

Effect of L-arginine on endothelial injury and hemostasis in rabbit endotoxin shock

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Received 20 March 2000; accepted in final form 12 June 2000

Wiel, Eric, Qian Pu, Delphine Corseaux, Emmanuel Robin, Régis Bordet, Niels Lund, Brigitte Jude, and Benoît Vallet. Effect of L-arginine on endothelial injury and hemostasis in rabbit endotoxin shock. J Appl Physiol 89: 1811–1818, 2000.—To investigate whether impaired endothelial function was related to alteration of nitric oxide (NO) formation during endotoxic shock, we studied the effects of supplementation of L-arginine (L-Arg), d-arginine (D-Arg), and N^G-nitro-L-arginine methyl ester (L-NAME), on endothelial function and structure in a rabbit model. Endotoxic shock was induced by a single lipopolysaccharide bolus (0.5 mg/kg iv, Escherichia coli endotoxin). Coagulation factors and expression of monocyte tissue factor were determined by functional assays. Endothelium-dependent vascular relaxation was assessed by in vitro vascular reactivity. Immunohistochemical staining (CD31) was performed to assess damaged endothelial cell surface of the abdominal aorta. These parameters were studied 5 days after the onset of endotoxic shock and were compared under three conditions: in absence of treatment, with L-Arg or D-Arg supplementation, or with L-NAME. Both L-Arg and D-Arg significantly improved endothelial-dependent relaxation and endothelial morphological injury. L-NAME did not alter endothelial histological injury induced by lipopolysaccharide. These data indicate that arginine supplementation nonspecifically prevents endothelial dysfunction and histological injury in rabbit endotoxic shock. Moreover, L-Arg has no effect on coagulation activation and expression of monocyte tissue factor induced by endotoxic shock.

endothelium; endothotic shock; lipopolysaccharide; N^G-nitro-L-arginine methyl ester; tissue factor; monocyte

ENDOTHELIAL DYSFUNCTION PLAYS a crucial role in the pathophysiology of septic shock due to gram-negative bacteria (8). Alteration in endothelial function, such as impaired release of endothelium-derived nitric oxide (NO), could alter physiological regulation of blood flow distribution by interfering with metabolic vasoconstriction in states of limited O2 supply (25). Indeed, endothelium-derived NO production has been reported to be reduced during endotoxemia (17, 28). This might be associated with the risk of multiple organ dysfunction syndrome (3). In contrast, enhanced inducible NO synthase (iNOS) production has been proposed as a mechanism to explain loss of vascular responsiveness to vasoconstrictor agents in endotoxemia (12, 22). In agreement with this, it has been suggested that inhibition of NO synthase is beneficial to restoring contractility (18, 23), and the use of NO synthase inhibitors has been proposed as a treatment. However, the inhibition of NO synthase was demonstrated to be detrimental during endotoxemia or sepsis (20, 21, 27). To block or to enhance NO production during sepsis or septic shock remains, therefore, a matter of controversy.

Our laboratory previously investigated endothelial function in a rabbit endotoxic shock model (14). In that study, it was reported that impaired endothelium-dependent relaxation of aorta in response to acetylcholine (ACh) was still present and almost maximal 5 days after lipopolysaccharide (LPS) injection. This endothelial dysfunction was associated with endothelial histological injury. Our results, along with those of others (13, 17, 19), suggest that an alteration in ACh receptor-NO synthase coupling and/or a reduced production of endothelium-derived NO causes this attenuated endothelium-mediated vasoconstriction. A similar type of endothelial dysfunction has been described in hypercholesterolemia and in angioplasty-associated vascular injury in rabbits (6, 11). Altered NO-mediated relaxation was prevented by administration of L-arginine (L-Arg), the precursor of NO synthase (6, 11). Interestingly, L-Arg has been shown to improve survival in septic animals (10, 15).

To explore further whether impaired endothelium-dependent relaxation of the aorta in a rabbit endotoxic shock model was related to alteration of NO formation, we examined the effect of L-Arg, its stereoisomer D-arginine (D-Arg), and of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase, on endothelial function and structure in a rabbit endothotoxemic shock model.
 MATERIALS AND METHODS

The animal experiments were approved by the French Agricultural Office for the care of animal subjects, and the care and handling of the animals were in agreement with the European legislation for animal research.

Animal Model

Experiments were carried out on male New Zealand White rabbits weighing 2.5–3.0 kg. All rabbits were obtained from the Charles River laboratory (St. Aubin-lès-Elbeuf, France). Animals were maintained throughout on a standard rabbit chow diet with food (200 g/day) containing 1.25% of arginine and water ad libitum. Rabbits were assigned to one of seven groups. For the endotoxin animals (4 groups), conscious animals were rapidly injected intravenously via a marginal ear vein with 0.5 mg/kg body wt of purified LPS endotoxin (Escherichia coli serotype O55:B5 from a single batch, Sigma Chemical, St. Louis, MO).

One group received LPS alone (LPS control group, n = 8), and the other three groups received LPS and were supplemented with either L-Arg (LPS+L-Arg group, n = 8), D-Arg (LPS+D-Arg group, n = 5), or L-NAME (LPS+L-NAME group, n = 6). For the sham animals (3 groups), animals were injected intravenously with saline and supplemented with either L-Arg (L-Arg group, n = 6) or L-NAME (L-NAME group, n = 6) or were not supplemented (control group, n = 8). L-Arg or D-Arg (1,500 mg•kg⁻¹•day⁻¹) or L-NAME (15 mg•kg⁻¹•day⁻¹) supplementation in the drinking water was initiated 3 days before the onset of endotoxic shock and was maintained for 5 days afterward. Before the initiation of supplementation, 3 days after supplementation, and 5 days after LPS injection, blood samples were obtained from animals in each group for measurement of plasma arginine levels. Plasma was deproteinated with 10% sulfosalicylic acid and analyzed for free arginine with an automated amino acid analyzer (model LC 300, Biotronic Instruments, Berlin, Germany).

Four hours after LPS or saline injection, arterial blood gases were measured in the seven groups. Coagulation and hematological parameters were measured in all groups 5 days after LPS or saline injection.

All animals were killed on day 5 (D5). They were anesthetized with pentobarbital sodium (30 mg/kg iv; Specia, Paris, France) and injected with heparin (500 IU/kg iv; Panpharma, Fougeres, France) to prevent coagulation. Abdominal aortas were quickly removed, placed into cold (4°C) Krebs-Henseleit (KH) buffer (pH 7.40 at 37°C), and gassed with 5% CO₂ in O₂. Abdominal aortic rings were incubated at 37°C overnight with a mouse-prepared monoclonal primary antibody to CD31 (Dako, Carpinteria, CA) diluted 1:20 in PBS. After extensive washing in PBS (3 × 10 min), an anti-mouse biotinylated secondary antibody was applied for 1 h. After three washings with PBS, the sections were incubated for 1 h in a solution of avidin-biotin-peroxidase complex (ABC Vector, Vectastain kit, Vector Laboratories, Burlingame, CA). The peroxidase activity was revealed by using hydrogen peroxide and diaminobenzidine as a chromagen. Finally, sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Elancourt, France). In each experiment, negative controls without the primary antibody were included to check for nonspecific staining.

Vascular Responses

Each ring was mounted on two stainless steel wire supports inserted through the lumen. To determine the possible existence of iNOS, vascular endothelium of one ring of each aorta was previously mechanically removed with a small wooden applicator. Rings were suspended in organ chambers (Radnoti Glass Technology, Monrovia, CA) filled with 40 ml KH solution, warmed (37°C), and continuously oxygenated (95% O₂:5% CO₂). One support was fixed, and the other was connected to an isometric force-displacement transducer. Incoming signals from the transducers were amplified by signal conditioners and sent to an Intel 486-based computer, after analog-to-digital conversion. This system allowed the continuous screen visualization and computer storage of changes in isometric tension.

Over a 60-min “step-tension” period, the vascular rings were stretched to a force of 8.0 g. In preliminary experiments, 8.0 g was determined to be the optimal point of ring length-tension relation. After a stabilization period of 60 min at the resting wall tension, all rings were preconstricted with 70 mM KCl to ensure their viability. The rings were contracted with 1-phenylephrine hydrochloride (PE; concentration range from 10⁻⁹ to 10⁻⁵ M; Sigma Chemical). Endothelium-dependent or -independent relaxant effects were established when the constrictor response to PE was stable. The relaxant response to ACh (concentration range from 10⁻⁹ to 3.10⁻⁵ M; Sigma) and to calcium ionophore A23187 (concentration range from 10⁻⁹ to 3.10⁻⁶ M; Sigma Chemical) was also measured. Similarly, the response to sodium nitroprusside (SNP, concentration range from 10⁻⁹ to 3.10⁻⁵ M; Sigma Chemical) was obtained. The same range of SNP concentrations was also applied to rings from which the vascular endothelium was removed.

Removal of the endothelium was verified by the failure of these rings to relax in response to submaximal concentrations of ACh (3.10⁻⁷ M). A final series of experiments was performed in L-NAME-treated rabbits that either received or did not receive endotoxin. Rings were incubated with L-Arg (10⁻² M) 30 min before the protocol of ACh-induced relaxation to avoid remnant effects of L-NAME and to separate LPS-induced endothelial dysfunction and L-NAME-induced NO synthase blockade.

Immunohistochemical Staining of Vascular Endothelium

Aorta segments were fixed with paraformaldehyde (4%), then cryoprotected by immersion in sucrose 30%. Tissues were embedded in optimal cutting temperature (methyl methacrylate) compound, frozen in isopentane, and stored at −70°C. Tissue sections were cut 6 μm thick. The endothelial cell layer was stained by using an antibody against the endothelium-specific intercellular adhesion molecule CD31-PECAM1. Briefly, frozen sections were air-dried for 1 h, incubated with peroxidase-blocking reagent, then rinsed in phosphate-buffered saline (PBS) for 10 min and blocked with 10% horse serum in PBS for 10 min. The sections were then incubated at 37°C overnight with a mouse-prepared monoclonal primary antibody to CD31 (Dako, Carpinteria, CA) diluted 1:20 in PBS. After extensive washing in PBS (3 × 10 min), an anti-mouse biotinylated secondary antibody was applied for 1 h. After three washings with PBS, the sections were incubated for 1 h in a solution of avidin-biotin-peroxidase complex (ABC Vector, Vectastain kit, Vector Laboratories, Burlingame, CA). The peroxidase activity was revealed by using hydrogen peroxide and diaminobenzidine as a chromagen. Finally, sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Elancourt, France). In each experiment, negative controls without the primary antibody were included to check for nonspecific staining.

For quantification of endothelial injury, three consecutive cross sections per aortic segment were microscopically photomicrographed (model Axioskop 20, Zeiss, Le Pecq, France). After photographic reconstruction of each tissue section, each picture was digitalized for computerized analysis (Color Image 1.32 Software). The surface area of endothelial cell injury (including three types: subendothelial vacuolization, detachment of endothelial cells, and endothelial
denudation) was measured and expressed as percentage of total circumference of each section.

Coagulation

Blood was sampled at day 1 (D1) and at D5 under sterile conditions from the ear artery. Samples collected on EDTA were used for blood cell counts (Coulter MAXM, Beckman, Fullerton, CA). The total white blood cell count was verified manually. Peripheral blood smears for differential white cell counts were stained with May Grünwald Giemsa. Each count was performed by three investigators, who were blinded to the treatment allocation. Factor II and V levels were determined in RPMI 1640 (GIBCO). Aliquots of cell preparations made by a method adapted from Carson (5, 7). After gradient centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO). Aliquots of cell preparations centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO). Aliquots of cell preparations centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO). Aliquots of cell preparations centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO). Aliquots of cell preparations centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO).

Measurement of Monocyte Tissue Factor Procoagulant Activity

The possible activation of monocyte tissue factor (TF) procoagulant activity after a single endotoxin injection was investigated in the LPS group, the LPS + L-Arg group, and the control group, respectively. Determination of TF activity was made by a method adapted from Carson (5, 7). After gradient centrifugation isolation, the mononuclear cells were resuspended in RPMI 1640 (GIBCO). Aliquots of cell preparations were cultured for 16 h at 37°C in a humidified 5% CO2 atmosphere, without or with stimulation by 1 ng/ml of endotoxin (LPS; 055:B5; Sigma Chemical). (Laboratoire de Fractionnement et des Biotechnologies, Les Ulis, France) as a source of factor VII (Biogénie, Maurin, France), the change in optical density at 405 nm was quantified with a microplate reader and converted to units of TF activity from log-log plots of serial dilutions of a rabbit brain thromboplastin (Cl-, Stago). Fibrinogen levels were measured by the Clauss technique (Biomérieux, Lyon, France).

Statistical Analysis

Results are expressed as means ± SE; n represents the number of rabbits. Hematology and coagulation results were compared by using unpaired Student’s t-test. Plasma arginine levels were compared via Wilcoxon’s test for paired groups and Mann-Whitney’s U-test for unpaired groups. Determination of the PE concentration eliciting 50% of the maximal constriction response (EC50) was calculated using a semilogistic regression. EC50 values were compared using the Mann-Whitney test. Relaxation responses were expressed as percent reduction of maximal vascular PE-induced tension. Mean intergroup differences were tested by repeated-measures ANOVA. If a significant value was found, Scheffé’s test for multiple comparisons was used. Significance was accepted at P < 0.05.

RESULTS

Clinical Parameters

There was a significant body weight loss of ~5–9% in the endotoxic animals compared with the control group at D1 and D5, respectively. The overall mortality for the nonsupplemented septic group was 40%, whereas it was 21.4% in the LPS + L-Arg group (P < 0.05 vs. LPS group), 20% in the LPS + L-NAME group (P < 0.05 vs. LPS group), and 0% in the LPS + D-Arg group (P < 0.05 vs. LPS group), respectively. Five-day survivors were considered permanent survivors because all animals were killed at 5 days.

Ex Vivo Measurements

Arterial blood gas analysis. Arterial blood-gas analysis was performed 4 h after LPS injection. Metabolic acidosis confirmed endotoxic shock (bicarbonate = 11.40 ± 0.92 in LPS group vs. 23.90 ± 0.81 in control group, P < 0.05). Acidosis was not significantly different between the endotoxic animals with or without treatment (bicarbonate = 11.33 ± 0.63 in LPS + L-Arg group, 8.33 ± 0.39 in LPS + D-Arg group, 12.30 ± 3.29 in LPS + L-NAME group, not significant vs. LPS group).

Plasma arginine levels and hematological modifications. L-Arg and D-Arg supplementation increased plasma arginine levels 3 days (D0) after initiation of supplementation and remained elevated after 8 days (D5) in the L-Arg group, the LPS + L-Arg group, and the LPS + D-Arg group compared with at baseline. In contrast, 5 days after LPS injection, plasma arginine levels decreased significantly in the nonsupplemented LPS group but not significantly in the supplemented LPS group (Fig. 1).

![Fig. 1. Plasma arginine concentration. Baseline, before l-arginine (L-Arg) or d-arginine (D-Arg) supplementation; D0, 3 days after the initiation of L-Arg or D-Arg supplementation and before LPS injection; D5, 8 days after the initiation of L-Arg or D-Arg supplementation and 5 days after LPS injection; L-Arg, animals that received L-Arg alone; LPS, animals that received lipopolysaccharide (LPS) alone; LPS + L-arg, animals that received LPS and L-Arg; LPS + D-Arg, animals that received LPS and D-Arg. *P < 0.05 vs. Baseline.](http://jap.physiology.org/Downloadedfrom)
One day after LPS injection, an inflammatory syndrome was noticed, with significant increase in circulating polymonuclear neutrophils (8.7 ± 2.3 vs. 2.4 ± 0.3 \(10^7/\text{mm}^3\) at baseline, \(P < 0.05\)) and fibrinogen levels (8.10 ± 0.40 vs. 3.61 ± 0.20 g/l at baseline, \(P < 0.05\)). In parallel, a significant decrease in platelet count (224 ± 27 vs. 420 ± 25 \(10^9/\text{mm}^3\) at baseline, \(P < 0.05\)), coagulation factors II and V (factor II = 76 ± 3% and factor V = 54 ± 7% vs. factor II = 97 ± 6% and factor V = 132 ± 13%, respectively, at baseline, \(P < 0.05\)) and prothrombin index (84 ± 3% vs. 98 ± 3% at baseline, \(P < 0.05\)) was observed. Five days after LPS injection, the polymonuclear count returned to baseline levels, but the fibrinogen level remained elevated. Platelet count was corrected as well as the level of coagulation factors II and V. Prothrombin index became normal 5 days after LPS injection. These changes were not different between endotoxic shock animals with or without L-Arg, D-Arg, or L-NAME.

Five days after LPS injection, an increase in spontaneous monocyte TF expression (101 ± 18 vs. 32 ± 14 mU/10^3 mononuclear cells in the control group, \(P < 0.05\)) and after in vitro stimulation (188 ± 26 vs. 55 ± 17 mU/10^3 mononuclear cells in the control group, \(P < 0.05\)) was observed. L-Arg treatment did not alter this increased monocyte TF expression in endotoxic animals without (80 ± 14 mU/10^3 mononuclear cells) or with stimulation (175 ± 30 mU/10^3 mononuclear cells).

**Immunohistochemical staining.** In the control group, endothelial cells were stained by immunohistochemical label (PECAM1/CD31) and appeared intact. LPS injection induced three types of endothelial cell injury at D5: subendothelial vacuolization, detachment of endothelial cells, and endothelial denudation. In endotoxic rabbits, the surface area of endothelial cell injury was 23.3 ± 0.8% of the total endothelial surface area of the abdominal aorta (Fig. 2). Both L-Arg and D-Arg supplementation decreased LPS-induced endothelial cell injury (7.0 ± 0.8% and 10.1 ± 1.5%, respectively, \(P < 0.05\) vs. LPS group), whereas L-NAME did not alter LPS-induced endothelial cell injury (22.1 ± 1.9%).

**In Vitro Vasoreactivity**

Vascular contraction. In control animals, the response to PE was larger in endothelium-denuded rings than in intact rings; the PE EC_{50} was significantly increased in the presence of the endothelium. The same results were observed in the L-Arg, the LPS+L-Arg, and the LPS+D-Arg groups, respectively. In contrast, there was no longer any endothelium-dependent contraction modulation in the LPS, the L-NAME, and the LPS+L-NAME groups, respectively, because EC_{50} was similar in aortic rings in the presence and absence of endothelium (Fig. 3A).

In addition, sensitivity to PE was significantly modified after endotoxin injection (Fig. 3B). Five days after LPS injection, aortic rings without endothelium elicited a greater PE EC_{50} compared with control rings. This difference in sensitivity was abolished in the presence in vitro of L-NAME. No differences were observed on endothelium-denuded rings between LPS and LPS+L-Arg or LPS+D-Arg groups, respectively.

**Endothelium-dependent and endothelium-independent relaxation.** The in vitro relaxation response of aortic rings was tested after PE precontraction with ACh, A23187, and SNP. In the control group, the maximum relaxation induced by ACh was 80.6 ± 3.1%. The response to calcium ionophore A23187 was similar (79.0 ± 2.5%). SNP, an endothelium-independent relaxing agent, caused a relaxation response of 92.9 ± 2.4%. In contrast, LPS injection was associated with a loss of ACh-induced relaxation. At D5, ACh-induced relaxation was decreased (\(P < 0.05\) vs. control group), with a maximum relaxation of 50.0 ± 6.1%. Both L-Arg and D-Arg prevented this altered ACh-induced relaxation (\(P < 0.05\) vs. LPS group) (Fig. 4A). L-NAME did not aggravate LPS-altered ACh-induced relaxation. Altered ACh-induced relaxation in the L-NAME group was completely reversed by an excess of L-Arg but was only partially reversed in the LPS+L-NAME group (Fig. 5A).

LPS injection did not modify the relaxation response to A23187 (Fig. 4B). L-NAME alone significantly impaired A23187 relaxation both in the LPS and the LPS+L-NAME groups, respectively. This effect was significantly reversed by an excess of L-Arg (Fig. 5B). SNP relaxation was not significantly different between the groups (Figs. 4C and 5C).

**DISCUSSION**

In the present study, we confirmed that a single injection of endotoxin (\(E.\) coli, 0.5 mg/kg) was rapidly associated with metabolic acidosis and with prolonged activation of monocyte TF expression and endothelial cell injury and dysfunction (14). Decreased endothelium-dependent relaxation in response to ACh was still present 5 days after LPS injection, whereas SNP-induced endothelium-independent relaxation was not altered. These results suggest that the impaired endothelium-dependent relaxation is not due to a decreased ability of vascular smooth muscle to respond to NO. Our study data also demonstrate that L-Arg supplementation was able, as in other types of endothelial injury (6, 11), to prevent this altered endothelium-dependent relaxation to ACh. This improved function was associated with restoration of endothelial mor-
However, this effect of \( \text{L-Arg} \) is nonspecific because \( \text{D-Arg} \) gave, in this model, the same results on endothelium function and morphology.

These data suggest that the NO pathway is not centrally involved in the functional and structural improvements observed with \( \text{L-Arg} \) in large conduit vessels from endotoxic shock rabbits. These results are not in agreement with studies in which hypercholesterolemia or balloon vascular injury in rabbits was associated with impaired endothelium-dependent relaxation and improved by supplemented \( \text{L-Arg} \) diet (6, 11). It is important to emphasize, however, that neither Cooke et al. (6) nor Hamon et al. (11) used \( \text{D-Arg} \) as a control treatment.

Endothelial dysfunction is a common feature of sepsis and septic shock induced by endotoxin-containing gram-negative bacteria, and it may be induced by a single injection of bacterial endotoxin used to induce septic shock in animals (26). Impairment of endothelial function and structure was restored by both \( \text{L-Arg} \) and \( \text{D-Arg} \) supplementation. Therefore, an effect of \( \text{L-Arg} \) on the endothelial synthesis and release of NO through \( \text{L-Arg} \) availability and/or metabolism is unlikely.

In the present study, \( \text{L-Arg} \) supplementation did not prevent activation of coagulation and induction of monocyte TF expression. This result is different from that of a recent study in which \( \text{L-Arg} \) supplementation inhibited increased monocyte TF expression observed in the hypercholesterolemic rabbit after angioplasty (7). This may be due to differences in the type of vascular injury. It must be underlined also that, in this previous study, \( \text{D-Arg} \) was not used as control.

Our results also showed that plasma arginine levels were decreased in endotoxic animals. \( \text{L-Arg} \) or \( \text{D-Arg} \) supplementation increased plasma arginine levels 3 days after initiation of treatment. Arginine levels remained elevated for days after the onset of endotoxic shock, while supplementation in drinking water was maintained. The decrease of plasma arginine levels after endotoxic shock induction may be explained by an increased utilization of arginine for NO synthesis. This, however, does not seem to be the case because the results were identical whether \( \text{L-Arg} \) or \( \text{D-Arg} \) was used for supplementation. Another proposed explanation for decreased arginine levels in septic animals may be impaired intestinal arginine absorption. Madden et al. (16) demonstrated a 400% increase in plasma arginine level 30 min after gavage with 100 mg arginine in nonseptic animals, whereas this increase was not found in septic animals. Gardiner et al. (9) investigated intestinal arginine absorption in two different models of sepsis (cecal ligation and puncture, or intraperitoneal injection of LPS). They concluded that, in both models, sepsis resulted in impaired intestinal amino acid uptake. In our model, this mechanism may play a role and explain the decrease in arginine level observed at D5 in supplemented animals. This effect is, again, nonspecific because the results are comparable whether \( \text{L-Arg} \) or \( \text{D-Arg} \) was given for supplementation.
L-Arg restored the relaxation response to ACh after LPS injection without affecting endothelium-dependent relaxation in control animals. Because the same effect was observed with D-Arg supplementation, we propose that a nonspecific effect of L-Arg leads to improved endothelial function. Consistent with a non-NO-dependent mechanism, A23187 induced similar endothelium-dependent relaxation in both control and endotoxic rabbit aortic rings. The calcium ionophore A23187 directly stimulates conversion of L-Arg to NO in contrast to ACh, which requires muscarinic receptors to allow calcium entrance and calmodulin activation. Aoki et al. (2) and Kessler et al. (13) demonstrated that endotoxin or cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), were able to impair the receptor-mediated release of NO in cat or rabbit carotid arteries. Cycloheximide, an inhibitor of protein synthesis, blocked this TNF-α-induced impaired relaxation, suggesting that TNF-α or IL-1β induces synthesis of one or several key messenger proteins interfering with the ACh-receptor-NO synthase pathway (2, 13). Whether L-Arg or D-Arg can interfere with this protein synthesis was not clarified by our study.

It has also been proposed that cytokines induce generation of iNOS in the vascular wall, thus affecting the signal transduction cascade in endothelial cells by inhibiting receptor mechanisms (13). Because iNOS is L-Arg blood concentration dependent for NO synthesis, increased extracellular L-Arg should have resulted in iNOS-derived overproduction of NO (23) and increased endothelium-dependent relaxation dysfunction (13). We can speculate that, in our model, this is unlikely because L-Arg did not cause major endothelial dysfunction.

![Fig. 4. Dose-response relationship of endothelium-dependent to acetylcholine-induced (A) and to A23187-induced (B) relaxation and endothelium-independent relaxation to sodium nitroprusside (C) in isolated aortic rings. *P < 0.05 vs. control animals; NS, no significant difference.](image)

![Fig. 5. Dose-response relationship of endothelium-dependent to acetylcholine-induced (A) and to A23187-induced (B) relaxation and endothelium-independent relaxation to sodium nitroprusside (C) in isolated aortic rings. *P < 0.05 vs. Control animals.](image)
Our data show that ACh-mediated relaxation is inhibited by in vivo pretreatment with L-NAME in endotoxic, as well as control, rabbits. This effect of L-NAME is completely reversible by incubation of rings from nonendotoxic rabbits with in vitro excess L-Arg, demonstrating remnant effects of L-NAME in ex vivo rings from these animals. In contrast, L-Arg only partially reversed L-NAME blockade in rings from the endotoxic rabbits. It is important to emphasize, however, that in vivo L-NAME pretreatment did not aggravate either endothelial denudation or ACh-induced altered relaxation. This suggests, again, that altered NO production by NO synthase in LPS-induced septic shock is not responsible, by itself, for endothelial dysfunction and morphological injury.

In the present study, the sensitivity to PE was decreased in aortic rings after LPS injection. In various animal models, induction of iNOS after an endotoxin challenge is usually observed within 3–12 h (12, 22). In a rabbit model (14), our laboratory previously demonstrated impaired smooth muscle contractility in response to PE 24 h after 0.5 mg/kg LPS injection. Contractility to PE was restored in the presence of L-NAME, suggesting the presence of iNOS in smooth muscle cells. This effect was still present, although less consistent, 5 days after LPS injection. Whether endothelial cell dysfunction triggers iNOS expression, or the reverse, remains controversial (1, 13). Adeagbo and Triggle (1) demonstrated that removal or damage of endothelial cells triggers the induction of iNOS in vascular smooth muscle cells of rat aorta. However, Kessler et al. (13) showed that decreased endothelial NO synthase activity due to cytokine-induced sustained generation of iNOS affects the signal transduction cascade in endothelial cells. Our results show that supplying L-Arg or D-Arg restores the sensitivity of smooth muscle cells to PE. This might be related to restoration of endothelial structure and function, which might allow limited iNOS expression.

Actually, our study demonstrates that recovery of endothelial function is associated with morphological restoration. This preservation of endothelial cell structure is obtained by L-Arg as well as D-Arg. It is well known that arginine increases protein synthesis in sepsis. León et al. (15) showed that arginine supplementation improved fibrinogen and acute-phase protein synthesis during sepsis in the rat. This may be an explanation for the protective effect of L-Arg on endothelial morphology in our model. However, we did not notice a significant increase in fibrinogen synthesis in our supplemented animals.

We observed endothelial cell abnormalities, even in surviving animals at D5 after LPS injection, suggesting that mortality was not directly related to endothelial dysfunction in endotoxic shock animals. We also observed that L-Arg or D-Arg supplementation decreased the mortality rate of endotoxic animals. This is consistent with a study by Madden et al. (16). They showed that arginine supplementation, given before sepsis induction, significantly increased animal survival. The mechanism by which arginine improves survival in our model remains unknown. Arginine has been shown to have multiple beneficial effects on T cell-mediated immunity (4) and protein synthesis in sepsis (15). On the other hand, it has been suggested that administration of NO synthase inhibitor increases (18, 24) or decreases (19, 21, 27) survival in animal sepsis. Mortality and tissue damage were well linked in these previous reports (19, 21, 27). Our present study showed that administration of L-NAME aggravated neither mortality nor endothelial morphologic injury in endotoxic rabbits.

It should be noted that the present study focused on the mechanisms involved in vascular endothelial cell dysfunction during endotoxic shock. This study was limited to arteries that are not involved in the microcirculatory regulation of blood flow. Results might be different at the microvascular level. The severity of septic shock might have been lower in surviving animals, and the absence of resuscitation may have modified our results.

In summary, our results indicate that, in endotoxic shock rabbits, 1) L-Arg or D-Arg supplementation prevents endothelial dysfunction by restoration of endothelial histological injury, which probably improves coupling between endothelial ACh receptor and NO synthase in a nonspecific fashion (i.e., in a manner independent of NO synthase substrate availability); 2) L-Arg or D-Arg supplementation decreases mortality; 3) L-Arg has no effect on coagulation activation and expression of monocyte TF; and 4) L-NAME does not aggravate endothelial histological injury and mortality rate.

REFERENCES
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