Adrenoceptor 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\(^6\)-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 adrenoceptor 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 ■ adrenoceptors

Sepsis is still associated with a high mortality rate and remains a major therapeutic challenge despite improved intensive care therapy.\(^1\) 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,\(^2\) resulting in hemodynamic instability and shock.

A variety of substances and mediators is released by activated blood cells and endothelial cells during sepsis.\(^2,3\) 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.\(^4\) 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\(^5\) 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.\(^6–8\) Experiments with iNOS knockout animals\(^9\) and animals treated with iNOS antisense oligonucleotides\(^10\) 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.\(^11\) 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 \(N^2\)-monomethyl-L-arginine (L-NMMA) mitigated hypotension in septic patients.\(^12\) 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.\(^13\) Systemic administration of NOS inhibitors is also limited by severe side effects, such as pulmonary hypertension and reduced cardiac output.\(^14\)

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. 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 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 acclimatize 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 [Nitrional, G. Pohl Boskamp GmbH], at 4, 8, and 16 nmol/min), acetylcholine (ACH [Clinalfa], at 25, 50, and 100 nmol/min), and N⁶-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 mg) 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.
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 $5.4\pm0.4 \times 10^9$/L at baseline to $11.4\pm0.8$ and $10.9\pm0.6 \times 10^9$/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 $\approx$4 hours after LPS administration. In a pooled analysis for all subjects, mean arterial pressure decreased significantly from 85±3 mm Hg at baseline to 78±2 and to 72±2 mm Hg ($P<0.05$), and pulse rate was elevated from 70±2 bpm to 87±2 and to 76±3 bpm ($P<0.00001$) after 4 and 8 hours, respectively.

PE caused a significant and dose-dependentpressor 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 ($P<0.05$) and systolic blood pressure ($P<0.00005$ at the highest dose only) but not diastolic blood pressure were significantly blunted in subjects receiving 2 ng/kg LPS (Figure 1), whereas the reduced responsiveness did not reach the level of significance in subjects administered 1 ng/kg LPS. Eight hours after LPS administration, the pressor response to PE was nearly restored to the predose level, except for PE-induced changes in the pulse rate, which was still slightly impaired in subjects administered 2 ng/kg LPS ($P<0.05$ versus maximal effect before LPS dosing).

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 any significant changes in forearm blood flow on the treatment arm from 4.0±0.2 mL/100 mL per minute at baseline to 6.4±0.2 mL/100 mL per minute ($P<0.05$) at 210 minutes. Hand warming increased basal forearm blood flow (mean from both hands) from 5.1±0.2 mL/100 mL per minute at baseline to 6.4±0.2 mL/100 mL per minute ($P<0.05$) at 210 minutes. Hand warming increased basal forearm blood flow on the treatment arm from 4.0±0.3 mL/100 mL per minute by 58%, ie, to 6.3±0.5 mL/100 mL per minute ($P<0.001$, Wilcoxon), but there was no change in the contralateral arm. Vasoconstriction to NA or L-NMMA was not changed by hand warming (data not shown).

Under baseline conditions, the drugs under study exerted the expected dose-dependent and significant changes in forearm blood flow (Figure 2). After LPS, vasoconstriction to NA was significantly reduced ($P<0.01$). No change in responsiveness to L-NMMA was seen. Endothelium-dependent vasodilatation to ACh was impaired ($P<0.05$), whereas GTN-induced endothelium-independent vasodilatation was unaltered.

Control experiments were conducted in 4 subjects to study the time course of LPS-induced changes in sensitivity to NA and L-NMMA. At baseline and every 2 hours for 6 hours after LPS administration, a dose of 240 pmol/min NA or 4 μmol/min L-NMMA was infused into the brachial artery, and
ACh on skin blood constriction was seen in the absence of iNOS expression in healthy subjects that was associated with an inflammatory reaction in the site of purulent areas 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, no ready explanation is available for this effect in the forearm, which has already been observed in vivo after vaccination with Salmonella typhi. 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. However, there was a substantial variability of data, which has also been reported by other investigators. 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 endotoxin shock in animal experiments. Unfortunately, selective iNOS inhibitors

**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.

![Figure 4: iNOS protein and actin control in PMNs at baseline and after LPS.](Image)

**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 blood and was not associated with an altered vascular effect of NO synthesis or NO-mediated dilatation. These findings are in agreement with clinical studies that have described iNOS solely at the site of putrescent areas 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.

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### Table: C<sub>r</sub> of iNOS and GAPDH Control mRNA Isolated From Whole Blood at Baseline and After LPS Administration

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<tr>
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<th>iNOS&lt;sub&gt;C&lt;sub&gt;r&lt;/sub&gt;&lt;/sub&gt;</th>
<th>GAPDH&lt;sub&gt;C&lt;sub&gt;r&lt;/sub&gt;&lt;/sub&gt;</th>
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<tr>
<td>Baseline</td>
<td>30.04±1.83</td>
<td>21.72±2.40</td>
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<tr>
<td>4 h after LPS</td>
<td>33.49±1.44&lt;sup&gt;*&lt;/sup&gt;</td>
<td>22.46±0.83</td>
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<tr>
<td>6 h after LPS</td>
<td>32.71±2.99&lt;sup&gt;*&lt;/sup&gt;</td>
<td>23.41±0.54</td>
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Values are mean±SEM (n=10). C<sub>r</sub> indicates threshold cycle.

<sup>*</sup>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 dysfunc-
tion and in which NOS inhibitors ameliorate the hemody-
namic 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 dis-
apparenting results of large-scale multicenter trials with NOS inhibitors in septic shock patients. Furthermore, the expres-
sion 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 Ca2+-handling mech-
isms in myofilaments directly,28,29 and activates vascular potassium channels.29 Internalization of vascular α-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 (O2·−). The production of O2·− is increased in sepsis as in other inflammatory conditions and could play a role in the pathogenesis of hemodynamic instability and organ dys-
function during septic shock.32 O2·− autoxidizes catechol-
a mines, including dopamine and NA.33 In a recent study in rats,34 the LPS-induced hyporeactivity to NA was im-
proved by a mimic of superoxide dismutase, which en-
hanced degradation of O2·−. 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.

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