ADP (Figures 1A and 1B), LPS did not affect vasodilator effects of substance P (Figure 1C). To ensure that the endothelium-dependent action of substance P was due to NO and not a cyclooxygenase-derived prostanoid, additional experiments were done with L-NAME (300 μM) and INDO (5 μM). INDO failed to affect either the efficacy or potency of substance P. Maximal vasodilation produced by substance P (10⁻⁶ M) in the absence and presence of INDO was 98±1% and 96±2%, respectively, in control aortas (n=8, p>0.05) and 96±5% and 97±2%, respectively, in LPS aortas (n=6, p>0.05). The IC₅₀ for substance P in the absence and presence of INDO was 0.60±0.29 and 0.56±0.26 nM, respectively, in control aortas (p>0.05) and 0.49±0.17 and 0.62±0.21 nM, respectively, in LPS aortas (p>0.05).

In contrast to the lack of effect of INDO on actions of substance P, L-NAME markedly inhibited vasodilator responses to substance P in control and LPS aortas. Maximal vasodilation to substance P in the absence and presence of L-NAME was 98±1% and 37±8%, respec-
A23187. In contrast to LPS-induced inhibition of vasodilator responses to ACh and ADP (Figures 1A and 1B), LPS did not reduce vasodilator effects of the Ca²⁺ ionophore A23187 (Figure 1D). Similar to results with substance P, the vasodilator response to A23187 was unaffected by INDO but markedly inhibited by L-NAME. The maximal vasodilator response to 10⁻⁵ M A23187 in control (n=8) and LPS (n=10) vessels with and without 5 μM INDO was between 88±2% and 101±3% in all four groups (p>0.05). In contrast, 300 μM L-NAME inhibited the vasodilator response to 10⁻⁵ M A23187 by 80–95% in both control and LPS groups (p<0.05).

Nitroprusside. Nitroprusside produced concentration-dependent vasodilator actions that were virtually identical in control and LPS vessels (Figure 8). The vasodilator response to nitroprusside (10⁻⁵ M) was similar in endothelium-intact (101±6% relaxation, n=10) and endothelium-denuded (106±8% relaxation, n=11) vessels. Similarly, neither INDO nor L-NAME affected the vasodilator effects of nitroprusside in either LPS or control vessels (Figure 8).

Discussion
Present findings with an E. coli endotoxemia model indicated that bacterial LPS can produce a selective diminution of the endothelium-dependent vasodilator actions of ACh and ADP. Although the physiological relevance of ACh in regulating endothelial function is...
unclear, ADP is a key endogenous purinoceptor agonist believed to play a pivotal role in modulating communications between endothelium, VSM, and thrombogenic elements. Present observations have important implications not only for exclaiming potential adverse pharmacodynamic actions of NO inhibitors during gram-negative sepsis, but also for improving understanding of the complex interrelations between LPS and endothelium-dependent control of VSM contraction–relaxation properties.

LPS and Contractile Properties of VSM

Length–force relations in the isolated blood vessels indicated that absolute circumferential lengths at Lₘₐₓ (aorta) and Lₒₒ (coronary arteries) were statistically indistinguishable in respective LPS and control groups. Since contractile responsiveness and activation states of VSM demonstrate an important component of fiber-length dependency, it was important to ensure that resting VSM fiber length was equivalent in LPS and control populations before intergroup comparison of mechanical responses to contraction–relaxation stimuli.

This study focused on endothelium-dependent vasodilation, but as a subsidiary finding we also confirmed previous observations that LPS can inhibit VSM reactions to certain vasoconstrictor agents. There currently is no consensus for the reduced vasoconstrictor responsiveness of blood vessels after their exposure to LPS, but proposed mechanisms include activation of guanylyl cyclase with increased VSM synthesis of cGMP, enhanced sensitivity of VSM to cGMP-modified receptor-coupled transduction mechanisms controlling VSM contraction, and enhanced synthesis of endogenous vasodilator(s) that exerts nonselective physiological antagonism against vasoconstrictor stimuli. The reduced vasoconstrictor responses to NE and PGF₂α in the present LPS groups could not be explained simply by nonselective mechanisms, because vasosconstriction produced by K⁺ was not inhibited by LPS. Contractile response of VSM to K⁺ depolarization depends on an opening of voltage-gated Ca²⁺ channels spanning the sarcolemmal membrane, with the resulting influx of Ca²⁺ activating the contractile protein assembly. Since LPS did not affect K⁺ responses in aorta and actually augmented K⁺ responses in coronary arteries, it seems unlikely that LPS exerted a major deleterious effect either on voltage-dependent sarcolemmal Ca²⁺ channels or subsequent Ca²⁺-dependent activation of VSM contractile filaments. Rather, since NE and PGF₂α receptors share activation of phospholipase C with its resulting effects on inositol trisphosphate and intracellular Ca²⁺, it seems plausible that LPS in some way adversely affects this transduction cascade in guinea pig VSM. Such a mechanism was suggested previously on
the basis of biochemical analyses of polyphosphoinositides in rat aorta and hepatic tissues. In any case, to circumvent the methodological problem introduced by LPS-associated changes in vasoconstrictor reactivity, we used either K+ contractions (not reduced by LPS) or equieffective concentrations of PGF2α or NE to ensure that absolute vasoconstrictor tone was comparable in control and endotoxemic tissues before we made intergroup comparisons with vasorelaxant measures.

**LPS and Endothelium-Independent Relaxation of VSM**

The vasorelaxant response to nitroprusside was not influenced by removal of endothelium, by L-NAME, or by INDO. These findings confirm that nitroprusside is a directly acting vasodilator that does not require endothelium-derived factors, NOS, or cyclooxygenase products of arachidonic acid. Rather, nitroprusside undergoes tissue-catalyzed reduction yielding NO; the latter directly activates the cytosolic fraction of VSM guanylyl cyclase with subsequent intracellular increases in the endogenous vasodilator cGMP. Previous studies have indicated that although LPS can affect cGMP concentrations in VSM under some conditions, LPS nevertheless spares the ability of VSM to respond to endothelium-independent vasodilator agents that act through the guanylyl cyclase–cGMP pathway. In the present study, endotoxemia affected neither the potency (IC50) nor the efficacy (maximal response) of nitroprusside, resulting in concentration–dependent vasodilator curves in endotoxemic vessels indistinguishable from control curves. We interpret the nitroprusside data as evidence that this LPS model failed to affect either the ability of NO to activate guanylyl cyclase–cGMP or the sensitivity of the VSM contractile apparatus to cGMP-mediated actions.

**LPS and Endothelium-Dependent Relaxation of VSM**

In contrast to the direct VSM relaxant action of nitroprusside, the vasodilator mechanism for ACh requires a muscarinic receptor-dependent release of endothelium-derived NO synthesized from an N⁰-guanidino nitrogen of L-arginine through the action of NOS. ADP also activates endothelial NOS through the P2Y subclass of purinergic receptors. Endothelium-derived NO migrates rapidly to nearby VSM, where it conveys activation to the catalytic component of guanylyl cyclase, accelerating conversion of GTP to cGMP with subsequent activation of the cGMP-dependent vasorelaxant mechanism. Since LPS consistently inhibited vasodilator responses to ADP and ACh but did not alter vasorelaxant effects of the directly acting cGMP-dependent nitroprusside, present data are consistent with the interpretation that endotoxia inhibited the synthesis of endothelium-derived NO rather than affecting NO–guanylyl cyclase interactions or sub-
sequeent cGMP-dependent mechanisms in the VSM cells.

Previous studies have shown convincingly that exogenous L-arginine, but not its D enantiomer, restores endothelium-dependent vasodilator activity to blood vessels depleted of endogenous arginine.41 In the present study, a maximally effective concentration of L-arginine did not countermand the LPS-induced inhibition of vasodilator reactions to ACh and ADP, indicating that production of NO by these tissues was probably not limited by availability of precursor.

The mechanism responsible for LPS inhibition of the endothelium-dependent vasodilator actions of ACh and ADP could involve heterogeneous perturbation of muscarinic and purinergic receptor populations of endothelial cells, disruption of signal-transduction elements coupling endothelial receptors to NOS, or pathogenic denuding of intimal structure. Another possibility is that LPS increased the inducible isofrom of NOS,7 which then led to suppression of the receptor-coupled constitutive isofrom of NOS. However, if endotoxemia nonselectively suppressed NOS activity or provoked anatomic loss of endothelium, we would have expected that all vasodilators that depend on endothelium-derived NO would be nonspecifically inhibited in the LPS groups. This expectation was voided by the finding that endotoxemia affected neither A23187- nor substance P-induced vasodilation. Thus, both NOS activity and endothelial integrity were sufficient to respond normally to both a receptor-independent Ca2+-ionophore activator of NOS (A23187)42 as well as a receptor-dependent agonist (substance P) that does not use cholinergic or purinergic receptors.43

The vasoactivity of ADP is complex and includes the release of an endothelium-derived factor as well as direct contraction and/or relaxation of VSM.29-30 Present studies with guinea pig aorta provided a new facet of the pharmacodynamic profile of ADP; in this tissue, the dominant response to ADP was vasodilation. Either L-NAME or denuding of endothelium prevented the vasodilator response to ADP, confirming the dependency of this action on increased synthesis of endothelium-derived NO. Unexpectedly, we also found that L-NAME not only inhibited the vasodilator response to ADP; it converted it to a robust vasoconstrictor action that was blocked individually by a cyclooxygenase inhibitor (INDO), a thromboxane synthase inhibitor (dazoxiben), and a prostaglandin H2/thromboxane A2 receptor antagonist (SQ29548).44 These findings indicate that ADP can release both endothelium-derived NO and an endothelium-derived cyclooxygenase-dependent eicosanoid that is most likely thromboxane A2.
Synthesis of thromboxane A₂ is expected to be more a function of platelets than of endothelium. However, in recent studies with canine basilar arteries, ADPproduced endothelium-dependent vasconstriction that was blocked individually by a cyclooxygenase inhibitor (aspirin), a thromboxane A₂ synthase inhibitor (OKY-046), and a thromboxane A₂ receptor antagonist (ONO-3708). In the present study with guinea pig aorta, inhibition of endothelial NOS was necessary to ablate the dominant ADP-induced vasodilation before the normally subservient thromboxane A₂-dependent vasoconstrictor action of this purine nucleotide could be expressed. Thus, although prostacyclin is the cyclooxygenase-derived eicosanoid released typically from endothelial cells, present findings indicate that endothelium in some blood vessels can also yield thromboxane A₂ in response to purinergic agonists.

Integration With Other LPS Studies

The literature describing interactions between LPS and EDRF/NO is controversial, with some studies indicating enhanced release of NO-like agents and other studies indicating just the opposite. Part of this controversy is being resolved by the discovery of two distinct isoforms of NOS: a constitutive enzyme (cNOS) prototypically present in endothelial cells and certain neurons and an inducible enzyme (iNOS) prototypically present in macrophages and other inflammatory cells only after exposure to inducing agents. The cNOS is Ca²⁺/calmodulin dependent, and it is activated within seconds by agonists such as ACh; whereas, the iNOS is Ca²⁺ independent, and it requires several hours for expression. Clearly, LPS and certain cytokines exhibit positive cooperativity in inducing gene expression controlling de novo synthesis of iNOS in VSM, macrophages, and other cell types. Induction of NOS in VSM is believed to account for altered contractile responses observed in some studies with endothelium-denuded blood vessels exposed to LPS. However, it is important to note that in the same studies suggesting LPS induction of iNOS in endothelium-denuded vessels, the vasodilator response to ACh was concomitantly inhibited by LPS in endothelium-intact vessels, just as endotoxemia diminished ACh and ADP vasodilation in endothelium-intact aorta in the present study. Present findings are unlikely to be restricted only to aorta and coronary arteries of guinea pigs. Studies with
coronary,16 renal, mesenteric, and femoral arteries19 from dogs likewise showed that LPS can inhibit vasodilator reactions that depend on rapid synthesis of endothelium-derived NO. Experiments with coronary arterioles from endotoxemic pigs suggest that disruption of endothelial–VSM communication by LPS is not restricted to conduit arteries but may also extend into resistance arteriolar networks.48 Myers et al.20 reported recently that incubation of bovine aortic endothelial cells with LPS inhibited release of EDRF bioactivity and NO as measured by the NO-chemiluminescent technique. Thus, although LPS cooperates in expressing iNOS in the response of different cell types to inflammatory/immunomodulatory stimuli,21 it now seems clear that LPS can also inhibit the cNOS isoform in vascular endothelial cells. Present data indicate that this inhibition does not entail a nonspecific response to all activators of NO synthesis but may be unique to agonists that share transduction mechanisms in common with ACh and ADP. Since the Ca2+-dependent activation of cNOS by ADP and certain other receptor agonists may involve a G protein/phospholipase C/inositol trisphosphate mechanism,49,50 it is tempting to speculate that LPS disrupts a key step in this transduction pathway in endothelial cells. Further study will be necessary to define the cellular loci where LPS acts to modulate NO synthesis in endothelium.

**Implications and Conclusions**

Although extrapolation from a guinea pig model of experimental endotoxemia should be approached cautiously, present data may have some important implications. The current findings with coronary arteries are consistent with the previous caveat that loss of endothelium-dependent vasodilation in coronary arteries from endotoxemic dogs could signal increased propensity for coronary vasospasm and myocardial ischemia during sepsis.16 This prediction was fulfilled by Wright et al.13 who showed that an NOS inhibitor exacerbated signs of myocardial ischemia and markedly increased mortality in endotoxemic rabbits. Although pharmacological inhibition of NOS increased peripheral vascular resistance and blood pressure in two patients with septic shock, one patient experienced a dramatic fall in cardiac index from 4.5 to 2.7 l·min⁻¹·m⁻²; this patient died after 2 days with adult respiratory distress syndrome and disseminated intravascular coagulation.14

A fall in cardiac index would not be beneficial during sepsis, especially if it is accompanied by (or due to) a combination of coronary vasospasm and an increase in peripheral vascular resistance with its attending elevation of cardiac workload and myocardial oxygen demand. Present findings add an important new candidate to this paradigm: ADP. This purine nucleotide is re-
leased by degranulating platelets, and it can further provoke thrombogenesis by autocrine activation of the platelets.20–23 However, ADP can paradoxically antagonize thrombogenesis through its separate endothelium-dependent release of NO. NO is not only a vasodilator; it also is an antiaggregating compound owing to its ability to activate guanylyl cyclase--cGMP within the platelet.24–26 Inhibition of NOS would not only convert ADP-induced vasodilatation to thromboxane A2-dependent vasoconstriction, as we now report in a guinea pig aorta model; it might also exacerbate thrombogenesis through an inhibition of the antiplatelet action of NO concomitant with unmasking platelet-aggregating actions of the nascent thromboxane A2.27–31 Theorically, therefore, NOS inhibition during sepsis could provoke arterial vasospasm and tissue ischemia while concomitantly inducing platelet aggregation, leading perhaps to disseminated intravascular coagulation. These possibilities caution that NOS inhibitors should not be used clinically until further studies in animals better define the pharmacodynamic profile and potential toxic side effects of these complex agents.

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