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Research Article

Treatment of volunteers or animals with endotoxin in vivo results in reduced vascular reactivity to catecholamines. Endotoxin also causes liberation of the vasoactive cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) from vascular smooth muscle and endothelial cells in culture. This study tested whether defects in contractility could be induced in isolated vascular tissue by prolonged exposure to endotoxin (1-100 ng/ml) in vitro, and whether IL-1 and TNF release by blood vessels is altered during the establishment of endotoxin induced contractile dysfunction. A concentration of endotoxin as low as 1 ng/ml suppressed contractions to norepinephrine (NE) and KCl; aortic sensitivity to NE also decreased. The presence of serum constituents or an intact endothelium were not necessary for endotoxin-induced vascular suppression. Aortas incubated with endotoxin liberated IL-1 and TNF in a dose-dependent fashion. The addition of dexamethasone or indomethacin during the incubations generally suppressed release of the cytokines and improved tissue reactivity to NE. The endotoxin-induced diminished vascular contraction and augmented IL-1 and TNF liberation required de novo protein synthesis; tissue incubated with endotoxin plus actinomycin D was completely shielded from the influence of endotoxin on vascular reactivity to NE. The association between endotoxin-induced vascular cytokine release and diminished contraction suggests a possible role for cytokines derived from the vasculature in the regulation of contractile function.

Prolonged Exposure of Rat Aorta to Low Levels of Endotoxin In Vitro Results in Impaired Contractility

Association with Vascular Cytokine Release

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Abstract

Treatment of volunteers or animals with endotoxin in vivo results in reduced vascular reactivity to catecholamines. Endotoxin also causes liberation of the vasoactive cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) from vascular smooth muscle and endothelial cells in culture. This study tested whether defects in contractility could be induced in isolated vascular tissue by prolonged exposure to endotoxin (1–100 ng/ml) in vitro, and whether IL-1 and TNF release by blood vessels is altered during the establishment of endotoxin-induced contractile dysfunction. A concentration of endotoxin as low as 1 ng/ml suppressed contractions to norepinephrine (NE) and KCl; aortic sensitivity to NE also decreased. The presence of serum constituents or an intact endothelium were not necessary for endotoxin-induced vascular suppression. Aortas incubated with endotoxin liberated IL-1 and TNF in a dose-dependent fashion. The addition of dexamethasone or indomethacin during the incubations generally suppressed release of the cytokines and improved tissue reactivity to NE. The endotoxin-induced diminished vascular contraction and augmented IL-1 and TNF liberation required *de novo* protein synthesis; tissue incubated with endotoxin plus actinomycin D was completely shielded from the influence of endotoxin on vascular reactivity to NE. The association between endotoxin-induced vascular cytokine release and diminished contraction suggests a possible role for cytokines derived from the vasculature in the regulation of contractile function. (*J. Clin. Invest.* 1990. 86:160–168.) Key words: endothelium • endotoxemia • interleukin 1 • sepsis • tumor necrosis factor

Introduction

Septic patients frequently exhibit increased plasma concentrations of epinephrine and norepinephrine (NE), but show a decrease in the positive correlation that exists between catecholamine concentrations and systolic blood pressure (1). This disordered relationship between catecholamines and vascular tone is reflected by the diminished peripheral systemic vascular resistance that frequently occurs in sepsis (2, 3). Depressed vascular reactions to pressor agents are also present in animal models of experimental sepsis. Septic rats display diminished pressor responses to both NE and angiotensin II in vivo (4). The sepsis-induced decrease in vascular reactivity persists when tissue is removed from septic rats and tested with NE and vasopressin in vitro (5).

Endotoxin is present in the circulation during sepsis and in early stages of the development of the disorder has been found to be an accurate predictor for subsequent septicemia, even more so than the documentation of blood cultures positive for Gram-negative bacteria (6). Increased plasma concentrations of endotoxin could contribute to the curtailed sensitivity to catecholamines by acting directly on the vasculature, by induction and/or release of circulating vasoactive mediators, or by a combination of the two actions. In volunteers, intravenous injection of 1 μg of *Escherichia coli* endotoxin results in diminution of vascular reactivity to NE 3–4 h after the injection (7). Intravenous injection of 2 mg/kg endotoxin in cats also results, 2–3 h later, in impaired vasoconstrictor responses to NE (8). The endotoxin-induced decrease in pressor responses persist when aortas from endotoxin-injected rats (6 mg/kg) are removed and tested in vitro (9). It is probable, however, that intravenous injection of milligram per kilogram quantities of endotoxin results in a short-term exposure of the vasculature to endotoxin concentrations that exceed actual pathophysiological levels by at least three orders of magnitude. Reported levels for biologically active endotoxins in sepsis vary widely, reflecting differences in endotoxin standards, assays, diseases, and models utilized. In humans, maximal reported concentrations of endotoxin in plasma range from > 0.1 (6), 0.85 (10), and 5 ng/ml (11), to as high as tens of nanograms per milliliter for lipooligosaccharides in systemic meningococcal disease (12). Plasma endotoxin concentrations reach levels slightly > 1 ng/ml during infusion of a lethal dose of *E. coli* into primates (13).

The present study was designed to investigate whether long-term exposure of rat aortic tissue to pathophysiologically relevant concentrations of endotoxin in vitro was sufficient to impart contractile defects similar to those documented in sepsis or after exposure to endotoxin in vivo. In addition, the influence of pharmacologic interventions on endotoxin-induced alteration in vascular function were investigated. Finally, because we have previously shown that IL-1 and tumor necrosis factor (TNF)[1] are implicated in inducing defective vascular contraction to pressor agents (5, 14, 15), the release of these cytokines by the rat aorta in response to endotoxin and during pharmacologic intervention was monitored.

Methods

Tissue preparation and incubation. Male Sprague-Dawley rats (150–250 g, Taconic Farms, Germantown, NY) were decapitated, the thoracic aorta and associated adventitia were dissected, and four rings

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1. *Abbreviations used in this paper:* PDB, phorbol 12,13-dibutyrate; TNF, tumor necrosis factor.

(3.5 mm in length) were cut from each aorta. Some aortas were deendothelialized before sectioning into rings. Aortas were deendothelialized by perfusion with phosphate-buffered saline (PBS) containing 2% sodium deoxycholate and air bubbles for 20 s, followed by a PBS and bubble rinse for 5 min. Removal of the endothelium was confirmed by scanning electron microscopy (*vide infra*). Individual aortic rings were placed in Petri dishes containing 1.2 ml Dulbecco's modified Eagle's medium (DME) supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin, 1% fetal calf serum (FCS) and variously treated with indomethacin (50 μM), dexamethasone (0.1 or 1.0 μM), actinomycin D (25 μM), cycloheximide (50 μM), or their vehicles. Endotoxin was diluted from a stock preparation (1 mg/ml in DME, aliquotted and stored at −80°C), into DME. The endotoxin preparation was sonicated for 3 min at maximum power in a cup horn sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) and dispensed into the incubation media at final concentrations of 1–100 ng/ml. The media and rings were rotated gently for 16 h in an incubator at 37°C under a 95% O_2-5% CO_2 atmosphere.

Measurement of aortic ring contraction. After incubation, rings were mounted between two stainless steel hooks in 10-ml jacketed tissue baths. One hook was fixed in the bath and the other hook attached to a strain guage (Kulite Semiconductor, Ridgefield, NJ) for measures of ring tension. Contractions by eight rings were measured simultaneously and recorded on multichannel strip chart recorders. The rings were maintained in Krebs-Ringer bicarbonate buffer (KRB; millimolar composition: NaCl 118; KCl 4.7; $CaCl_2$ 1.3; $MgSO_4.7H_2O$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.0; glucose 11.7) at pH 7.4 while being continually bubbled with 95% O_2-5% CO_2. The rings were stretched to a resting tension of 2.4 g and allowed to equilibrate for 30 min. The rings were then contracted by two applications of NE (0.1 μM); acetylcholine (1 μM) was added to the baths at maximal contraction to the first dose of NE to test for the functional integrity of the vascular endothelium (16). The tissue baths were flushed with fresh KRB between applications of NE until ring tension returned to resting values. Ring contractions were then induced by stepwise cumulative additions of NE (10^{-9} to 3×10^{-5} M) or by sequentially adding KCl to the bath (10–90 mM); in some experiments phorbol 12,13-dibutyrate (PDB; 1 μM) was added to the baths after maximal contraction to NE was attained and any additional increments in tension recorded. All rings were blotted and weighed after experiments were completed.

Vascular cytokine release and assays. Rat aortic rings were prepared and incubated in DME supplemented with antibiotics, anti-inflammatory agents and endotoxin as described above, with the exception that incubation volumes were doubled; therefore two rings were allocated into each 2.4 ml of medium. After 16 h of incubation, the rings were removed and weighed, and the conditioned medium was desalted (Econo-Pac 10DG, Bio-Rad Laboratories, Richmond, CA) and lyophilized. The lyophilized samples were redissolved in RPMI 1640 and cytokine activities measured. IL-1 was measured with a mouse thymocyte comitogenic assay as described elsewhere (5). Sample activities were compared to those of human recombinant IL-1α utilized as a standard in this assay. The sensitivity of this bioassay had a range of 3–6 pg/ml. TNF activity was measured by an in vitro cell cytotoxicity assay utilizing actinomycin D–treated murine fibroblast L929 cells (American Type Culture Collection, Rockville, MD). L929 cells were plated into 96-well microtiter plates at 50,000 cells per well and incubated 24 h at 37°C in 5% CO_2. After incubation, the medium was aspirated and 100 μl of fresh medium was added to all wells. Recombinant human TNFα was diluted into medium for standards. 50 μl of standards or samples plus an additional 50 μl of medium were pipetted in duplicate into the first column of wells and then serially diluted across the plate. 100 μl of medium containing 10 μg/ml actinomycin D was added to all wells and the cells incubated 24 h. Cytotoxicity was detected by a tetrazolium dye technique (17). The plates were read at 570 nM on a microtiter plate reader (model 650, Dynatech Laboratories, Inc., Alexandria, VA) against *n*-propyl alcohol blanks. TNF activity was quantitated by an area under the curve method as described by Hewlett et al. (18). The sensitivity of this bioassay had a range of 100–200 pg/ml.

Measures of protein synthesis. 12 aortic rings (1.7 mm in length) were prepared from each rat aorta and incubated in DME supplemented with antibiotics, anti-inflammatory agents, and endotoxin as described above. After 16 h of incubation, rings were rinsed twice in PBS and transferred to RPMI 1640 that was deficient in L-methionine and L-cysteine and supplemented as described above. The medium also contained 10 μCi/ml of mixed ^{35}S-labeled methionine and cysteine. The rings were incubated for an additional 2 h, rinsed twice in PBS, and frozen until processed. The rings were powdered under liquid N_2, dissolved in lysis buffer (1% sodium deoxycholate, 1% Nonidet NP 40, and 1 μM leupeptin in PBS), and the protein was precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10%. The precipitate was washed once with 10% TCA, once with acetone, dried, and taken up in sample buffer (10% [wt/vol] sodium dodecyl sulfate [SDS], 10% [vol/vol] glycerol, in 0.06 M Tris-HCl, pH 6.8). Protein content of the samples was determined by bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) and the incorporated radioactivity was measured by scintillation spectrophotometry.

Scanning electron microscopy. Aortic rings were fixed in 2% glutaraldehyde/1% paraformaldehyde in 0.1% sodium cacodylate buffer (pH 7.5) for 60 min. After three washings in the buffer, the rings were cut into 2-mm^2 pieces, dehydrated in graded ethanol solutions, and dried by a critical-point drying method using liquid CO_2. Samples were then mounted luminal surface up on metal stubs, coated with a thin film of gold-palladium, and examined in a scanning electron microscope (JSM-35CF, JEOL USA, Peabody, MA).

Statistics. Aortic ring contractile performance to NE and KCl was characterized by deriving E_{max} (maximum generated tension) and EC_{50} (concentration of agonist causing a half-maximal contraction) values with a nonlinear regression analysis program (19) that fitted a third-order polynomial to ring dose-responses. Tests for differences among treatment means were by randomized blocks analysis of variance; if significant differences were indicated, then pairs of means were tested by Student-Newman-Keuls *a posteriori* tests (20). Tests for differences between EC_{50} values were based on log EC_{50} values (21). Percentage data were normalized by arcsin transformation before analysis. The influence of anti-inflammatory agents and endotoxin on protein synthesis was also assessed by the same protocol. Differences in cytokine release by aortic tissue were tested by paired *t* tests. Differences with probabilities of 0.05 or less were accepted as significant. All data are expressed as the mean±SEM.

Materials. HBSS, RPMI 1640, FCS, and actinomycin D were purchased from Gibco, Grand Island, NY. Norepinephrine bitartrate, indomethacin, dexamethasone, cycloheximide, PDB, and leupeptin were obtained from Sigma Chemical Co., St. Louis, MO. Sodium deoxycholate, SDS, and acetylcholine chloride were purchased from Calbiochem-Behring Corp., San Diego, CA. Penicillin/streptomycin and PBS were bought from Quality Biological, Gaithersburg, MD. Endotoxin (*E. coli* lipopolysaccharide serotype 055:B5) was purchased from Difco, Detroit, MI. Tran^{35}S label labeling reagent was obtained from ICN, Irvine, CA. Recombinant human TNFα was obtained from Genzyme, Boston, MA. Stock dilutions of NE were made up in 0.01 N HCl. Actinomycin D and indomethacin were dissolved in ethanol, and PDB and dexamethasone were dissolved in dimethylsulfoxide (DMSO) before addition to medium. The remaining reagents were dissolved directly in medium.

Results

Characteristics of endotoxin-induced suppression of vascular contractile responses. Incubation of aortic rings with endotoxin for 16 h resulted in significantly impaired contractile function. Maximal contractions to NE were progressively diminished after incubation with higher concentrations of endotoxin (Fig. 1, *left panel*). In addition, all endotoxin-treated

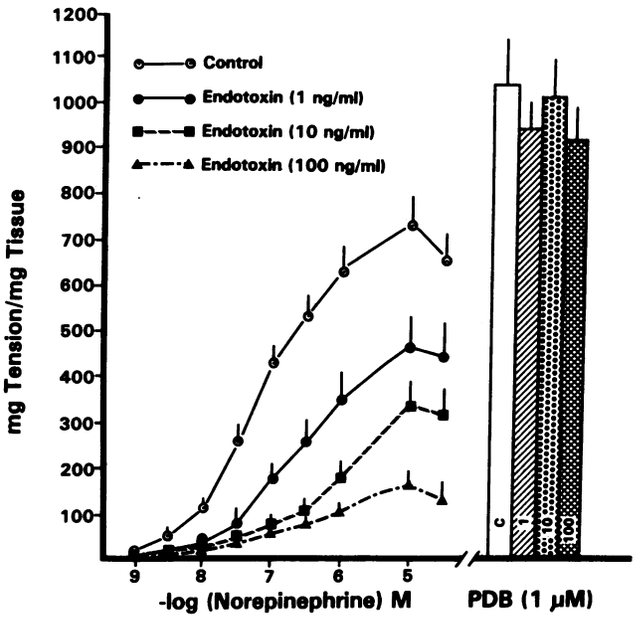

Figure 1. (*Left panel*) Contraction to cumulative doses of NE by rat aortic rings incubated for 16 h in control medium, or media with 1, 10, or 100 ng/ml endotoxin. The responses are plotted as means + SEM with curves fitted by hand. (*Right panel*) Maximum contraction by the same rings to PDB applied after completion of NE dose–response measures. Numbers on bases of histograms refer to endotoxin concentration (ng/ml) of incubation media. Four rings were prepared from each rat and one ring allocated into each medium preparation. $n = 9$ rats.

rings expressed elevated EC_{50} values (Table I). Adding PDB to the organ baths after the maximum contraction to NE was attained returned endotoxin-treated ring contractions to normal (Fig. 1, *right panel*). Rings incubated with endotoxin also exhibited an impaired contraction to KCl (Fig. 2). The presence or absence of FCS in the medium during incubation did not alter the negative actions of endotoxin on aortic ring contraction to NE (data not shown).

Removal of the vascular endothelium before incubation with endotoxin improved sensitivity and maximal response to NE in comparison to endotoxin-treated rings with an intact endothelium (Fig. 3, Table I). However, E_{max} and EC_{50} of endotoxin treated deendothelialized rings remained significantly impaired in comparison to endothelium-intact or deendothelialized control rings (Fig. 3, Table I). Successful removal of the endothelium of the aortic rings was confirmed by the absence of vasodilatory responses to acetylcholine and by scanning electron microscopy (Fig. 4).

Altered influence of endotoxin on aortic ring contraction after coincubation with anti-inflammatory agents and inhibitors of protein synthesis. Aortic rings incubated with endotoxin and dexamethasone or indomethacin exhibited a significant improvement in sensitivity and maximal contraction to NE compared to rings incubated only with endotoxin (Fig. 5, Table I). However, the rings continued to manifest suppressed E_{max} values in comparison to rings incubated in control medium (Fig. 5). Rings incubated with endotoxin and a combination of dexamethasone (0.1 μM) and indomethacin (50 μM) did not exhibit improvements in E_{max} (Fig. 5) or EC_{50} (567±189 nM) in comparison to rings incubated with endotoxin and either anti-inflammatory agent alone. Finally, incubation with a 10-fold higher concentration of dexamethasone did not further improve ring responses to NE (data not shown).

In contrast to the moderate protection attributable to the actions of anti-inflammatory agents, rings coincubated with endotoxin and actinomycin D manifested striking, completely normal contractile responses to NE (Fig. 6, *left panel*). Treat-

Table I. EC_{50} Values for NE-stimulated Rat Aortic Rings after Incubation for 16 h with Control or Endotoxin-treated Media and Anti-inflammatory Agents

Dose responses to endotoxin ($n = 9$)		Modulation by endothelium ($n = 10$)	
Treatment	EC_{50}*	Treatment	EC_{50}
	nM		*nM*
1. Control	90±19	Control	190±28
2. 1 ng/ml endotoxin	339±88‡	Control (deendothelialized)**	24±4‡
3. 10 ng/ml endotoxin	1,171±516‡	Endotoxin¶	2,031±419‡§
4. 100 ng/ml endotoxin	573±226‡	Endotoxin¶ (deendothelialized)	1,501±714‡§‖
Indomethacin ($n = 8$)		**Dexamethasone ($n = 12$)**	
Treatment	EC_{50}	Treatment	EC_{50}
	nM		*nM*
1. Control	74±8	Control	67±16
2. Control + IM#	60±5	Control + DMSO	63±3
3. Endotoxin¶ + IM	249±105	Endotoxin¶ + DEX§§	566±189‡§
4. Endotoxin¶	445±9‡§‖	Endotoxin¶	2,442±471‡§‖

* Log EC_{50} values (concentration of agonist causing a half-maximal contraction) were utilized for determination of significant differences between treatment groups; the data were then converted to arithmetic means and standard errors to allow the EC_{50} values to be expressed as nanomolar concentrations. ‡ $P < 0.05$ vs. 1; § $P < 0.05$ vs. 2; ‖ $P < 0.05$ vs. 3. ¶ Rings incubated with 10 ng/ml endotoxin. ** Rings deendothelialized by treatment with deoxycholate. # IM, indomethacin, 50 μM. §§ DEX, dexamethasone, 0.1 μM.

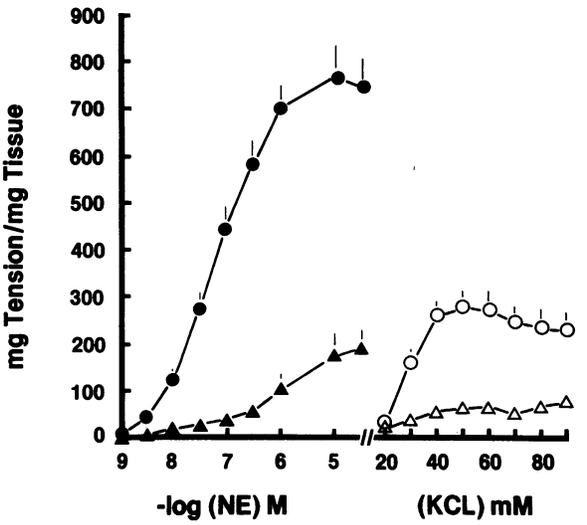

Figure 2. (*Left panel*) Contraction to cumulative doses of NE by rat aortic rings incubated for 16 h in control medium (*circles*), or medium with 10 ng/ml endotoxin (*triangles*). (*Right panel*) Contraction to sequential doses of KCl by sister rings subjected to the same incubation protocol. Four rings were prepared from each rat and two rings allocated into each medium preparation; thereafter one member of each pair was tested with NE and the other member tested with KCl. Means and errors presented as in Fig. 1. n = 8 rats.

ment of the rings with actinomycin D for 16 h did not interfere with a normal contraction to PDB (Fig. 6, *right panel*). Coincubation of rings with endotoxin and cycloheximide also resulted in completely normal contractile responses to NE (data not shown). Incubation of aortic rings for 16 h with actinomycin D resulted in a significant decrease in incorporation of label into protein (Table II). The presence or absence of endotoxin, dexamethasone, indomethacin, or their vehicles had no significant effect on gross protein synthesis (Table II).

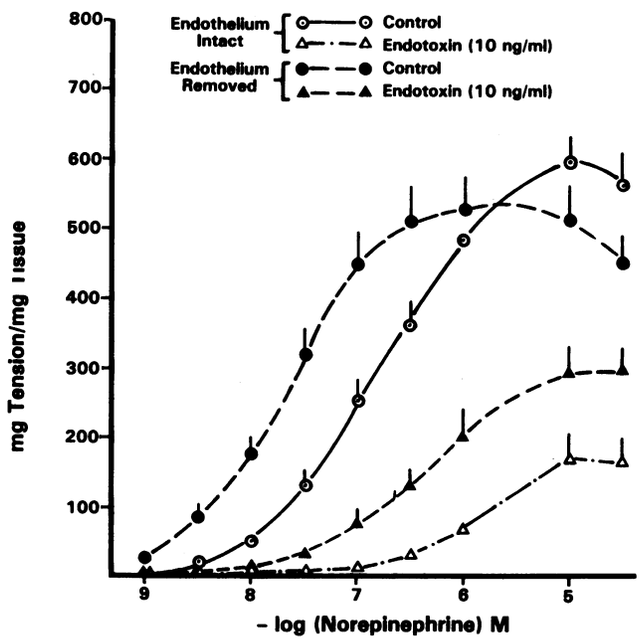

Figure 3. Contraction to cumulative doses of NE by rat aortic rings with intact (*open symbols*) or ablated (*closed symbols*) endothelium. Rings were incubated for 16 h in control medium (*circles*) or medium with 10 ng/ml endotoxin (*triangles*). Four rings were prepared from each rat and one ring allocated into each combination of medium and state of endothelium. Means and errors presented as in Fig. 1. n = 10 rats.

Effect of endotoxin on vascular cytokine release. Liberation of IL-1 by aortic rings was positively correlated with exposure to endotoxin. Removal of the endothelium prior to incubation with endotoxin yielded a small, nonsignificant, reduction in IL-1 release. Dexamethasone, indomethacin, and actinomycin D all significantly reduced endotoxin-stimulated release of IL-1 (Table III).

Aortic rings released TNF in response to endotoxin in a dose-dependent fashion. In contrast to endotoxin-stimulated IL-1 release from deendothelialized rings, removal of the endothelium before incubation with endotoxin halted release of TNF. Another contrast with the pattern of endotoxin-stimulated release of IL-1 was that indomethacin treatment did not suppress the liberation of TNF by endotoxin-treated rings. Dexamethasone and actinomycin D significantly reduced endotoxin-stimulated release of TNF (Table III).

Discussion

Incubation of rat aortic tissue with pathophysiologically relevant concentrations of endotoxin for an extended time resulted in major impairments in tissue response to α_1-adrenoceptor-mediated and non–receptor-mediated pressor agents. It is unlikely that the impaired contraction to NE and KCl simply reflects a toxic effect of endotoxin on vascular cell energy metabolism or contractile elements because the rings can be forced to exhibit normal magnitudes of contraction when exposed to two pressor agents together, i.e., NE and PDB (Figs. 1 and 6). α_1-Adrenoceptors act via the hydrolysis of phosphatidylinositol 4,5-bisphosphate into two compounds that possess second messenger activity. Inositol trisphosphate mobilizes intracellular calcium, and diacylglycerol activates protein kinase C (PKC) to phosphorylate proteins (see Berridge [22] for review). PDB can substitute for diacylglycerol to activate PKC (23) and cause vascular contraction by a mechanism that is not completely understood (see Rasmussen et al. [24] for review). Chiu et al. (25) demonstrated that a portion of PDB-induced contraction in the rat aorta is independent of activation of the calcium-calmodulin pathway. These observations suggest that simultaneous input of stimuli that activate different avenues leading to contraction allow tissue that has been incubated with endotoxin to overcome a relatively specific lesion in the contraction mechanism.

The proximate cause of the sepsis or endotoxin-induced diminution in vascular contractile performance is not known. It is likely that impaired calcium availability to the contractile mechanism or an inability to efficiently utilize calcium contributes to the dysfunction. The importance of Ca^{2+} availability during sepsis or after exposure to endotoxin is demonstrated by improvements in vascular contractility that occur after enhanced access to Ca^{2+}. A subset of patients with bacterial sepsis develop both hypocalcemia and hypotension; these patients exhibited a restoration of normal blood pressure after augmentation of their ionized serum calcium by calcium infusion (26). Stimulation of vascular potential–operated calcium

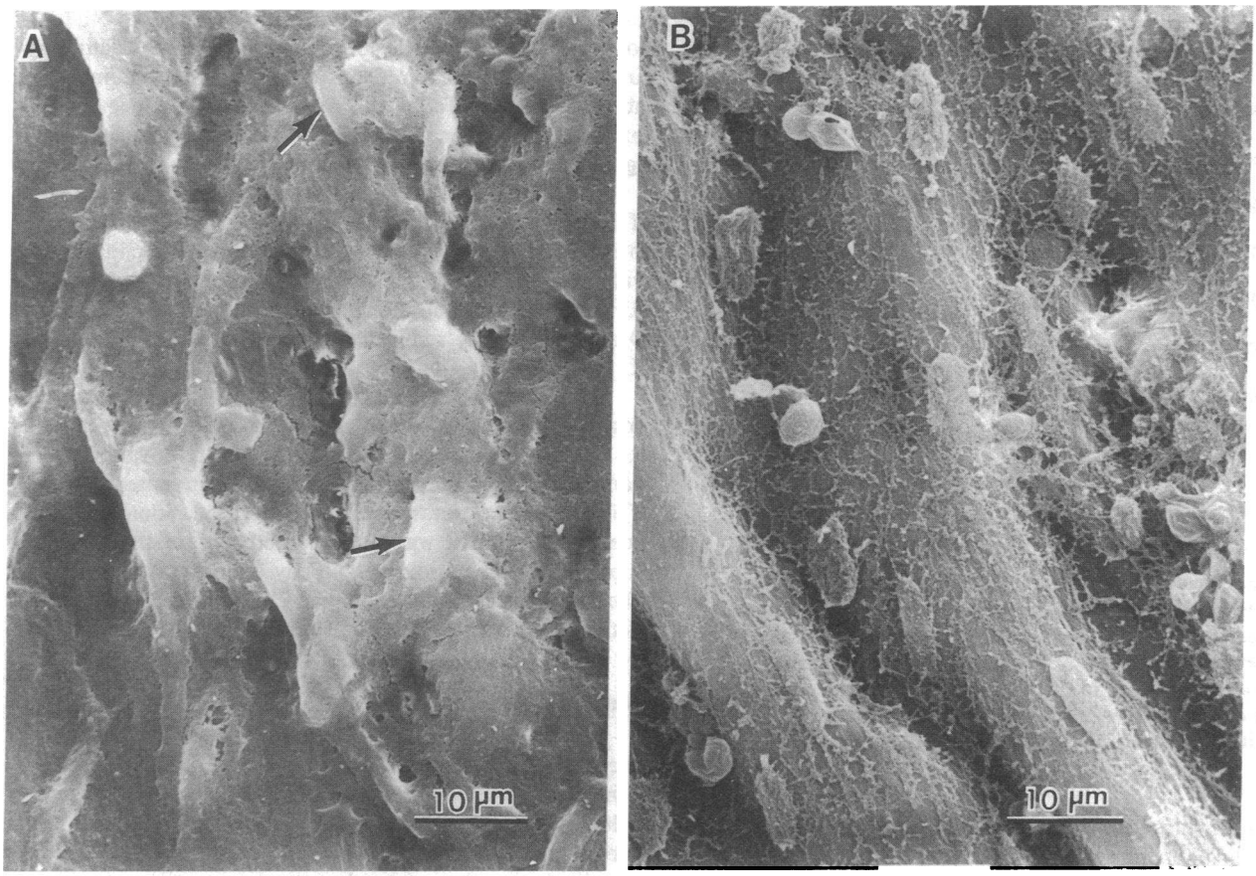

Figure 4. Scanning electron micrographs of endothelium of control and deoxycholate-treated rat aortas. (*A*) Luminal surface of control aorta shows intact endothelial cell layer. Arrows denote endothelial cell nuclei. (*B*) Luminal surface of deoxycholate-treated aorta exhibits endothelial cell loss.

channels by the calcium channel agonist BAY k 8644 promoted a rapid recovery in the mean arterial blood pressure of endotoxin-shocked rats (27). In aortas isolated from endotoxin-injected rats, changing the tissue bath Ca^{2+} concentration from 2.5 to 7.5 mM during KCl-induced contraction resulted in normal contraction by tissue that previously manifested diminished contractile performance (28).

Tissue incubated with endotoxin exhibits rightward-shifted and suppressed dose–response curves during stimulation with NE that are similar to the pattern observed after irreversible blockade of catecholamine receptors in the rabbit aorta (29). Indeed, $alpha_1$ receptor number is reduced in aortas isolated from septic rats (30), and aortic rings from septic rats exhibit reduced $^{45}Ca^{2+}$ influx when stimulated by NE (31). The diminished calcium influx via receptor operated calcium channels may contribute to impaired contraction in response to receptor agonists such as NE. However, diminished vascular contraction in response to KCl stimulation implies that a significant portion of the contractile defect resides at mechanisms other than those engaged by α_1-adrenoceptor activation. This conclusion is based on the fact that KCl-induced contractions in the rat aorta are mediated by Ca^{2+} influx subsequent to membrane depolarization, and not by phosphoinositide hydrolysis (32). The defect in KCl-induced contraction cannot be attributed to an impaired Ca^{2+} influx through potential operated calcium channels. Aortas from septic rats showed normal calcium influx during stimulation by KCl (31). Aortas from endotoxin-injected rats exhibited $^{45}Ca^{2+}$ uptake that was greater than that of control tissue (28); this observation led the authors to suggest that a disturbance of intracellular calcium utilization rather than defects in potential operated calcium channels cause diminished blood vessel contractility.

Vascular endothelial and smooth muscle cells have the capacity to synthesize and release biologically active IL-1 in response to endotoxin (33, 34). In this study, the release of IL-1 by aortic rings in response to endotoxin was monitored because we have documented that IL-1 is a potent suppressor of vascular reactivity to pressor agents (5, 14, 15). Others have recently reported similar results (35). A striking congruence exists between profiles of vascular suppression by IL-1 (5, 14, 15, 35) and endotoxin; i.e., after incubation with either agent, aortic rings exhibit decreased E_{max} and increased EC_{50} for NE, diminished response to KCl, the reversal of suppression by PDB, and a complete shielding of the tissue from suppressive influences by coincubation with actinomycin D.

The mouse thymocyte and L929 cytotoxicity bioassays used to measure aortic ring IL-1 and TNF release are susceptible to interference by cytokines released from vascular endothelium and smooth muscle cells. The bioassays were used in this study because more specific assays that require antibodies against rat cytokines are not available. Human endothelial cells secrete significant amounts of IL-6 in response to TNFα, IL-1β, and bacterial endotoxin (36). Recombinant murine IL-6 induces proliferation by phytohemagglutinin-activated

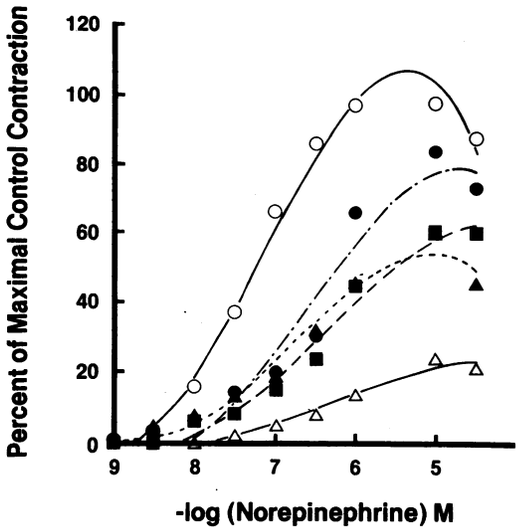

Figure 5. Contraction to cumulative doses of NE by rat aortic rings incubated for 16 h in control medium (○) or rings incubated in media containing 10 ng/ml endotoxin variously supplemented with no supplements (△), 0.1 μM dexamethasone (●), 50 μM indomethacin (▲) and the two agents together (■). Four rings were prepared from each rat and one ring always allocated to control medium, one ring always allocated to medium containing endotoxin, and the remaining rings allocated to variously supplemented medium as described above. Responses by all treated rings were expressed as a percentage of the maximum contraction by sister rings isolated from the same aorta and incubated in control medium. The responses are plotted as means; SEM were small and do not extend beyond the symbols. The curves are best fit of the data by nonlinear least squares technique to a polynomial model. n = 8–12 rats per treatment group.

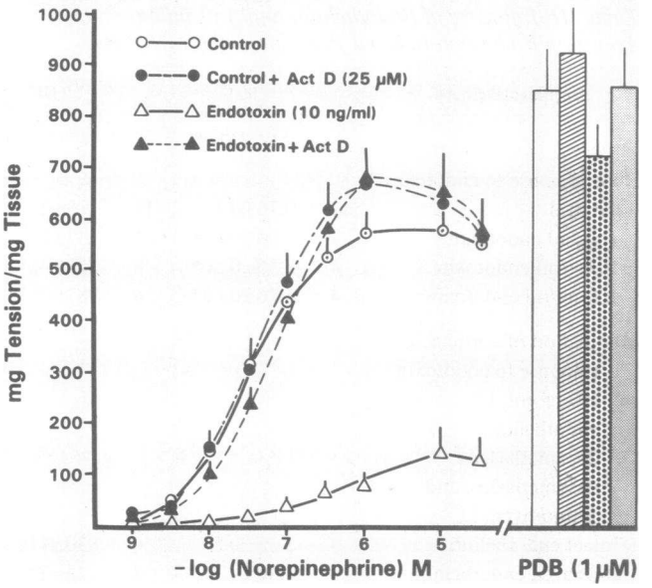

Figure 6. (*Left panel*) Contraction to cumulative doses of NE by rat aortic rings incubated for 16 h in control medium or control medium plus 25 μM actinomycin D (*circles*) or incubated with 10 ng/ml endotoxin without and with actinomycin D (*triangles*). (*Right panel*) Maximum contraction by the same rings to PDB applied after completion of NE dose–response measures. Rings were incubated in control medium (*open histogram*), control plus actinomycin D (*striated*), endotoxin only (*stippled*), and endotoxin plus actinomycin-D (*cross-hatched*). Four rings were prepared from each rat and one ring allocated into each medium preparation. Means and errors presented as in Fig. 1. n = 12 rats.

thymocytes (37); therefore it is possible that rat endothelium-derived IL-6 could be measured as IL-1 activity. It is unlikely that a major IL-6 influence on the thymocyte assay contributed to the measured IL-1 activity in the current experiments because deendothelialized rings exhibited no significant decline in release of IL-1 activity during exposure to endotoxin (Table III). An additional attribute of the bioassays is that IL-1 and TNF can mutually interfere with assays for the other. IL-1 is cytocidal against some strains of L929 cells (38), and TNF can augment thymocyte proliferation in the presence of phytohemagglutinin (39). However, these interactions require IL-1 and TNF concentrations greater than those measured in the current experiments (38–40). Therefore, the values in Table III are probably accurate estimates of IL-1 and TNF concentrations, but errors resulting from the relative nonspecificity of the bioassays cannot be completely excluded.

The amount of IL-1 activity liberated by endotoxin-treated rings reflects the magnitude of suppressed contraction to NE (Fig. 1, Table III); when endotoxin-treated rings were simultaneously treated with modulators of IL-1 synthesis (indomethacin, dexamethasone, actinomycin D) they manifested improved reactivity to NE and reciprocal changes in the release of IL-1 activity (Figs. 5 and 6, Table III). The diminished liberation of IL-1 by rings in the presence of indomethacin and endotoxin was unexpected because treatment of human vascular endothelial cells with indomethacin results in enhanced, not reduced, release of IL-1 after stimulation by exogenous IL-1 (41). The reason for the different response by rat aortic rings is unknown. It is possible that the difference in responses could relate to attributes of the aortic ring preparation because the rings consist of a composite of endothelial and smooth muscle cells in comparison to a preparation that consists of multiply passaged endothelial cells. Alternatively, the difference could reflect differences in stimuli (endotoxin vs. IL-1), or species or tissue examined. The observations from this study suggest that products of cyclooxygenase transduce a

Table II. Influence of Endotoxin and Anti-inflammatory Agents on Vascular Protein Synthesis

Treatment	No endotoxin	10 ng/ml endotoxin
	cpm/μg	
Control	7,177±910*	6,281±501
Ethanol (0.6%)	7,221±464	6,529±141
DMSO (0.4%)	5,818±551	6,622±357
Dexamethasone (0.1 μM)	5,230±1,202	5,789±1,158
Indomethacin (50 μM)	6,799±608	6,880±635
Actinomycin D (25 μM)	1,304±268‡	1,378±76‡

* n = three rings per treatment group; values are expressed as counts per minute per microgram of protein.
‡ $P < 0.05$ in comparison to either untreated or endotoxin-treated control.

Table III. Influence of Endothelium and Anti-inflammatory Agents on Endotoxin-induced Vascular Cytokine Release

Experimental groups	IL-1		TNF	
	n	*pg/mg tissue*	*n*	*pg/mg tissue*
Dose response to endotoxin				
Control	12	0.76±0.17	18	5±2
1 ng/ml endotoxin	4	1.00±0.09*	4	37±18^{ns}
10 ng/ml endotoxin	4	2.36±0.50*	8	150±40*
100 ng/ml endotoxin	4	4.76±0.68*	6	981±307*
Modulation of aortic ring response to endotoxin (10 ng/ml) by endothelium, dexamethasone, indomethacin, and actinomycin D				
Intact endothelium	4	3.35±0.55	4	830±116
Removed endothelium	4	2.40±0.17^{ns}	4	5±3*
DMSO (vehicle control)	11	2.89±0.82	4	422±74
Dexamethasone (0.1 μM)	11	1.53±0.49*	4	26±10*
Ethanol (vehicle control)	7	2.11±0.54	6	522±166
Indomethacin (50 μM)	7	0.48±0.13*	6	797±336^{ns}
Ethanol (vehicle control)	5	7.45±2.56	8	136±16
Actinomycin D (25 μM)	5	0.30±0.15*	8	3±1*

* $P < 0.05$ in comparison to paired control.

portion of the action of endotoxin on vascular tissue contractile function and IL-1 release.

Dexamethasone can interfere with the synthesis and release of IL-1 at several points. Dexamethasone blocks transcription of IL-1 mRNA and posttranscriptional synthesis of IL-1 in U937 cells (42). Dexamethasone also inhibits prostanoid synthesis via suppression of transcription of mRNA for cyclooxygenase (43). Because products of cyclooxygenase action are necessary for the expression of at least a part of the endotoxin-induced vascular suppression and release of IL-1 activity, interference with cyclooxygenase synthesis by treatment with dexamethasone could simultaneously diminish the influence of endotoxin on vascular contraction and IL-1 release. Indomethacin and dexamethasone may counter endotoxin action via the same mechanism, because endotoxin-induced suppression of aortic reactivity to NE is not inhibited by the agents in an additive fashion (Fig. 5).

The rat aorta releases TNF in response to endotoxin. The source of the TNF is apparently the endothelial cells, because deendothelialized rings do not exhibit enhanced release of TNF in the presence of endotoxin (Table III). Others (44) have shown that vascular smooth muscle cells have the capability of releasing TNF, but only under a superinduction protocol. It is unlikely that TNF directly mediates endotoxin-induced vascular contractile suppression because endotoxin causes suppression in deendothelialized rings despite the absence of TNF (Fig. 3, Table III). In addition, incubation of rings with indomethacin partially ameliorates endotoxin-induced vascular suppression in the absence of any significant decrease in TNF release (Fig. 5, Table III). A possible function for TNF in this vascular system is to synergize with IL-1 to enhance IL-1 action. For instance, IL-1 and TNF synergize to augment the ability of IL-1 to induce additional IL-1 release by vascular endothelial cells (45).

Suppression of vascular contractile function by endotoxin requires *de novo* synthesis of protein. The identity and number of protein entities that are responsible for the vascular suppression are unknown. If endotoxin-induced vascular IL-1 contributes to the suppression, then blockade of synthesis of this cytokine would improve vascular contractile function. We have found, however, that vascular suppression by IL-1 itself requires *de novo* synthesis of protein (5); whether the new protein is simply additional IL-1, because IL-1 induces more IL-1 in vascular smooth muscle and endothelial cells (41, 46), or some other protein(s) is not known. Another protein that is a candidate for both transduction of the suppressive action of endotoxin and for susceptibility to inhibition by actinomycin D is cyclooxygenase. The mRNA for cyclooxygenase is very short-lived, and as a result, inhibition of RNA synthesis by actinomycin D rapidly results in reduced prostaglandin production (47).

Exogenous IL-1 suppresses vascular contractile function, and an association between the suppressive effects of endotoxin and liberation of endogenous IL-1 implies that IL-1 behaves as a vasoactive autacoid in vascular tissue. However, definitive proof of the involvement of IL-1 in endotoxin-induced vascular suppression in the rat aortic ring model must await access to agents such as neutralizing antibodies or antagonists to IL-1 receptors that directly bar IL-1 action on this tissue.

If altered vascular contraction to NE during prolonged exposure to endotoxin is a constitutive property of vascular tissue, then what function could such a trait serve? The answer may lie in the time-dependent interactions of the microvasculature and endotoxin. Initial exposure of vascular beds to endotoxin and catecholamines in the rabbit and rat result in an intense, sustained vasoconstriction (48); others have shown prolonged arteriolar vasoconstriction by rat cremaster muscle after endotoxin infusion (49). Conscious rats infused with *E. coli* bacteria exhibited progressive intestinal microvascular vasoconstriction over 2 h and the authors of that study suggested that the altered intestinal perfusion could contribute to tissue injury during sepsis (50). Altura et al. (51) reported that metarterioles constricted by endotoxin are refractory to vasodilator agents. It is possible that persistent vasoconstriction results in inadequate perfusion of tissue, and that a compensatory mechanism allowing the vasculature to escape from inappropriate vasoconstriction would be beneficial. Zweifach et al. (48) demonstrated that mesoappendix vessels from endotoxin-injected rats altered responses to epinephrine over time, so that tissue that initially exhibited enthusiastic contractile responses to the catecholamine became progressively less responsive until a refractory state was attained. These authors also demonstrated that rats rendered tolerant to endotoxin by repeated daily injections of small doses of endotoxin manifested no enhanced reactivity to epinephrine. If the tolerizing process included induction of the protein synthesis–dependent state described in this study, then the vasculature should indeed be less responsive to catecholamine influence.

During prolonged exposure to endotoxin it may, unfortunately, be possible to overcompensate. A retrospective exami-

nation of records from patients with septic shock indicated that those that died had less capability to augment peripheral vascular resistance than did survivors (2). Numerous studies of animal models for endotoxin and septic shock and clinical observation of patients suggest that diminished systemic vascular resistance, to which diminished catecholamine reactivity would contribute, is deleterious to adequate tissue perfusion and oxygen delivery. Schumacker and Samsel (52) suggested that tissue in which microvascular perfusion defects occur may be particularly susceptible to hypoxic hypoxia. Altered distribution of cardiac output between organs occurs in sepsis and results in compromised blood flow to discrete organs (53). Maldistribution of cardiac output between organs or within tissues, or the action of both distributive disorders in concert, could result in diminished effective perfusion of organs as has been described in an animal model for sepsis (54).

In conclusion, prolonged exposure of rat aortas to low levels of endotoxin results in diminished reactivity to NE and KCl. The action of endotoxin on the aorta is direct, because potential blood-borne mediators are not necessary. Suppression of the rat aorta by endotoxin does not require the presence of an intact endothelium. The efficacy of pharmacologic intervention against endotoxin-induced vascular suppression implies that the action of endotoxin itself is mediated by mechanisms that require *de novo* protein synthesis, and to a lesser extent, by products of cyclooxygenase activity. An association between the endotoxin-stimulated vascular release of IL-1 and the extent of impaired contraction to catecholamines is present, and may reflect a vasomodulatory action for this cytokine.

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Animal Disclaimer

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, Department of Health and Human Services, Publication No. (NIH) 86-23 (1985).

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