Escherichia coli Endotoxin Inhibits Agonist-Mediated Cytosolic Ca\textsuperscript{2+} Mobilization and Nitric Oxide Biosynthesis in Cultured Endothelial Cells

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Abstract Altered release of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) has been proposed as a final common pathway underlying the abnormal vasodilator responses to gram-negative lipopolysaccharide (endotoxin). However, mechanisms responsible for lipopolysaccharide-induced changes in EDRF/NO release from endothelial cells have not been clarified. We evaluated direct effects of Escherichia coli endotoxin on agonist-stimulated cytosolic Ca\textsuperscript{2+} mobilization and NO biosynthesis in cultured bovine and porcine aortic endothelial cells (ECs). Two methods were used to assay for NO: (1) analysis of NO-induced endothelial levels of cGMP as a biological indicator of NO generation and (2) direct quantitative measurement of NO release (chemiluminescence method). Cytosolic free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) was evaluated using fura 2 fluorescence methodology (340/380-nm ratio excitation and 500-nm emission). Incubation of ECs with endotoxin (0.5 μg/mL, 1 hour plus 1-hour wash) significantly inhibited bradykinin (100 nmol/L)– and ADP (10 μmol/L)–mediated increases in endothelial cell cGMP to 37% and 22% of control responses, respectively. In contrast, endotoxin failed to inhibit the increase in cGMP produced by the non–receptor-dependent Ca\textsuperscript{2+} ionophore A23187 (1 μmol/L) or sodium nitroprusside (1 mmol/L). Similarly, incubation with endotoxin inhibited ADP-stimulated increases in NO release and EDRF bioactivity to 55% and 56% of control values, respectively, but did not affect A23187-stimulated increases in NO release or EDRF bioactivity. Endotoxin produced significant decreases in both transient and sustained [Ca\textsuperscript{2+}] responses to bradykinin and ADP. For example, the initial rapid increase in bovine EC [Ca\textsuperscript{2+}], in response to bradykinin was reduced to 31% of the initial increases in control cells, and the secondary plateau phase was reduced to only 3% of respective control responses. Concentration-response relation to endotoxin (10\textsuperscript{-3} to 10\textsuperscript{6} μg/mL) indicated high correlation and similar IC\textsubscript{50} values (0.025 and 0.021 μg/mL, respectively) for inhibitory effects on cGMP and [Ca\textsuperscript{2+}]. Endotoxin had no effect on inositol trisphosphate formation ([\textsuperscript{3}H]myo-inositol incorporation) and intracellular Ca\textsuperscript{2+} release ([Ca\textsuperscript{2+}]), responses in Ca\textsuperscript{2+}-free medium) induced by bradykinin. However, agonist-stimulated Mn\textsuperscript{2+} quenching (index of Ca\textsuperscript{2+} influx) was significantly attenuated by endotoxin treatment. These studies demonstrate that endotoxin directly decreases agonist (bradykinin and ADP)–mediated biosynthesis and release of EDRF/NO from ECs. These effects can be explained by altered [Ca\textsuperscript{2+}] mobilization mechanisms, which in turn produce subsequent decreases in activity of the Ca\textsuperscript{2+}-calmodulin–dependent constitutive isoform of NO synthase and, ultimately, impairment of agonist-mediated NO release and endothelium-dependent vasodilation. (Circ Res. 1994;75:659–668.)

Key Words • lipopolysaccharide • endothelium-derived relaxing factor • nitric oxide synthase • cGMP • fura 2

Vascular dysfunction during endotoxemia and septic shock is expressed as a heterogeneous pattern of peripheral vasodilation and vasoconstriction in different organs, culminating in decreased systemic vascular resistance and hypotension. Mechanisms responsible for endotoxin-induced disruptions in arterial function remain unclear. Recent studies have suggested that endotoxin and/or its endogenous biological mediators modulate the activity of endothelium-derived relaxing factor (EDRF). EDRF is believed to be nitric oxide (NO)\textsuperscript{1} and/or a closely related nitroso compound.\textsuperscript{2} NO is derived from the guanidino nitrogen atom of L-arginine through a reaction catalyzed by NO synthase (NOS)\textsuperscript{3–6}; two major classes (three isoforms) of NOS have been identified.\textsuperscript{4–6} Constitutively expressed NOS (cNOS) isoforms I and III are found primarily in neurons and endothelial cells, respectively. Both cNOS isoforms are Ca\textsuperscript{2+}-calmodulin dependent; agonist-mediated release of NO from endothelial cells depends on increased intracellular Ca\textsuperscript{2+} and subsequent activation of cNOS. Another isoform (type II) is inducibly expressed (iNOS) in a variety of cell types (macrophages, vascular smooth muscle, etc), is Ca\textsuperscript{2+}-calmodulin independent, and requires hours for induction.\textsuperscript{4–6} On release from the endothelium, EDRF/NO migrates rapidly to adjacent vascular smooth muscle fibers and elicits vasorelaxation by activation of cytosolic guanylyl cyclase and subsequent increases in cellular cGMP.\textsuperscript{4–7}

We have recently reported evidence for impaired release of EDRF in experimental models of guinea pig\textsuperscript{8,9} and canine\textsuperscript{10} endotoxemia. Recently, loss of endothelium-dependent relaxation in pulmonary vessels has been suggested to support sustained pulmonary hypertension after exposure to endotoxin in sheep.\textsuperscript{11} Myers and colleagues\textsuperscript{12,13} reported evidence for decreased
basal and bradykinin-stimulated NO release and EDRF bioactivity after incubation of endothelial cells with endotoxin. These studies and others have suggested that altered vasorelaxation during endotoxemia is due to inhibitory effects of lipopolysaccharide and/or secondary mediators on cNOS-derived NO. On the other hand, it has been proposed that endotoxin and/or associated cytokine mediators promote release of both endothelium and non-endothelium-derived NO. This concept has been based on several lines of evidence, including the observation that pharmacologic inhibition of NO synthesis antagonizes the hypotensive response to endotoxin and cytokines in intact animals. Accordingly, since endotoxin and cytokines have been reported to induce expression of iNOS in both vascular smooth muscle and endothelial cells, NOS inhibitors have been proposed as therapeutic interventions in the clinical management of septic shock. Because of these disparate reports about the effects of endotoxin on release of EDRF/NO, we conducted the present study to increase our understanding of direct actions of endotoxin on endothelial cNOS and biosynthesis of NO.

Precise interactions between one agent and one type of cell are difficult to discern when many other cell types and many other biologically active agents are also present as uncontrolled variables. Therefore, we used an in vitro approach to this problem and tested the direct effects of endotoxin alone on cultured vascular endothelial cells isolated from bovine and porcine aorta. The objectives of the present study were to (1) evaluate the effects of in vitro endotoxin on agonist-stimulated biosynthesis of NO as indicated by endothelial cGMP levels and direct NO measurements and (2) evaluate intracellular mechanisms of endotoxin-mediated impairment of NO formation in endothelial cells by analysis of cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) by fura 2 measurements.

**Materials and Methods**

**General Approach**

Direct effects of endotoxin on cultured endothelial cells were evaluated by using a protocol similar to that previously described. Briefly, monolayers of endothelial cells were preincubated for 1 hour in the absence or presence of endotoxin (concentrations indicated in “Results”) in culture medium without fetal calf serum (FCS) at 37°C. Cells were then washed twice and reequilibrated for an additional 60 minutes in culture medium (without FCS) before determination of cGMP levels, EDRF bioactivity, and NO production. We also determined changes in [Ca\(^{2+}\)]\(_{i}\), in response to endotoxin by using fura 2 methodology.

**Primary Cell Cultures**

Primary endothelial cell cultures were obtained by incubation of freshly harvested bovine or porcine aorta for 20 minutes with 200 U/mL collagenase, 2 mg/mL bovine serum albumin, 1 mg/mL soybean trypsin inhibitor type II-S, essential and nonessential amino acids, and vitamins in Dulbecco’s minimum essential medium. After centrifugation, cells were collected, resuspended in Opti-MEM containing 5% FCS, and seeded in plastic dishes or six-well clusters. Unless otherwise indicated, experiments were performed with cells from first passage. Cell culture techniques have been previously described.

**Determination of Endothelial cGMP as Marker for EDRF Formation**

Graier, Kukovetz, and colleagues and others have previously documented the activity of endothelial cell cGMP levels as a measure of NO generation. Intracellular cGMP levels were measured by radioimmunoassay as described previously. Briefly, confluent endothelial cells were preincubated as described below with or without endotoxin in serum-free culture medium. Cells were washed twice and reequilibrated in culture medium without serum. After 45 minutes, medium was aspirated, and cells were preincubated with HEPES-buffered solution containing 2.5 mmol/L CaCl\(_2\), 1 mol/L 3-isobutyl-1-methylxanthine, and 10 μmol/L indomethacin for an additional 15 minutes. Experiments were started by the addition of the drug to be tested for 4 minutes, and incubation was stopped by removing incubation buffer and the addition of 2 mL of 0.01 mol/L HCl. Cells were stored frozen until cGMP assay.

**NO Measurement (Chemiluminescence Method) and Bioactivity**

For NO measurements, endothelial cells (passages 3 through 10) were grown to confluence in T150 flasks, harvested, and equally dispersed to siliconized spinner flasks. Microcarrier beads (0.27 g) were placed in each flask, which on the average was calculated to yield 1.24x10\(^6\) cells per flask if cells were confluent. Since the number of cells on any one experimental day could vary, three identical flasks were prepared, and one flask served as the control, allowing expression of data as percent control. In general, 1.24x10\(^6\) cells would yield a measured basal NO concentration of \(\approx 2.3 \times 10^{-4}\) mol/L. After bradykinin stimulation, the NO would increase to \(\approx 6.8 \times 10^{-4}\) mol/L. The absolute value of NO measured could vary from experiment to experiment since the number of cells could minimally vary.

**Preparation of NO Standard**

Standard solutions of pure NO were prepared fresh to construct standard curves for conversion of the NO signal (output measured in volts) to molar concentration. Pure NO gas (6 μL) was dissolved in 25 mL distilled water that had previously been bubbled at least 0.5 hour with helium; this yielded a 10 μmol/L NO stock solution. This solution was maintained in a gas-tight Hamilton syringe and used within 1 hour.

**Measurement of NO**

NO was quantitatively determined by an NO analyzer (Dasibi model 2108). The principle of measurement uses a specific chemiluminescence method and has been previously applied to determination of NO in biological systems. Briefly, cell effluent was immediately transferred (without storage) to a reflux chamber containing glacial acetic acid–1% sodium iodide. The mixture was refluxed at 55°C while the chamber was continually flushed with nitrogen gas, which in turn was transferred to the NO analyzer, where the NO reacted with ozone to produce light. The amount of light was measured by a photomultiplier tube and converted into voltage recorded on a standard flatbed pen recorder. This signal was then used to construct a voltage-concentration standard curve by using known concentrations of pure NO. The molar amount of NO from the cells was then determined by using the standard curve. Typically, there were two phases to the NO release after bradykinin stimulation, characterized as a rapid release followed by a secondary sustained release; NO was recorded as that concentration measured during the sustained plateau phase, normally =5 minutes of agonist exposure. All measurements were made during the steady-state signal. Near-simultaneous determination of the effluent for both NO release and EDRF bioactivity was performed in a paired fashion by selective distribution of the effluent to the NO analyzer and then to the biodetector vascular ring.
**Determination of EDRF Bioactivity**

Bioassay vascular rings (biodetectors) to quantify EDRF bioactivity were prepared from 3-mm-long segments of porcine proximal circumflex coronary arteries. EDRF bioactivity was determined by superfusion of the effluent from the cultured cells (control-incubated or endotoxin-incubated) over the biodetector ring. Segments were denuded of endothelium by gently rubbing the intimal surface with the closed tips of forceps. Biodetectors were mounted on three wire stirrups, one of which was stationary and the other attached to a Gould FT03C force transducer for measurement of isometric force. The rings were superfused at 4 mL/min with Krebs' physiological saline (37°C) previously bubbled with 95% O2/5% CO2. Each biodetector was passively stretched to its optimal length as determined by serial exposure to 100 mM KCl. The biodetector was then preconstricted to 4 to 6 g active tension with prostaglandin F2α (1 x 10⁻⁶ to 5 x 10⁻⁹ mol/L). Removal of the endothelium was confirmed by the absence of vasorelaxation after directly superfusing the biodetector with 2 x 10⁻⁸ mol/L bradykinin. Bioactivity was expressed as percent relaxation of the preconstricted biodetector.

**[Ca²⁺]** Measurements

[Ca²⁺], was measured by using fura 2 methodology as described previously. Confluent endothelial cells were harvested with 0.02% trypsin and 0.02% EDTA (2 minutes), centrifuged, and resuspended in culture medium without FCS. After 1 hour at 37°C with or without endotoxin, cells were centrifuged, washed twice, and further resuspended in culture medium without FCS, containing 2 μmol/L fura 2-AM for 45 minutes, followed by an equilibration period for 15 minutes in HEPES buffer (mmol/L: NaCl, 145; KCl, 5; CaCl₂, 2.5; MgCl₂, 1; and HEPES acid 10, adjusted with NaOH to pH 7.4). Experiments were performed at 37°C, and [Ca²⁺]i was calculated from the fura 2 fluorescence ratio (340/380-nm excitation and 500-nm emission) according to the method of Grynkiewicz et al; autofluorescence was corrected according to Hallam et al.

**Mn²⁺ Quench Experiments**

Experiments were performed according to the method described previously. Briefly, cells were loaded with fura 2-AM as described above and stimulated in the presence of 2.5 mmol/L MnCl₂ with the compound to be tested. Mn²⁺ entry was detected by the decrease of fura 2 fluorescence, measured at 360-nm excitation and 500-nm emission.

**Measurement of Inositol Trisphosphate Levels**

Bovine aortic endothelial cells (passages 4 through 6) were seeded onto six-well tissue-culture plates (Corning) and grown to confluence (66 to 68 hours) at 37°C in a 5% CO₂ atmosphere in medium 199, with Earle's salts and 20% FCS, supplemented with neomycin (GIBCO) and containing [³H]myo-inositol (10 μCi/ml). After growing 3 days in [³H]myo-inositol, all cells of one plate were incubated in the absence or presence of endotoxin (0.5 μg/ml) as described above. [³H]myo-inositol and endotoxin were then rinsed out, and cells were incubated for an additional hour and equilibrated at room temperature for 10 minutes in serum-free medium 199 containing indomethacin (5 μmol/L) and HEPES (25 mmol/L) and buffered to pH 7.3 with NaOH. Endothelial cells in three wells of each plate were incubated for 2 minutes in bradykinin (18 nmol/L, added as a 20-μL stock of 1.8 μmol/L), and the reaction was stopped by aspiration of the solution and addition of 1 mL ice-cold trichloroacetic acid (TCA, 10% with 0.1 mmol/L EDTA). Inositol phosphates were extracted from endothelial cells for 1 hour at 4°C. The plates were then scraped with a rubber spatula, and the cell extract was rinsed (0.5 mL TCA) once more and transferred to a 2-mL Eppendorf tube. Protein was removed by centrifugation (16,000 g). The supernatant was extracted four times with water-saturated diethyl ether (4 mL), diluted to 20 mL with water, and applied to a 1-mL AG1-XS column (formate form) equilibrated with water. Inositol phosphates were separated as previously described by using a modification of the procedure of Dowen and Michell. Briefly, free myo-inositol was eluted from the column in the void and then followed with a 20-mL wash of ddH₂O. Glycerol inositol phosphate was eluted with 10 mL of 60 mmol/L ammonium formate in 5 mmol/L sodium borate (pH 5.0) and discarded. Inositol monophosphate (IP₁), inositol bisphosphate (IP₂), inositol trisphosphate (IP₃), and inositol tetrakisphosphate (IP₄) were batch-eluted with 10 mL each of 150 mmol/L, 400 mmol/L, 800 mmol/L, and 1 mol/L ammonium formate in 0.1 mol/L formic acid buffered to pH 5.0 with ammonium hydroxide. Aliquots (1 mL) of each fraction were added to scintillant (Ultima-Flu AF, Packard Instrument Co), and inositol phosphates were quantified by scintillation spectroscopy.

**Drugs and Supplies**

Endotoxin was purchased as purified *Escherichia coli* endotoxin (lipopolysaccharide W, 0127:B8, Difco Laboratories). Fura-2 AM was purchased from LAMBDA Fluorescence Technology, and ADP, A23187, bradykinin, collagenase, EGTA, HEPES acid, indomethacin, 3-isobutyl-1-methylxanthine, sodium nitroprusside, trypsin, and soybean trypsin inhibitor type II-S were from Sigma Chemical Co. Cell culture materials were obtained from Boehringer Mannheim, culture media were purchased from Gibco/BRL, and FCS was obtained from Sebak. Six-well clusters were purchased from Costar.

**Statistics**

All experiments were performed in triplicate with at least three different batches of endothelial cell preparations. The results are expressed as mean±SEM. Statistical significance was evaluated with one- or two-way ANOVA, including Scheffé's F test. For IP₃ studies, significance was determined by a Kruskal-Wallis one-way ANOVA on ranks, with multiple comparisons determined by a Student-Newman-Keuls test. In all experiments, the level of significance was defined as P<.05.

**Results**

**Effects of Endotoxin on Endothelial cGMP Levels**

Concentrations of cGMP were measured as a biological marker for generation of EDRF/NO. Under control conditions (without endotoxin), bradykinin (100 nmol/L) and ADP (10 μmol/L) increased endothelial cGMP levels from basal levels of 0.89±0.04 to 2.54±0.12 (n=15) and 2.11±0.09 (n=9) pmol/L cGMP per 10⁶ cells, respectively (Fig 1). These concentrations produce maximal responses for both agonists. Incubation of bovine aortic endothelial cells (Fig 1A) with 0.5 μg/ml *E. coli* endotoxin (1-hour incubation plus 1-hour wash) did not alter basal cGMP levels. However, endotoxin reduced both bradykinin and ADP-induced increases in cGMP to 37% and 22% of control increases (P<.05), respectively. For instance, incubation with endotoxin produced significant reductions in the cGMP responses to bradykinin and ADP from basal levels of 0.83±0.04 pmol/L cGMP per 10⁶ cells to agonist-stimulated values of only 1.45±0.04 (P<.05 versus basal) and 1.10±0.04 (P<.05 versus basal) pmol/L cGMP per 10⁶ cells, respectively (Fig 1A). In contrast to results with bradykinin and ADP, endotoxin failed to inhibit increases in cGMP produced by the non–receptor-dependent Ca²⁺ ionophore A23187 (1 μmol/L). Similarly, the cGMP increase produced by nitroprusside (1 mmol/L), which stimulates soluble guanylyl cyclase di-

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**Endotoxin Impairs Endothelial EDRF/NO Synthesis**

Graier et al

661
Effects of Endotoxin on Endothelial NO Release and EDRF Bioactivity

As indicated in Fig 2, incubation of bovine endothelial cells with endotoxin (0.1 and 0.5 μg/mL) decreased both basal and ADP-stimulated increases in EDRF bioactivity (Fig 2A) and NO production (Fig 2B). At 0.5 μg/mL endotoxin, EDRF bioactivity and NO production following stimulation with ADP averaged only 56% (P<.05) and 55% (P<.05) of respective control values. In contrast to ADP, A23187-stimulated EDRF bioactivity and NO production were not impaired by endotoxin (Fig 2). (Results of similar experiments with bradykinin were recently published.) Results are expressed as percent of NO release obtained for control cells. Approximate correlations with absolute levels of NO release in the identical flasks of confluent cells for control and experimental groups are presented in “Materials and Methods.”

Intracellular Ca²⁺ Responses

Since agonist-induced EDRF formation in vascular endothelial cells depends on increases in [Ca²⁺], we investigated whether endotoxin affects agonist-induced Ca²⁺ responses in cultured bovine aortic endothelial cells. Results of a typical representative experiment are illustrated in Fig 3. Under control conditions (without endotoxin) (n=16), bradykinin (100 nmol/mL) produced a rapid and transient increase in [Ca²⁺] from values averaging 149±3.2 to 678±11.9 nmol/mL (P<.05). After the transient phase, there was a secondary sustained plateau phase, which was constant for at least 10 minutes (Fig 3, top, and summarized in the Table). In endotoxin (0.5 μg/mL)-treated cells (n=12), the initial rapid increase in [Ca²⁺] induced by bradykinin was significantly reduced to 31% of initial increases in control cells (P<.01), and the bradykinin-induced secondary plateau phase was not sustained and returned to near basal values (within 3%, P>0.05) within 10 minutes (Fig 3, top, and Table). Similar to bradykinin, the effect of ADP on [Ca²⁺] was strongly diminished by endotoxin. As shown in a representative tracing in Fig 3, bottom, addition of 10 μmol/L ADP to untreated cells (n=9) increased [Ca²⁺], from overall values of 153±5.6 to 486±10.7 nmol/L, followed by a sus-

Fig 1. Bar graphs showing the effects of endotoxin (Etx) on bradykinin (100 nmol/L)–, ADP (10 μmol/L)–, Ca²⁺ ionophore A23187 (1 μmol/L)–, and sodium nitroprusside (SNP, 1 mmol/L)–induced endothelium-derived relaxing factor formation, measured as increases in intracellular cGMP levels in cultured bovine aortic endothelial cells (A) and porcine aortic endothelial cells (B). Cultured cells were exposed for 1 hour to culture medium without serum with or without 0.5 μg/mL Etx and washed twice, which was followed by a further wash period for 1 hour in culture medium without Etx. Experiments were performed as described in “Materials and Methods.” Each column represents mean±SEM (for panel A, n=15; for panel B, n=9).

Fig 2. Bar graphs showing the effects of endotoxin (Etx) on ADP-stimulated endothelin-derived relaxing factor bioactivity (A) and nitric oxide production (B) in cultured bovine aortic endothelial cells. Bioactivity was determined by using vasorelaxation responses in endothelium-denuded biotector rings (porcine coronary arteries) precontracted with prostaglandin F₂α. Nitric oxide production was quantified by a chemiluminescence technique (see “Materials and Methods”). Data are presented as effects observed in cells incubated with Etx (0.5 μg/mL) as a percentage of response of same-batch control cells incubated without Etx.
Effects of Endotoxin (0.5 μg/mL, 1 hour Plus 1-hour Wash) on Agonist (Bradykinin and ADP)-Stimulated Increases in [Ca\(^{2+}\)]\(_i\), in Cultured Bovine Endothelial Cells

<table>
<thead>
<tr>
<th>[Ca(^{2+})](_i), nmol/L</th>
<th>Control</th>
<th>Endotoxin</th>
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<tbody>
<tr>
<td>Bradykinin (n=16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>149±3.2</td>
<td>152±9.9</td>
</tr>
<tr>
<td>Initial spike</td>
<td>678±11.9</td>
<td>318±6.4*</td>
</tr>
<tr>
<td>Sustained plateau</td>
<td>407±6.6</td>
<td>159±4.3*</td>
</tr>
<tr>
<td>ADP (n=9)</td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>153±5.6</td>
<td>147±4.1</td>
</tr>
<tr>
<td>Initial spike</td>
<td>486±10.7</td>
<td>268±1.7*</td>
</tr>
<tr>
<td>Sustained plateau</td>
<td>301±4.6</td>
<td>152±1.4*</td>
</tr>
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</table>

Values are mean±SEM. Values obtained for sustained plateau measurements were obtained after 4-minute exposure to maximally effective concentrations of bradykinin (100 nmol/L) or ADP (10 μmol/L).

*P<.01 vs corresponding control value.
Effect of Endotoxin on Bradykinin-Stimulated IP₃ Levels

As indicated in Fig 6, both control and endotoxin-treated endothelial cells responded to bradykinin (18 nmol/L), with significant (P<.05) increases in IP₃ levels. However, incubation with endotoxin (0.5 μg/mL) had no significant effect on either basal or bradykinin-stimulated increases in IP₃ (Fig 6). Bradykinin-stimulated increases in IP₃ were also observed, although the magnitude was less than that measured for IP₃ after 2 minutes of incubation in bradykinin. IP₃ levels were unaffected by bradykinin under these experimental conditions (data not shown).

[Ca²⁺]ᵢ Responses in Nominally Ca²⁺-Free Solution

The role of Ca²⁺ influx from extracellular sites was determined by evaluation of bradykinin-stimulated [Ca²⁺]ᵢ responses in the absence of extracellular Ca²⁺ (Fig 7). After 1-minute incubation in zero (nominally free) Ca²⁺, addition of bradykinin (100 nmol/L) produced a small but significant rise in [Ca²⁺]ᵢ, in both groups. Basal [Ca²⁺]ᵢ values averaged 106±5 and 102±7 nmol/L (P>.05), respectively, in control (n=6) and endotoxin-treated (n=4) cells. Maximal bradykinin-stimulated increases in [Ca²⁺]ᵢ in zero Ca²⁺ were not significantly different (217±21 versus 203±18 nmol/L, P>.05) in control and endotoxin-treated cells. Importantly, Ca²⁺ entry by the readdiction of Ca²⁺ (2.5 mmol/L) to these cells was markedly diminished in endotoxin-treated cells compared with control cells (Fig 7).

Effects of Endotoxin on Mn²⁺ Quenching of [Ca²⁺]ᵢ

As shown in representative tracings in Fig 8, Mn²⁺ entry induced by bradykinin (27±4.6% of control values, n=9, P<.05) or ADP (33±6.7% of control values, n=7, P<.05) was significantly attenuated in cells preincubated with endotoxin.

Discussion

Vascular endothelium has been implicated as a key intermediary in the pathophysiological response to endotoxemia and gram-negative sepsis. Findings from the
present study indicate that endotoxin directly inhibits agonist-stimulated biosynthesis and release of NO from endothelial cells. These results suggest that the responsible mechanism(s) involves altered mobilization of intracellular Ca\(^{2+}\) and, in turn, subsequent decreases in activity of the Ca\(^{2+}\)-calmodulin–dependent cNOS. To our knowledge, the present study represents the first investigation into the relation between endothelial cell [Ca\(^{2+}\)], and NO formation (direct measurements) after extended (1-hour) exposure to endotoxin.

**Methodological Considerations**

Two methods were used to assay for NO: (1) measurements of NO-induced endothelial cell cGMP levels and (2) direct quantitative measurement of NO release. The biological indicator of endothelial cell NO biosynthesis was analysis of endothelial cGMP.\(^{23-37,43}\) Measurement of endothelial cGMP levels is a highly sensitive indicator of intracellular NO biosynthesis.\(^{27-33}\) For instance, increases in endothelial cell cGMP levels in response to agonists (eg, bradykinin) normally exceed corresponding increases in measured NO release.\(^{45}\)

Unlike measurements of NO release, NO-mediated increases in endothelial cell cGMP appear minimally affected by secondary influences known to alter post-synthesis inactivation of NO (eg, free radical scavengers).\(^{4,5,14}\) Importantly, agonist-mediated increases in endothelial cGMP are completely blocked by inhibitors of NOS (L-arginine analogues), confirming the specificity of this determination for NO-generated activation of guanylyl cyclase and corresponding increases in cGMP.\(^{43}\)

The present study also used a sensitive and specific chemiluminescence technique to measure NO and provide an important correlative evaluation of EDRF bioactivity and NO production and release. The application of the chemiluminescence technique to quantify NO derived from biological systems has been described in detail previously.\(^{12,13}\) This technique was adapted to provide parallel and paired quantitative measurements of both EDRF vasodilator bioactivity and NO concentrations from the same cell population. Determination of both of these variables is important because the exact identity of EDRF remains controversial. Some studies indicate that EDRF is authentic NO,\(^{1,4}\) whereas other data are more consistent with the interpretation that EDRF is a NO-containing compound more potent than authentic NO.\(^{2}\) The chemiluminescence assay for NO measures total NO and requires a strong reducing environment to release NO from organic compounds containing a nitroso moiety or moieties; this assay system therefore does not distinguish between free NO released directly from a cell versus NO cleaved ex vivo from a parent nitroso compound. For these reasons, we were careful throughout the study to address both EDRF bioactivity and NO production.

**Effects of Endotoxin on NO Production**

Taken in concert, our combined studies now document that incubation of endothelial cells with endotoxin results in reductions of NO biosynthesis (endothelial cell cGMP content) and NO release (chemiluminescence technique) in response to bradykinin and ADP. The incubation period for the present study was designed to correlate with that of the recent study by Myers et al.\(^{12}\) Those studies documented that endotoxin (0.01 to 1.5 \(\mu\)g/mL) did not have an immediate effect on EDRF bioactivity or NO production but, rather, exerted a time-dependent cellular effect that resulted in impaired responses to bradykinin. Endotoxin was reported to decrease EDRF bioactivity and NO production under bradykinin-stimulated conditions after extended (1-hour incubation plus 1-hour wash) but not acute exposure.\(^{12}\) Those studies also documented that although high concentrations of endotoxin (>5 \(\mu\)g/mL) were cytotoxic, endotoxin concentrations <1 \(\mu\)g/mL resulted in minimal endothelial cell death not significantly different from the control level. Therefore, evaluation of effects of endotoxin on cGMP and [Ca\(^{2+}\)], in the present study was conducted by using the endotoxin concentrations and incubation periods described by Myers et al.\(^{12}\) Our studies now document that both ADP- and bradykinin-mediated generation and release of endothelial cell NO are inhibited by 1-hour endotoxin exposure.

Mechanisms involved in the inhibitory effect of endotoxin on bradykinin- and ADP-mediated NO production could hypothetically involve a number of target sites including the following: perturbation of bradykinin and purinergic receptor populations of endothelial cells, disruption of signal-transduction elements involved in coupling endothelial receptors to NOS, nonspecific cytotoxic effects on NOS activity, downregulation of endothelial NOS mRNA similar to that recently reported in response to cytokines,\(^{3}\) or even nonselective suppression of activity of the endothelial guanylyl cyclase resulting in reductions in cGMP not necessarily associated with reduced biosynthesis of NO. However, present experiments allowed us to discount several of these possible mechanisms. For instance, if endotoxin altered intrinsic activity (including downregulation) of NOS or guanylyl cyclase, we would have expected that all agents that increase [Ca\(^{2+}\)], or activate guanylyl cyclase would be similarly inhibited by endotoxin. However, since the cGMP increase produced by non–receptor-dependent Ca\(^{2+}\) ionophore A23187 was unaffected by endotoxin, this indicates that NOS activity was sufficient to respond normally to increases in [Ca\(^{2+}\)], mediated through receptor-independent mechanisms. Thus, it is unlikely that endotoxin-mediated inhibition of NO biosynthesis involves intrinsic enzyme impairment, downregulation of NOS, or decreased NOS mRNA levels. Similarly, nitroprusside undergoes tissue (cell)-catalyzed reduction yielding NO (bypassing NOS); NO then directly activates cytosolic guanylyl cyclase with subsequent increases in cGMP. Since increased cGMP levels in response to nitroprusside were unaffected by endotoxin, guanylyl cyclase also appears spared by endotoxin. These findings are analogous to those reported by Parker and Adams,\(^{8}\) who used ex vivo vascular tissue (aorta and coronary arteries) isolated from a guinea pig model of endotoxemia. In those studies, endothelium-dependent vasorelaxation to the receptor agonists acetylcholine and ADP was significantly impaired by endotoxemia, whereas relaxation responses to A23187 and nitroprusside were unaffected. Thus, it appears that both in vitro endotoxin and in vivo endotoxemia selectively alter receptor-dependent activation of cNOS and release of NO, apparently via modulation of endothelial
[Ca\(^{2+}\)], regulatory mechanisms, while sparing intrinsic activity of cNOS and guanylyl cyclase.

**Effects of Endotoxin on Endothelial Cell [Ca\(^{2+}\)]**

Increases in endothelial [Ca\(^{2+}\)], are well established to be responsible for agonist-mediated formation of NO by cNOS. Autacoid-induced elevations in endothelial [Ca\(^{2+}\)] are generally biphasic and have been attributed to several mechanisms, including the IP\(_3\)-mediated release of intracellular stores of Ca\(^{2+}\) as well as activation of a ligand-stimulated voltage-independent Ca\(^{2+}\)-entry mechanism. Certain agonists (e.g., bradykinin) can also alter K\(^+\) channel activity and endothelial membrane potential, affecting Ca\(^{2+}\) entry via changing of its electrochemical gradient. In general, the initial spike increase in [Ca\(^{2+}\)], following agonist stimulation reflects phospholipase C activation and subsequent IP\(_3\)-mediated Ca\(^{2+}\) release, whereas the secondary sustained phase is the result of enhanced Ca\(^{2+}\) influx from extracellular sites. However, we and others previously reported that the initial increase not only reflects intracellular Ca\(^{2+}\) release but also Ca\(^{2+}\) entry. This is indicated by the decrease of the Ca\(^{2+}\) transient in the absence of extracellular Ca\(^{2+}\) compared with the initial transient in the presence of extracellular Ca\(^{2+}\).

In the present studies, incubation with endotoxin was found to markedly inhibit both phases of the [Ca\(^{2+}\)]\(_i\) response to bradykinin and ADP in Ca\(^{2+}\)-containing solution (Figs 3 and 4). Although these data might initially suggest effects of endotoxin on both Ca\(^{2+}\) release and Ca\(^{2+}\) influx, our subsequent experiments evaluating IP\(_3\), zero-Ca\(^{2+}\) responses, and Mn\(^{2+}\) quench effects provide insight into more specific mechanisms of altered Ca\(^{2+}\) mobilization. First, bradykinin-stimulated increases in IP\(_3\), were unaffected by endotoxin, suggesting lack of effect on receptor-mediated phospholipase C activation and generation of IP\(_3\), an intracellular second messenger for Ca\(^{2+}\) release. This supposition is supported by comparable bradykinin-stimulated [Ca\(^{2+}\)]\(_i\) release (initial Ca\(^{2+}\) response) in the absence of extracellular Ca\(^{2+}\), where differences in Ca\(^{2+}\) influx mechanisms are minimized or abolished. Interestingly, on readdition of exogenous Ca\(^{2+}\) to nominally Ca\(^{2+}\)-free medium, [Ca\(^{2+}\)]\(_i\) responses of endotoxin-treated cells were markedly diminished, suggesting a primary effect of endotoxin on Ca\(^{2+}\) influx mechanisms.

The studies using the Mn\(^{2+}\) quench technique provide further support of endotoxin-mediated impairment of endothelial Ca\(^{2+}\) influx. This method is based on the ability of Mn\(^{2+}\) to enter endothelial cells through agonist-stimulated Ca\(^{2+}\)-entry pathways, thereby quenching the fura 2 fluorescence signal of [Ca\(^{2+}\)]. Although bradykinin and ADP increased Mn\(^{2+}\) entry and decreased fura 2 fluorescence, cells incubated with endotoxin exhibited markedly reduced effects of the agonists on Mn\(^{2+}\) entry through Ca\(^{2+}\)-influx pathways (Fig 8). Effects of endotoxin on endothelial Ca\(^{2+}\) influx may have particular functional relevance. Luckoff et al and Graier and colleagues provided evidence that formation of EDRF in cultured vascular endothelial cells depends more on agonist-stimulated Ca\(^{2+}\) entry than on IP\(_3\)-mediated Ca\(^{2+}\) release. Release of intracellularly stored Ca\(^{2+}\) by IP\(_3\) results in a rapid increase in [Ca\(^{2+}\)], necessary for a quick response of agonist-induced EDRF formation. However, Ca\(^{2+}\) influx is a more sustained response important for basal and long-lasting EDRF formation involved in the regulation of vascular tone. In the present experiments, bradykinin- and ADP-mediated [Ca\(^{2+}\)] responses were not fully sustained after exposure to endotoxin (Figs 3 and 4), returning to basal values within minutes, despite continued exposure to the agonists. In control endothelial cells not exposed to endotoxin, significant Ca\(^{2+}\) influx sustained [Ca\(^{2+}\)] during the entire period of study (>10 minutes). These studies suggest significantly impaired Ca\(^{2+}\)-influx mechanisms by endotoxin in endothelial cells and further implicate the potential presence of reduced [Ca\(^{2+}\)]-dependent agonist-mediated cNOS activity in vivo in endotoxic shock states, even in the presence of exposure to endothelium-dependent vasodilator autacoids. Precise mechanisms and second messengers involved in endothelial cell Ca\(^{2+}\) entry have not been elucidated. However, since membrane hyperpolarization has been shown to control the electrochemical gradient for agonist-induced Ca\(^{2+}\) entry (for review see Reference 44), one might speculate that endotoxin-mediated inhibition of Ca\(^{2+}\)-activated K\(^+\) channels is potentially responsible for the decreased Ca\(^{2+}\) entry in endotoxin-treated endothelial cells. Thus, a direct inhibition of Ca\(^{2+}\)-entry mechanisms, endotoxin-mediated inhibition of agonist-stimulated Ca\(^{2+}\) entry could also be related to an inhibition of endothelial K\(^+\) channels.

**Implications and Conclusions**

Our findings provide direct support for the contention that endotoxin at relatively low concentrations and after 1-hour exposure significantly decreases agonist-stimulated production of NO by endothelial cells. The present study, combined with the recent studies of Myers and colleagues, suggests that sustained systemic hypotension resulting from endotoxia may not be due to direct effects of endotoxin to promote the acute release of endothelium-derived NO. On the other hand, studies using intact animal models have suggested that the early (within 30 minutes) hypotensive response to intravenous endotoxin may involve enhanced formation of NO due to activation of endothelial cNOS. Those lines of evidence have been based primarily on pressor and/or vasodepressor response to inhibitors of NO (N\(^\text{G}\)-nitro-l-arginine methyl ester and N\(^\text{G}\)-mono-methyl-l-arginine) rather than direct measurements of NO generation. Studies using NOS inhibitors in vivo to examine endotoxin-mediated blood pressure responses cannot exclude the possibility of direct inhibitory actions of endotoxin itself on Ca\(^{2+}\) mobilization and cNOS activity, as we now report with endothelial cells. Furthermore, it is possible that endotoxin may trigger the release of a variety of circulating mediators (bradykinin, histamine, serotonin, and catecholamines) that may secondarily release NO from endothelium or other cell types. Indeed, Fleming et al reported that cultured endothelial cells produce kinins that can stimulate endothelial NO production in an autocrine manner. Although the present in vitro studies rule out direct endotoxin activation of cNOS (after 1-hour exposure), we cannot exclude the possibility of indirect activation of cNOS in vivo produced via endotoxin-mediated release of secondary mediators. Larger concentrations of endotoxin (30 \(\mu\)g/mL) have been reported to produce a
small short-lived (<5 minutes) increase in [Ca\(^{2+}\)], in cultured endothelial cells.\(^{27}\) In contrast, the present study is the first to document impaired agonist-mediated [Ca\(^{2+}\)]\(_i\), mobilization and decreased biosynthesis and release of NO (using direct measurements) after more prolonged exposure to low noncytotoxic concentrations (0.5 μg/mL) of endotoxin. Furthermore, our results indicate that impaired Ca\(^{2+}\) influx (rather than Ca\(^{2+}\) release) plays an important mechanistic role in altered Ca\(^{2+}\) mobilization produced by endotoxin.

Present findings also provide an explanation for earlier unexplained reports that endotoxin shock reduces endothelium-dependent relaxation in isolated arterial preparations.\(^{8-11,14-16}\) This endothelial dysfunction occurs relatively early\(^{10,16,48}\) and is sustained through later stages of endotoxin shock.\(^{8,9,13}\) Moreover, the inhibitory effects of in vitro endotoxin on release of EDRF in cultured endothelial cells mimic the impaired endothelium-dependent relaxation observed in ex vivo vascular preparations removed from experimental models of endotoxemia. For instance, we have now shown that both forms of impairment are receptor dependent and do not involve altered responses to Ca\(^{2+}\) ionophore (direct activation of eNOS by increased [Ca\(^{2+}\)]\(_i\)) or to sodium nitroprusside (direct relaxation of vascular smooth muscle bypassing endothelial eNOS).

Clearly, endotoxin and certain cytokines exhibit positive cooperativity in inducing gene expression controlling de novo synthesis of iNOS in vascular smooth muscle, macrophages, and other cell types.\(^{4-6,21-25}\) Induction of iNOS in vascular smooth muscles is believed at least partially to account for altered contractile responses of endothelium-intact and -denuded blood vessels exposed to endotoxin.\(^{20,23,25,28,50,51}\) Excess NO produced from iNOS has gained considerable attention as a mechanism responsible for the sustained hypotension and vascular hyporesponsiveness observed in endotoxemia and septic shock. Induction of iNOS in vascular smooth muscle occurs over prolonged periods (2 to 24 hours) and is unlikely to be involved in the present studies.

In summary, present findings provide direct evidence for inhibitory effects of endotoxin on endothelial cell Ca\(^{2+}\)-influx mechanisms, decreased [Ca\(^{2+}\)]\(_i\), reduced Ca\(^{2+}\)-dependent activation of endothelial eNOS, and, ultimately, decreased NO biosynthesis and release from endothelial cells. These mechanisms may play a role in impaired endothelium-dependent vasodilation in endotoxemia and sepsis.

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