Adrenocceptor Hyporeactivity Is Responsible for Escherichia coli Endotoxin–Induced Acute Vascular Dysfunction in Humans


Abstract—Impaired response to catecholamines contributes to the altered hemodynamics in sepsis, which has been attributed to excessive NO formation. We have studied the systemic hemodynamic and local forearm responses and inducible NO synthase (iNOS) expression during experimental endotoxemia in humans. Escherichia coli endotoxin (lipopolysaccharide [LPS]) was administered at doses of 1 or 2 ng/kg to healthy volunteers. In 10 subjects, the systemic pressor effect of phenylephrine was assessed before and after the administration of LPS. In 9 further subjects, forearm blood flow responses to intra-arterial noradrenaline, acetylcholine, glyceryl trinitrate, and N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) were studied at baseline and after LPS administration. Peripheral blood was collected and analyzed for iNOS mRNA and protein. Four hours after LPS, the response of systolic blood pressure (P < 0.0005) and heart rate (P < 0.05) to phenylephrine was significantly reduced. In the forearm, noradrenaline-induced vasoconstriction was also reduced by ~50% (P < 0.01), but L-NMMA responsiveness was unchanged. iNOS mRNA or protein was not increased. Marked vascular adrenocceptor hyporeactivity is detectable in the absence of increased NO activity or iNOS expression in endotoxemia, arguing against major involvement of vascular iNOS activity in the acute systemic vasodilation to LPS. (Arterioscler Thromb Vasc Biol. 2002;22:95-100.)

Key Words: inducible nitric oxide synthase ■ nitric oxide ■ sepsis ■ lipopolysaccharide ■ adrenocceptors

Sepsis is still associated with a high mortality rate and remains a major therapeutic challenge despite improved intensive care therapy.

One of the key clinical aspects of sepsis, which is responsible for the poor hemodynamic state of these patients, is inappropriate vasodilation and impaired response to catecholamines, resulting in hemodynamic instability and shock.

A variety of substances and mediators is released by activated blood cells and endothelial cells during sepsis. Many in vitro and in vivo animal experiments have indicated that excess formation of nitric NO in the vasculature may play the key role in the systemic vasodilation in sepsis and endotoxemia. These data suggest that most of the NO produced is formed after the induction of an inducible form of NO synthase (iNOS), which is not expressed in the vasculature under normal conditions. iNOS can be induced in a variety of cells, such as vascular endothelial cells, vascular smooth muscle cells, and white blood cells (WBCs) after in vitro stimulation with endotoxin and remains present for several days. Inhibition of NO synthesis in animals by NO synthase inhibitors reduces Escherichia coli endotoxin (lipopolysaccharide [LPS])–induced hypotension and vascular leakage and improves mortality. Experiments with iNOS knockout animals and animals treated with iNOS antisense oligonucleotides corroborated these pharmacological studies.

Data on iNOS expression in humans are limited. A recent study did not detect iNOS in cells or vessels of the systemic circulation in septic patients but only in tissue at the site of acute putrid inflammation. This has led to the speculation that vascular iNOS expression could be compartmentalized in patients. However, this observation is not compatible with the proposed mechanism of vascular hyporeactivity, which is thought to be caused by systemic excessive NO formation. Indeed, inhibition of NO synthesis with intravenous N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) mitigated hypotension in septic patients. However, a subsequent large-scale human trial failed to confirm a clinical benefit of NO synthase (NOS) inhibition apart from reduction of catecholamine requirement, as already shown in earlier experiments. Systemic administration of NOS inhibitors is also limited by severe side effects, such as pulmonary hypertension and reduced cardiac output.

Thus, although several lines of evidence argue for an involvement of iNOS in LPS-induced vasodilation in animal experimental models, the data from human studies are at variance, and the role of NO and iNOS in sepsis is not understood. The lack of understanding is also due to technical and ethical limitations of studying vascular reactivity to...
NO-modulating agents in septic patients. Aware of this background, we investigated LPS-induced vascular hyporeactivity in humans after the administration of a low dose of LPS to healthy volunteers. This experimental model has been shown to reproduce many of the pathological alterations observed in clinical sepsis, including hemodynamic changes and coagulation activation. In an effort to assess the contribution of adrenoceptor reactivity and NO formation in this setting, we have studied the systemic and local responsiveness to adrenoceptor agonists, NO synthase inhibition, and control drugs. Furthermore, iNOS mRNA and protein expression were studied in peripheral venous blood.

Methods

Study Population
Twenty-six healthy male subjects from whom informed consent was obtained before enrollment were included in the present study. The subjects were aged between 20 and 33 years. All of the subjects claimed not to have ingested any prescribed medications or “over-the-counter” drugs containing nonsteroidal anti-inflammatory drugs from 2 weeks before screening until the study follow-up investigation was complete. The present study was approved by the Ethics Committee of the University of Vienna and conforms with the principles outlined in the Declaration of Helsinki, including current revisions and the Good Clinical Practice (GCP) guidelines. All subjects were given a complete health examination (including physical examination, ECG, and laboratory screening) within 14 days before the study day. Subjects were studied after overnight fasting. All studies were started between 8:00 and 9:00 AM. Studies were conducted at an ambient temperature of 22°C in a quiet room with complete resuscitation facilities.

Systemic Hemodynamic Effects of LPS
Systemic effects of LPS (National Reference Endotoxin, E coli, United States Pharmacopeia Convention Inc) and phenylephrine (PE, Neo Synephrine, Winthrop Breon Laboratories) were assessed in a double-blind randomized parallel group trial. Ten volunteers (aged 24±4 [mean±SD] years) received either 1 or 2 ng/kg IV LPS. The pressor response to cumulatively increasing doses of PE (saline and 0.5, 1.0, 2.0, and 4.0 µg·kg−1·min−1 IV PE) was measured at baseline and 4 and 8 hours after LPS administration. Blood pressure and heart rate were measured noninvasively (by Hewlett Packard CMS patient monitor) at the end of each consecutive infusion step. Tympanic temperature (Thermoscan pro, Braun GmbH) was measured at frequent intervals. Venous blood for quantification of WBC count and for extraction of iNOS mRNA (polymerase chain reaction [PCR]) and protein (Western blot) was drawn before and at 4 and 8 hours after LPS administration.

Regional Hemodynamic Effects of LPS
The effects of LPS on forearm and skin blood flow were studied simultaneously in 9 volunteers (aged 26±5 years.). A fine needle (27-gauge needle, Sterican, B. Braun) was inserted into the brachial artery of the nondominant arm, and physiological saline was infused at 1 mL/min. Subjects were allowed to acclimate to the needle for at least 15 minutes before drug infusion. Forearm blood flow was measured as described previously. Briefly, strain gauges, placed on the forearms, were connected to plethysmographs (EC-6, D.E. Hokanson), and traces were analyzed by using the NIVP3 software (version 5.25, Hokanson). Bilateral plethysmography, expressing the ratio of responses in the intervention arm and in the control arm, was used; the baseline ratio was defined as 100%. Cuffs were placed around both upper arms and inflated to 45 mm Hg by a rapid cuff inflator (AG 101, Hokanson) during the measurements to occlude venous outflow. Wrist cuffs were inflated to suprasystolic pressures during each measurement to exclude circulation of the hands. To compensate for the expected higher flow rates after LPS administration, we have chosen a relatively long deflation period to ensure adequate venous emptying. Therefore, flow measurements were recorded for 9 seconds (longest analyzing period of the NIVP3 software) at 30-second intervals during drug infusion.

For assessment of skin blood flow, a laser probe was placed on the forearm (DPIT-V2-skin probe with DRT4 Monitor, Moor Instruments). Skin blood flow was recorded at frequent intervals.

Baseline measurements of forearm and skin blood flow were taken for 5 minutes. Responses to intra-arterial infusions of increasing doses of noradrenaline (NA [Arterenol, Hoechst AG], at a dose of 60, 120, 240 pmol/min), glyceryl trinitrate (GTN [Nitrusal, G. Pohl Boskamp GmbH]), at 4, 8, and 16 nmol/min, acetylcholine (ACH [Clinalfa], at 25, 50, and 100 nmol/min), and Nω-monomethyl-L-arginine (L-NMMA [Clinalfa], at 1, 2, and 4 µmol/min) were then obtained. The vasodilators (ACH and GTN) were infused cumulatively for 3 minutes per dose level, and the vasoconstrictors (NA and L-NMMA) were infused for 5 minutes.

After baseline readings, a bolus of LPS at a dose of 2 ng/kg was infused intravenously. Four hours after LPS administration, responses of forearm and skin blood flow to NA, ACh, GTN, and L-NMMA were repeated in an identical fashion. Venous blood was drawn at baseline and at 4 and 8 hours after LPS administration for assessment of iNOS expression and WBC count.

Concentration of endotoxin in the blood was determined as described previously in 6 subjects before and 4 and 8 hours after LPS administration.

To exclude a potential hemodilution effect of increased basal forearm blood flow, responses to the vasoconstrictor drugs in the present study were also measured at baseline and during increased forearm blood flow, as induced by local hand warming (40°C) with a water bath (N=3). Effects of the vasoconstrictors were calculated as changes versus baseline.

Western Blot Analysis
Platelets and peripheral blood mononuclear cells (PMNs) were extracted in lysis buffer, and protein concentrations were determined. Equal amounts of protein (10 μg) were subjected to electrophoresis and then incubated with mouse monoclonal antibodies against human iNOS (Transduction Laboratories) or rabbit polyclonal antibodies against actin (Sigma Chemical Co), followed by detection of reactive bands by chemoluminescence.

Positive Controls
A positive control was prepared from raw mouse macrophages. Mouse macrophages were simulated with 100 IU/mL interferon-γ and 10 μg/mL LPS for 12 hours, and cells were then extracted in lysis buffer and handled accordingly.

Reverse Transcriptase–PCR
RNA was isolated from blood samples, and control RNA was isolated from in vitro stimulated fibroblasts, which are known to produce iNOS mRNA. Negative controls were genomic RNA. The quality of the resulting cDNAs was determined by using the housekeeping gene GAPDH as an internal standard. Amplification, data acquisition, and data analysis were carried out by using an ABI Prism 7700 Sequence Detector (Perkin Elmer). During PCR, the calculated normalized reporter signals increase with amplicon copy numbers until the reaction approaches a plateau. The threshold cycle indicates the point of an increase in signal, which represents exponential growth of the PCR product.

Statistical Analysis
Systemic hemodynamics (pulse rate, systolic blood pressure, diastolic blood pressure, and mean arterial pressure) were expressed as absolute values or percent changes from baseline. Forearm and skin blood flow measurements were expressed as percent change from baseline. The effects of NA, ACh, GTN, and L-NMMA at baseline and after the administration of LPS were assessed by ANOVA for repeated measurements with use of the Statistica software package (Release 4.5, StatSoft Inc). Friedman ANOVA was used for reverse transcriptase–PCR results. A value of P<0.05 was considered significant. Values are expressed as mean±SEM, unless indicated otherwise.
Results

LPS and the drugs under study were well tolerated, and no side effects apart from the expected flulike symptoms were reported after endotoxin administration. These transient symptoms were only mild, and all subjects were discharged symptom-free from our unit after ~9 hours.

Systemic Responses to LPS

Temperature and WBC count were significantly elevated ($P<0.00001$) after the administration of endotoxin. Body temperature increased to $>37^\circ C \approx 2$ hours after 1 or 2 ng/kg LPS and remained elevated until 6 hours. Maximum body temperature was seen at 4 hours, with $37.6\pm0.2^\circ C$ and $38.0\pm0.3^\circ C$ after 1 and 2 ng/kg LPS, respectively. WBC count increased from $5.5\pm0.8$ and $5.4\pm0.4 \times10^9/L$ at baseline to $11.4\pm0.8$ and $10.9\pm0.6 \times10^9/L$ at 4 hours after 1 and 2 ng/kg LPS, respectively. No significant differences were observed between the dosage groups. LPS blood levels were $0.059\pm0.013$ IU/mL at baseline and $0.066\pm0.014$ and $0.052\pm0.012$ IU/mL at 4 and 8 hours after LPS administration, respectively ($n=6$, $P=NS$).

LPS altered baseline hemodynamics. This effect was less pronounced after administration at 1 ng/kg, but there was no statistical difference between groups. After a brief hypertensive reaction, systemic hypotension slowly developed, with a plateau maximum effect at ~4 hours after LPS administration. In a pooled analysis for all subjects, mean arterial pressure decreased significantly from $85\pm3$ mm Hg at baseline to $78\pm2$ and to $72\pm2$ mm Hg ($P<0.05$), and pulse rate was elevated from $70\pm2$ bpm to $87\pm2$ and to $76\pm3$ bpm ($P<0.00001$) after 4 and 8 hours, respectively.

PE caused a significant and dose-dependent pressor effect in all subjects under study and increased systolic and diastolic blood pressure and decreased pulse rate ($P<0.00001$, ANOVA; Figure 1). Four hours after LPS administration, the systemic response to PE was impaired: effects on pulse rate

Figure 1. Effects of increasing doses of PE on systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse rate before (open symbols) and 4 hours after 2 ng/kg LPS (solid symbols). Systemic responses are expressed as percent change from baseline values and are presented as mean±SEM ($n=5$). LPS significantly impaired the dose-dependent effects of PE. *$P<0.05$ between dose-response curves (ANOVA).

Regional Blood Flow Responses to LPS

Because a more pronounced attenuation of vasoconstriction to systemically administered PE was seen after 2 ng/kg LPS and because this dose was well tolerated, studies on regional blood flow were performed after the higher dose of LPS only. None of the drugs infused into the brachial artery caused a significant and dose-dependent vasodilation to ACh before LPS (Figure 2). After LPS, vasoconstriction to NA or L-NMMA still slightly impaired in subjects administered 2 ng/kg LPS ($P<0.05$ versus maximal effect before LPS dosing).

Figure 2. Dose-response curves of NA, L-NMMA, GTN, and ACh on forearm blood flow before (open symbols) and 4 hours after 2 ng/kg LPS (solid symbols). Percentages of baseline ratio (intervention/control arm) are presented as mean±SEM ($n=9$). LPS significantly decreased NA-induced vasoconstriction. *$P<0.05$ between dose-response curves (ANOVA).
ACh on skin blood flow was seen in the absence of iNOS expression in healthy subjects that was associated with an inflammatory reaction in patients with peritonitis or cellulitis, but the findings failed to show enhanced iNOS expression or NO formation in other vascular beds. This supports the compatibility of the present human sepsis model and demands a different explanation of the systemic vascular hyporesponsiveness in septic shock. Sensitivity to adrenoceptor agonists was reduced; this response is similar to that seen in septic patients. Therefore, we consider 2 ng/kg LPS adequate to mimic typical alterations of sepsis. However, we cannot exclude the possibility that higher LPS doses could have yielded different results regarding the magnitude of the vascular effects of iNOS expression.

ACh-induced vasodilation was reduced by LPS in the present experiments. Although it has been reported that muscarinic receptor-mediated signal transduction mechanisms are inhibited by LPS,22 no ready explanation is available for this effect in the forearm, which has already been observed in vivo after vaccination with Salmonella typhi.23 The different effect of ACh on skin blood flow compared with forearm blood flow might result from the nonhomogeneous distribution of ACh receptors on the forearm, inasmuch as the only vasodilatory cholinergic receptors in cutaneous vessels are muscarinic.24 However, there was a substantial variability of data, which has also been reported by other investigators.25 Therefore, the results obtained by using skin laser Doppler flux should be interpreted with caution.

LPS caused the expected hemodynamic and symptomatic effects in our cohort and reduced the systemic and local responses to PE and NA, respectively. L-NMMA was administered to assess the involvement of NO synthesis after LPS. Although not specific for the different NO synthases, L-NMMA has been shown to influence endotoxic shock in animal experiments. Unfortunately, selective iNOS inhibitors were not significantly altered.

iNOS Expression
No measurable amounts of iNOS protein were found at baseline or at 4 or 8 hours after 2 ng/kg LPS in isolated platelets or PMNs (Figure 4). Expression of iNOS mRNA tended to increase slightly after LPS, but the changes over baseline were in the range of those observed for GAPDH controls (Table). No significant increase in iNOS mRNA was detectable in blood incubated with an equivalent concentration of LPS over 4 hours in vitro.

Discussion
LPS produced a systemic hypotensive and inflammatory reaction in healthy subjects that was associated with an impaired vascular response to adrenoceptor agonists. This profound and transient reduction in adrenoceptor-mediated constriction was seen in the absence of iNOS expression in the reduction of forearm blood flow was measured. At baseline, forearm blood flow was decreased by NA by a mean of 39% and subsequently by 28%, 24%, and 20% after 2, 4, and 6 hours, respectively, whereas the decrease in forearm blood flow by L-NMMA was almost unaltered (39% at baseline and subsequently 39%, 37%, and 33%). On the basis of these data, assessments of forearm blood flow were scheduled between 4 and 6 hours after LPS.

The effects of the drugs under study on forearm blood flow were paralleled by changes in skin blood flow (Figure 3). There was a substantial variability of data; therefore, the signal-to-noise ratio of skin blood flow measurements was limited. Of note, the vasodilatation to ACh was less pronounced in skin blood flow compared with forearm blood flow. After LPS, the NA-induced reduction in skin blood flow was significantly impaired (P<0.05). Again, no change in L-NMMA–induced vasoconstriction was detectable. The vasodilator effect of ACh on skin blood flow was significantly reduced at doses of 60 and 120 pmol/min after LPS (P<0.05, ANOVA; Figure 3); responses to GTN were not significantly altered.

### Table

<table>
<thead>
<tr>
<th>Time</th>
<th>iNOS, Ct</th>
<th>GAPDH, Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>30.04±1.83</td>
<td>21.72±2.40</td>
</tr>
<tr>
<td>4 h after LPS</td>
<td>33.49±1.44*</td>
<td>22.46±0.83</td>
</tr>
<tr>
<td>6 h after LPS</td>
<td>32.71±2.99*</td>
<td>23.41±0.54</td>
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Values are mean±SEM (n=10).

Ct indicates threshold cycle.

*P<0.05 vs baseline (Friedman ANOVA).
are not available for human studies. However, the effects of L-NMMA on forearm and skin blood flow were unchanged by LPS, rendering the anticipated major role of increased NO synthase activity in this acute situation unlikely. This is in good agreement with a previous study, in which L-NMMA could not reverse local hyporesponsiveness to NA after local instillation of LPS into a dorsal hand vein.26 Contrary to animal experiments, in which much smaller concentrations of LPS are sufficient to induce vessel dysfunction and in which NOS inhibitors ameliorate the hemodynamic effects, the functional alterations in the healthy humans under study were not influenced by the administration of L-NMMA. These species differences might explain the disappointing results of large-scale multicenter trials with NOS inhibitors in septic shock patients. Furthermore, the expression of iNOS seems to be influenced by the type of bacteria.11 The lack of iNOS mRNA and protein expression in the present in vivo experiments is in good agreement with these previous observations. This was also confirmed by the lack of iNOS mRNA in blood incubated >4 hours with a low dose of LPS. It is possible that the LPS stimulus in the present experiments was too small to induce iNOS expression in blood, as evidenced by the lack of a significant increase in blood endotoxin concentrations. We wanted to analyze iNOS expression in vascular tissue from skin pouch biopsies, but these specimens did not contain enough vessels to draw reliable conclusions to confirm this negative result (data not shown).

Our findings suggest that mechanisms such as altered or impaired adrenoceptor signal transduction are responsible for systemic vasodilation and hyporesponsiveness in the endotoxin model. This concept should be critically tested before initiation of further clinical drug trials in sepsis. Possible explanations are that LPS reduces the myocardial L-type calcium current,27 influences Ca\(^{2+}\)-handling mechanisms in myofilaments directly,28 and activates vascular potassium channels.29 Internalization of vascular \(\alpha\)-adrenoceptors could also contribute to the hypodynamic states, as shown in rat hearts30 or platelets.31 Another explanation for our finding of decreased responses to catecholamines after LPS could be that catecholamines are inactivated by free radicals, including superoxide anions (\(O_2^-\)). The production of \(O_2^-\) is increased in sepsis as in other inflammatory conditions and could play a role in the pathogenesis of hemodynamic instability and organ dysfunction during septic shock.32 \(O_2^-\) autoxidizes catecholamines, including dopamine and NA.33 In a recent study in rats,34 the LPS-induced hyporeactivity to NA was improved by a mimetic of superoxide dismutase, which enhanced degradation of \(O_2^-\). This concept has not yet been confirmed in human experiments.

In conclusion, the present results demonstrate that vascular adrenoceptor hyporeactivity is detectable in the absence of increased NO activity or iNOS expression in endotoxemia. These findings argue against an involvement of vascular iNOS activity as the primary mediator of acute systemic hyporeactivity to LPS.

Acknowledgments
The experiments were supported by a grant from the “Hochschuljubiläumsstiftung der Stadt Wien.” We are grateful for the skillful assistance and administrative work of Carola Fuchs, RN.

References
11. Annane D, Sanquer S, Sebille V, Faye A, Djuranovic D, Raphael JC, Gajdos P, Bellissant E. Compartmentalised inducible nitric-oxide synthase seems to be influenced by the type of bacteria.11 The lack of iNOS mRNA and protein expression in the present in vivo experiments is in good agreement with these previous observations. This was also confirmed by the lack of iNOS mRNA in blood incubated >4 hours with a low dose of LPS. It is possible that the LPS stimulus in the present experiments was too small to induce iNOS expression in blood, as evidenced by the lack of a significant increase in blood endotoxin concentrations. We wanted to analyze iNOS expression in vascular tissue from skin pouch biopsies, but these specimens did not contain enough vessels to draw reliable conclusions to confirm this negative result (data not shown).

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