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Research Article

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Activated Macrophages Depress the Contractility of Rabbit Carotids via an L-Arginine/Nitric Oxide–dependent Effector Mechanism

Connection with Amplified Cytokine Release

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Abstract

Inflammatory mediators released by macrophages (MΦ) are believed to be involved in septic vasoplegia. To investigate the effect of MΦ on vascular reactivity, excised rabbit carotids were exposed intraluminally either to peritoneal rabbit MΦ, activated by 18 h of incubation with 1 μg/ml lipopolysaccharide, or to the supernatants (SPN) derived from them. The contractile responses to phenylephrine (PE, 10^{-6} M) were determined by measuring changes in diameter using an ultrasonic microdimensiometer 1, 2, and 3 h after the first control contraction. In control arteries (n = 12), PE-induced contractions were, respectively, 102.9±3.3%, 95.2±4.1%, and 89.7±3.8% of the first contraction, after 1, 2, and 3 h. Activated MΦ significantly reduced PE-stimulated contractions after as little as 1 h of carotid exposure (percentage of controls at 1, 2, or 3 h: 74.1±5.6, 57.2±5.2, and 34.2±5.6, n = 10, $P < 0.001$). The activated macrophage-derived SPN took longer to diminish carotid contractility than the MΦ themselves, and became significant only after 2 h. The greater effect of MΦ might be due to cooperation between MΦ and vascular cells, as suggested by the amplified interleukin-1 release observed after MΦ infusion. The presence of the endothelium partially protected carotid contractility from depression by activated MΦ. Extraluminal addition of N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthesis prevented this depression in arteries with or without endothelium. No products of the oxidative pathway of L-arginine were detected in rabbit activated MΦ. These results suggest that activation of this pathway in smooth muscle cells seems to be involved in vascular hypocontractility. (*J. Clin. Invest.* 1992. 89:851–860.) Key words: anion superoxide • cytokines • endothelium • lipopolysaccharide • nitric oxide

Introduction

Human septic shock is characterized by cardiocirculatory insufficiency in which vascular hyporesponsiveness is a major determinant of mortality (1). It has been shown that the lethal effect of bacterial lipopolysaccharide (LPS) originates from hematopoietic-derived cells and probably from macrophages (2). Macrophages play an essential role in host defense against infection, via vascular phagocytic clearance function and via many different secretory capacities when stimulated by LPS (3). Some of their products, including cytokines often called monokines such as tumor necrosis factor (TNF)[1] and interleukin 1 (IL-1), have been extensively studied and appear to induce the systemic acute phase responses of severe sepsis. Furthermore, TNF may be the major determinant of septic shock, not only through its direct toxicity but also as a proximal mediator capable of inducing a cascade of other humoral factors originating from both macrophages and vascular cells (4–6).

Our original hypotheses were that many products of the pluripotent macrophage contribute to the vascular defect occurring during sepsis, and that interactions between macrophages and vascular cells participate in this process. To address these questions, we undertook the present study using an in vitro model of excised rabbit carotid artery exposed to LPS-activated macrophages and contracted to phenylephrine. We found that LPS-activated macrophages depress carotid contractility, and we explored the role played by reactive oxygen and nitrogen intermediates in this depression as well as its connection with monokine release.

Methods

Artery preparation. Male New Zealand white rabbits (2–2.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.), tracheotomized, and artificially ventilated. After medium cervicotomy, both the right and left common carotid arteries were carefully dissected free, and the collateral arteries were ligated. An 18-gauge cannula was inserted retrogradely into the distal end of each 3-cm carotid segment and connected to a reservoir placed ~ 80 cm above the animal. The proximal end of each artery was ligated and a second cannula pointing distally was inserted. Using this procedure, pressure was continuously maintained within the vessel. The two cannulas were clamped into an adjustable rig that maintained the artery at its in vivo length and prevented its shortening upon excision. Afterwards, the carotids were immersed in a bath containing 10 ml of Krebs-Ringer solution (95% O_2, 5% CO_2, pH 7.4, 39°C). A 30-cm-long glass tube (0.5 cm in diameter) was filled with Krebs-Ringer solution containing 4% albumin and 0.03% Evans blue dye and connected to a manometer pressurized to 90 mm Hg and to the still-pressurized artery via a three-way stop cock. Once the stop cock was opened, the luminal pressure of the carotid equilibrated with that of the manometer. The artery was perfused discontinuously every 15 min. To change the intraluminal solution while keeping the artery pressurized, the stop cock was closed toward the artery and opened towards the glass tube. The solution to be tested was introduced into the glass tube, the connection was reestablished between the artery and the glass tube, and the carotid was then perfused.

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1. *Abbreviations used in this paper:* EDRF, endothelium-derived relaxing factor; LNMMA, N^G-monomethyl-L-arginine; PE, phenylephrine; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TNF, tumor necrosis factor.

The excision procedure preserved the endothelium intact, as assessed functionally by acetylcholine-induced relaxation of precontracted arteries, and structurally by the absence of blue staining of the luminal surface at the end of the experiment. The presence of Evans blue dye in the intraluminal solution did not affect the acetylcholine-induced relaxation. In addition, one set of carotids was fixed with glutaraldehyde and stained on the luminal surface with 0.05% silver nitrate, to verify the presence of a continuous layer of endothelial cells.

In another series of experiments, the endothelium was mechanically removed from the arterial segments by gentle luminal insertion and removal of an inflated 2 F-Fogarty catheter. This eliminated > 90% of the endothelium, as assessed by the absence of dilation when precontracted arteries were exposed to acetylcholine, and by the absence of silver nitrate staining of the luminal surface.

Macrophage preparation. Elicited peritoneal macrophages were obtained by intraperitoneal injection of 40 ml of sterile mineral oil and aseptic lavage of the peritoneum with 2% fetal bovine serum in RPMI medium 3 d later. Cells were then harvested and centrifuged twice for 10 min at 1,000 *g* and 4°C, and erythrocytes were lysed under hypotonic conditions. The remaining cells were resuspended in serum-free RPMI medium at a density of 5×10^6 cells/ml, plated in 75-cm^2 sterile flasks (15 ml), and incubated for 90 min at 37°C, 5% CO_2. Nonadherent cells were removed by vigorous washing and the flasks were replenished with serum-free RPMI medium. The resulting cell population included > 90% macrophages when examined by phase-contrast microscopy for cytomorphological characteristics and Wright staining. More than 95% of these macrophages were viable, as assessed by Trypan blue exclusion.

Macrophages were obtained by incubation for 18 h in 5% CO_2 at 37°C in a sterile atmosphere with 1 μg/ml LPS (LPS-activated macrophages), or without LPS (elicited macrophages). The cells were then separated from their supernatants, resuspended in serum-free RPMI medium with LPS (1 μg/ml) or without LPS, and infused intraluminally into the carotids at a concentration of 2×10^6 cells/ml. The supernatants from 18 h of incubation were kept at −40°C until infusion into the artery or measurement of IL-1 activity and nitrites.

In one series of experiments, paraformaldehyde-fixed macrophages were used to test membrane-bound cytokine activity. For this purpose, macrophages were activated by 18 h of incubation with LPS and fixed with 1% paraformaldehyde for 15 min at 20°C, washed extensively three times with RPMI medium, resuspended with 0.1 M ethanolamine for 15 min, washed again, and incubated at 4°C before use.

Since LPS tolerance might develop in activated macrophages, two series of experiments were conducted to assess the regulation of cytokine release: (a) Macrophages were incubated for 18 h in the presence of LPS, washed, and challenged by reincubation in RPMI medium with LPS for another 3 h. The challenge dose of LPS was the same as in the primary treatment (1 μg/ml). The supernatant resulting from the 3-h challenge with LPS was removed and assayed for IL-1. (*b*) LPS-activated macrophages were washed and resuspended in fresh LPS-RPMI medium, infused into the carotid lumen, and left there for 3 h. The carotid was then clamped at both ends and cut off from the cannulas. The intraluminal solution (80–100 μl) was then harvested by purging the artery, and the cell-free supernatant obtained by centrifugation was processed for IL-1 assay.

Vascular contractility assessment. After 1 h of equilibration, contraction of the carotid arteries was induced by adding 10^{-6} M phenylephrine (PE) to the external bath. Intraluminal pressure was maintained at 90 mm Hg, and the changes in arterial diameter were measured by continuous recording with an ultrasonic microdimensiometer (Application Electronique Mécanique, Montreuil, France). Mean carotid diameter was 2.19±0.03 mm (n = 52) before contraction, and it decreased by 41.5±1.2% (n = 52) after the first PE test. The first contractile response served as control. After removal of PE by washing out the external solution and complete relaxation of the carotids, the intraluminal solution was changed to an RPMI solution containing either LPS-activated macrophages or their supernatants, supplemented with 4% albumin. Carotids were perfused discontinuously every 15 min with this solution and the extraluminal solution (oxygenated Krebs-Ringer solution) was changed every 30 min. Contractile tests were repeated every hour for 3 h. Contractile responses were expressed as percentages of the first control contraction.

Role of free oxygen radicals. A series of experiments was carried out to assess the role of free radicals in vascular contractility. 30 min before the control contractile test with PE, the intraluminal solution was supplemented with radical scavengers: 100 or 300 U/ml superoxide dismutase (SOD) and/or 2,000 U/ml catalase. Radical scavengers were added to the LPS-activated macrophage intraluminal solution, as well. The experiments using radical scavengers were conducted both in intact and deendothelialized arteries.

Exploration of the oxidative pathway of L-arginine. L-Arginine is the precursor molecule for an oxidative pathway synthesizing inorganic nitrogen oxides and L-citrulline. Nitric oxide (NO) is the effector molecule of this pathway and decomposes rapidly in aerated solutions to form stable nitrite/nitrate products. Nitrite concentrations were determined and used as an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with the Griess reagent (7). Samples (0.2 ml) were mixed with 0.1 ml of 1% sulfanilamide-2.5% phosphoric acid and 0.1 ml of 0.5% naphtylethylenediamine dihydrochloride-2.5% phosphoric acid, and absorbance was read at 543 nm. Concentrations were determined from a linear standard curve between 2 and 100 μM sodium nitrite. The lower threshold of nitrite detection in this assay is ≥ 5 μM. Supernatants derived from LPS-activated macrophages, lysates of these macrophages obtained by hypotonic lysis, and supernatants derived from the activated macrophages exposed for 3 h to the carotids, were assayed.

As this method is relatively insensitive, we also explored the oxidative pathway of L-arginine by analysis of amino acid metabolites as described by Granger et al. (8). Briefly, 2,3,4,5-^{3}H-labeled L-arginine (Amersham, Bucks, England) and its metabolites citrulline and ornithine were separated by a reversed-phase ion-pairing HPLC on a 4.6 × 200-mm C_{18} mino-RPC column (Pharmacia, Uppsala, Sweden), using 1% methanol/99% aqueous sodium acetate (20 mM, pH 4.1)-sodium hexanesulfonate (10 mM) as isocratic mobile phase, and run at 1 ml/min. This labeling of L-arginine permitted detection of two radioactive metabolites, L-citrulline and L-ornithine, respectively produced by the oxidative pathway and arginase activity. The culture medium samples used for this analysis were supernatants derived from LPS-activated macrophages which had been incubated for 18 h in the absence or presence of 300 μM N^G-monomethyl-L-arginine (LNMMA).

The involvement of the L-arginine/nitric oxide (NO) pathway was evaluated by using LNMMA, a known competitive inhibitor of NO synthesis (9). To determine the role of this metabolic pathway in the artery wall, LNMMA was administered solely in the extraluminal solution at the concentration of 3×10^{-4} M, 45 min before the first PE-induced contraction and experiments were conducted both in intact and deendothelialized arteries.

IL-1 biological assay. IL-1 activity was assessed by testing D10.G4 T cell proliferation, an IL-1–dependent growth process. Proliferation was measured by D10.G4 T cell [^{3}H]thymidine uptake after 72 h in culture (10). A standard curve relating cell growth to doses of recombinant human IL-1β (Biogen, Inc., Cambridge, MA; sp act 5×10^8 U/mg) was used to quantify the IL-1 activity in the supernatants respectively derived from the elicited macrophages and the LPS-activated macrophages. IL-1 activity was also examined in the supernatants from LPS-activated macrophages, which had been either reincubated in flasks or exposed to the carotids for a second 3-h challenge with LPS. The sensitivity of this bioassay was ~ 5 pg/ml and its variability did not exceed 10%. The values are given in nanograms of IL-1β per milliliter.

Histological studies. Rabbit carotids exposed for 3 h to LPS-activated macrophages were fixed with buffered 10% formalin solution and embedded in paraffin. Transverse sections were cut and stained with hematoxylin-eosin-safran and Masson trichrome for light microscopy examination.

Solutions and drugs. Krebs-Ringer solution contained the following (mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4,

Table I. Effect of LPS

	1 h	2 h	3 h
		%	
Krebs (n = 8)	103.5±5.8	92.7±3.7	86.3±2.6
LPS (n = 4)	108.7±6.9	100.4±10	96.4±10.2

Effect of LPS alone on the response of rabbit carotid arteries to 10^{-6} M extraluminal PE. The intraluminal Krebs-Ringer solution contained either no LPS (*Krebs*) or 1 μg/ml LPS (*LPS*). Data (means±SEM) are expressed as percentage of the initial contraction.

25 $NaHCO_3$, 0.026 CaEDTA, and 11.1 glucose. RPMI medium was RPMI 1640 (Gibco BRL, Cergy Pontoise, France) supplemented with 2 mM L-gluthamine, 10 mM Hepes, 5 U/ml heparin, and 50 U/50 μg penicillin/streptomycin per milliliter. PE, acetylcholine, bovine erythrocytes, SOD, and catalase were from Sigma Chemical Co., St. Louis, MO. LNMMA was from Calbiochem-Behring Corp., San Diego, CA. LPS from *Escherichia coli* (0.127:B8) was from Difco, Inc., Detroit, MI.

Statistical analysis. Results are means±SEM. Statistical analyses were performed using two-way ANOVA with repeated measurements (11). When significant variance was obtained ($P < 0.05$), multiple comparisons were done using the Bonferroni t test (11).

Results

Effect of macrophages on the vascular contractility of intact arteries

Effect of LPS alone, of elicited macrophages, or of their supernatants on PE-induced contraction. In the presence of control Krebs-Ringer solution, PE-induced carotid contraction did not change significantly after 1 and 2 h but decreased slightly after 3 h (Table I). Exposure of intact carotids to 1 μg/ml LPS alone did not change the PE-induced contraction observed without LPS (Table I). Consequently, the contraction values obtained with and without LPS were pooled and used as controls for subsequent comparisons. In addition, neither elicited macrophages nor their supernatants significantly affected the carotid reactivity (Table II), even though, after 3 h of exposure to elicited macrophages, carotid contraction was slightly smaller than that of control carotids.

Effect of LPS-activated macrophages or their supernatants. Unlike the elicited macrophages, both LPS-activated macrophages and their supernatants significantly depressed carotid contractility ($P < 0.001$). Furthermore, this contractility varied significantly owing to the interaction of time and the type of intraluminal solution ($P < 0.001$), indicating that different degrees of time-dependent depression were caused by the activated macrophages and by their supernatants. Thus, exposure of carotids to LPS-activated macrophages reduced the contractile response to PE significantly (Fig. 1), even after as little as 1 h (74.1±5.6% [n = 10] vs. 102.9±3.3% [n = 12] in controls, $P < 0.001$), whereas the depressive effect of the supernatants only became significant after 2 h (74.4±7.0 [n = 7] vs. 95.2±4.1 [n = 12] $P < 0.05$). The depression increased with time, and the difference between that caused by LPS-activated macrophages and their supernatants was no longer significant after 3 h (34.2±5.6 [n = 10] and 40.3±7.1 [n = 7], respectively, vs. 89.7±3.8% in controls [n = 12], $P < 0.001$, Fig. 1).

Effect of intraluminal LPS removal. In view of possible macrophage deactivation, we determined the contractile response to PE by carotids exposed to LPS-activated macrophages without LPS in the intraluminal solution, and found that the PE-induced contractions were not significantly different from those of controls (Table III).

Effect of paraformaldehyde fixation of activated macrophages. To assess the possible influence of bioactive membrane-bound monokines, LPS-activated macrophages were fixed with paraformaldehyde before infusion into the arteries. No significant effect on the contractile response to PE was seen (Table III).

Table II. Effects of Elicited Macrophages and Their Respective Supernatants

	1 h	2 h	3 h
		%	
Control (n = 12)	102.9±3.3	95.2±4.1	89.7±3.8
Elicited Mac. (n = 7)	93.2±4.1	87.5±5.3	76.3±7.7
Elicited SPN (n = 5)	99.2±3.4	97.1±4.5	91.3±5.8

Contractile response to 10^{-6} M extraluminal PE by rabbit carotid arteries exposed to elicited macrophages (*Elicited Mac.*) or to their supernatants (*Elicited SPN*). Data (means±SEM) are expressed as percentage of the initial contraction. ANOVA revealed no significant effect of elicited macrophages or their supernatants on carotid contraction in response to PE.

Role of the endothelium

Removal of the endothelium did not significantly modify carotid diameter (2.16±0.08 mm) or the initial PE-induced contraction (45.7±3.6%, n = 10). In a series of four deendothelialized carotids filled with control Krebs-Ringer solution, we determined that the contractile response to PE at 1, 2, and 3 h did not significantly vary from that of intact carotids.

The depression of contractility by LPS-activated macrophages in deendothelialized carotids was significantly greater

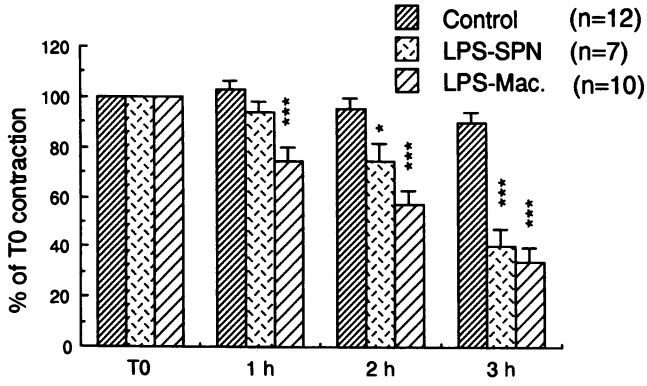

Figure 1. Contractile response to 10^{-6} M extraluminal PE by rabbit carotid arteries exposed to LPS-activated macrophages (*LPS-Mac.*) or to their supernatants (*LPS-SPN*). Data (means±SEM) are expressed as percentage of the initial contraction (*T0*). ANOVA revealed that activated macrophages and their supernatants both significantly reduced contractions in response to PE. The difference between LPS-Mac. and LPS-SPN was significant at 1 h ($P < 0.05$), and 2 h ($P < 0.05$), but not at 3 h. Asterisks indicate significant differences vs. the control (*$P < 0.05$; ***$P < 0.001$). Numbers of arteries are given in brackets.

Table III. Effect on Rabbit Carotid Contractility of LPS Removal and PFA Fixation of LPS-activated Macrophages

	1 h	2 h	3 h
		%	
Control (n = 12)	102.9±3.3	95.2±4.1	89.7±3.8
LPS-Mac.—LPS (n = 4)	87.1±3.5	86.1±3.9	78.9±6.7
PFA-Mac. (n = 4)	94.1±5.7	95.6±2.8	88.3±8.9

Contractile response to 10^{-6} M extraluminal PE after exposure to LPS-activated macrophages, either without LPS in the intraluminal solution (*LPS-Mac.—LPS*), or after LPS-activated macrophage fixation with PFA (*PFA-Mac.*) Data (means±SEM) are expressed as percentage of the initial contraction. ANOVA revealed that neither LPS-Mac.—LPS nor PFA-Mac. significantly diminished the contractions induced by PE.

than that observed in intact arteries (Fig. 2). After 1 h of exposure to these macrophages, the contractile response to PE was 52.6±7.6% in damaged carotids vs. 74.1±5.6% in intact arteries ($P < 0.05$, Fig. 2). After 2 h, the corresponding values decreased to 33.5±8.9% vs. 57.2±5.2% ($P < 0.02$), and further decreased after 3 h to 14.6±2.8% vs. 34.2±5.6% in intact vessels ($P < 0.02$).

The depressive effect of supernatants derived from LPS-activated macrophages in deendothelialized carotids relative to their effect on intact arteries (Table IV) only increased significantly after 1 h of exposure.

Involvement of metabolic pathways

Effect of antioxidants. To investigate the possible involvement of reactive oxygen intermediates, carotids were incubated in the presence of SOD or catalase. SOD alone did not affect the contractile responses of control carotids. In 11 intact carotids exposed for 1 h to LPS-activated macrophages, incubation with

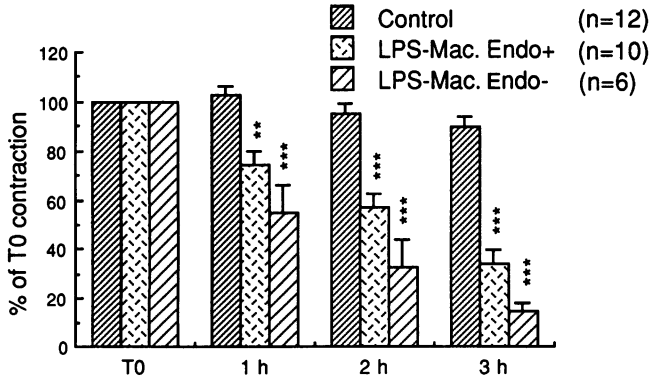

Figure 2. Contractile response to 10^{-6} M extraluminal PE by intact rabbit carotid arteries infused with LPS-activated macrophages (*LPS-Mac. Endo+*) and by deendothelialized vessels (*LPS-Mac. Endo−*) similarly infused. Data (means±SEM) are expressed as percent of the initial contraction (*T0*). ANOVA revealed that LPS-activated macrophages depressed contractility more markedly after removal of the endothelium. The difference between the depression in LPS-Mac. Endo+ and LPS-Mac. Endo− was significant at 1 h ($P < 0.05$), 2 h ($P < 0.02$), and 3 h ($P < 0.02$). Asterisks indicate significant differences vs the control (**$P < 0.01$; ***$P < 0.001$). Numbers of arteries are given in brackets.

Table IV. Effect on Rabbit Carotid Contractility of LPS-activated Macrophage Supernatants (SPN) after Removal of the Endothelium

	1 h	2 h	3 h
		%	
Control (n = 12)	102.9±3.3	95.2±4.1	89.7±3.8
LPS-SPN (Endo+ (n = 7)	93.5±4.1	74.4±7.1*	40.3±7.1§
LPS-SPN Endo− (n = 5)	72.3±7.9‡,‖	60.3±7.6‡	41.2±6.1§

Contractile response to 10^{-6} M extraluminal PE by intact carotids (*LPS-SPN Endo+*) and deendothelialized carotids (*LPS-SPN Endo−*) exposed to supernatants derived from LPS-activated macrophages. Data (means±SEM) are expressed as percentage of the initial contraction.

* Significantly different from control at $P < 0.05$.
‡ Significantly different from control at $P < 0.01$.
§ Significantly different from control at $P < 0.001$.
‖ Significantly different from LPS-SPN Endo+ at $P < 0.05$.

100 U/ml SOD preserved the contractile response (Table V). However, after 2 and 3 h, SOD did not maintain its protection. In view of the possible degradation of SOD in solution, a higher dose (300 U/ml) was tested in two carotids but did not provide greater protection.

SOD was also tested after removal of the endothelium. In five deendothelialized carotids, partial protection was observed at 1, 2, and 3 h (Table V). Moreover, SOD protected these arteries in such a way that their contractile responses were not significantly different from those of intact arteries exposed to LPS-activated macrophages without SOD (Table V).

When arteries (n = 3) were treated with catalase (2,000 U/ml) in addition to SOD (100 U/ml), no further improvement in contractility was observed compared to treatment with SOD alone.

Effect of LNMMA. To investigate the role of the L-arginine/NO pathway, we supplemented the extraluminal bath medium with LNMMA, which is known to inhibit this pathway, 45 min before the first PE-induced contraction. Under control condi-

Table V. Effect of SOD

		1 h	2 h	3 h
			%	
Endo+	LPS-Mac (n = 10)	74.1±5.6	57.2±5.2	34.2±5.6
	LPS-Mac + SOD (n = 11)	91.2±3.2*	61.7±6.3	35.5±3.6
Endo−	LPS-Mac (n = 6)	52.6±7.6	33.5±7.9	14.6±2.8
	LPS-Mac + SOD (n = 5)	87.4±6.3‡	47.6±4.9§	34.6±10.8§

Contractile response to 10^{-6} M extraluminal PE by carotid arteries with endothelium (*Endo+*) or without (*Endo−*) after exposure to LPS-activated macrophages with SOD (*LPS-Mac + SOD*) or without SOD (*LPS-Mac*) in the intraluminal solution. Data (means±SEM) are expressed as percentage of the initial contraction.

* Significantly different from LPS-Mac Endo+ at $P < 0.05$.
‡ Significantly different from LPS-Mac Endo− at $P < 0.01$.
§ Significantly different from LPS-Mac Endo− at $P < 0.05$.

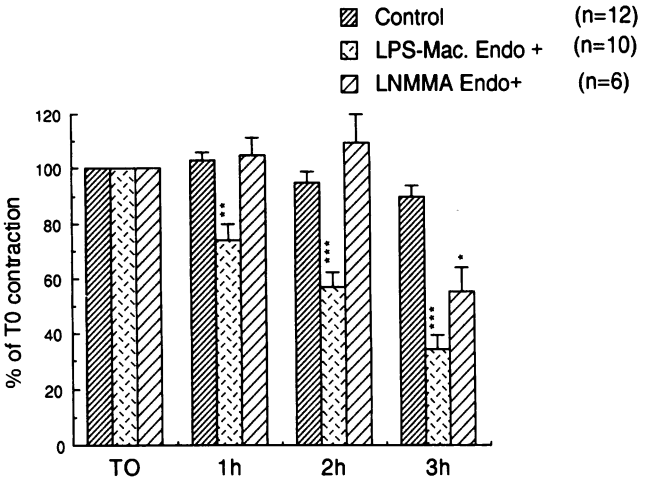

Figure 3. Contractile response to 10^{-6} M extraluminal PE by intact rabbit carotid arteries exposed to LPS-activated macrophages with LNMMA in the extraluminal solution (*LNMMA Endo+*) or without (*LPS-Mac. Endo+*). Data (means±SEM) are expressed as percentage of the initial contraction (*T0*). Asterisks indicate significant differences vs the control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Numbers of arteries are given in brackets.

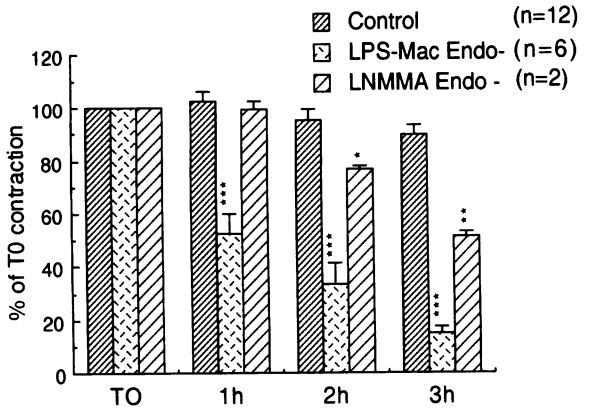

Figure 4. Contractile response to 10^{-6} M extraluminal PE by deendothelialized rabbit carotid arteries exposed to LPS-activated macrophages with LNMMA in the extraluminal solution (*LNMMA Endo−*) or without LNMMA (*LPS-Mac. Endo−*). Data (means±SEM) are expressed as percentage of the initial contraction (*T0*). Asterisks indicate significant differences vs the control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Numbers of arteries are given in brackets.

tions, 300 μM LNMMA significantly increased PE-induced contractions. Two-way ANOVA of the data obtained for intact carotids exposed to LPS-activated macrophages with or without extraluminal addition of LNMMA showed that LNMMA afforded significant protection, which was complete after 1 and 2 h, and only partial after 3 h (Fig. 3). Similar protection was obtained with extraluminal LNMMA in two deendothelialized carotids exposed to LPS-activated macrophages, indicating that the endothelium was not the target of LNMMA action (Fig. 4).

Mediator assays

Nitrite quantification. Nitrite content was in the low sensitivity range of the Griess colorometric method in supernatants from LPS-activated macrophages, lysates of these macrophages, and intraluminal solutions containing LPS-activated macrophages to which carotids had been exposed for 3 h.

HPLC analysis. Analysis of L-arginine metabolites using HPLC showed that the main product of L-arginine metabolism was ornithine, owing to arginase activity. Only 16% of the labeled L-arginine was consumed and produced ornithine (88%) and citrulline (6%). Supernatants of macrophages incubated with LNMMA showed no difference in their L-arginine metabolism, indicating the absence of detectable L-arginine nitrogen oxidation. The small fraction of labeled citrulline was probably due to ornithine transcarbamylase activity. By contrast, when supernatants from rat peritoneal macrophages were analyzed using the same method, we found that 78% of the labeled L-arginine was consumed, producing 15% ornithine and 80% citrulline. Moreover, citrulline production was inhibited by 70% after treatment of the rat macrophages with LNMMA. This suggests that rabbit macrophages, unlike rat macrophages, do not possess an active L-arginine oxidative pathway. In addition, it is noteworthy that arginase activity is not exaggerated in rabbit macrophages, which could have accounted for the absence of a detectable L-arginine oxidative pathway.

IL-1 quantification by biological assay. After 18 h in culture, the amounts of IL-1 respectively released by elicited and LPS-activated macrophages averaged 0.8±0.2 and 3.6±0.7 ng/ml ($n = 16$, $P < 0.01$).

When some of these activated macrophages were washed, resuspended in fresh RPMI medium, and challenged a second time with LPS, they continued to produce large quantities of IL-1 totalling 1.2±0.2 ng/ml over 3 h ($n = 7$). However, other LPS-activated macrophages were resuspended in fresh LPS-RPMI medium and left in the carotid for 3 h, the IL-1 concentration in the intraluminal solution was markedly higher than in the solution from the macrophages challenged in flasks with LPS only (44±14 ng/ml, $n = 4$).

Discussion

The present study demonstrates that LPS-activated rabbit peritoneal macrophages markedly inhibit the α-adrenergic vascular contraction of rabbit carotid arteries through the two cell-dependent pathways and important metabolic effector described below: (*a*) The presence of LPS-activated macrophages close to the vascular wall of the carotids depressed PE-induced contraction more than the presence of supernatants derived from these macrophages, possibly owing to amplified monokine release resulting from macrophage–vascular cell interactions. (*b*) The greater depression of contractility found in deendothelialized arteries suggests that, to some extent, the endothelium protects smooth muscle contractility from alteration by LPS-activated macrophages. (*c*) The L-arginine/NO pathway in vascular smooth muscle cells seems to be an important factor leading to the observed depression, as shown by the protection afforded by LNMMA.

Role of LPS activation. The depression of carotid contractility by LPS-activated macrophages or their supernatants was not due to direct action by LPS, because LPS alone had no significant effect throughout the time course of our experiments, in agreement with previous results (12). However, the activation of macrophages by LPS seemed necessary to trigger their deleterious action, in that elicited macrophages or their supernatants did not significantly affect the contractile response compared to that of control carotids. McKenna et al. (13) reported similar results in the rat aorta. The notion of macrophage activation is complex, and most authors now refer

to macrophage functional phenotypes. In the present work, quiescent resident macrophages were fully activated by LPS via a state of elicitation induced by intraperitoneal injection of sterile mineral oil. This elicitation led to detectable IL-1 release averaging 0.8 ng/ml, but was not sufficient to alter vascular contractility. The activated state subsequently induced by LPS, known to be one of the most potent macrophage agonists, is required to fulfill multiple secretory capacities, especially of monokines. In our study, the concentration of IL-1, which was assayed as an indicator of macrophage activation and monokine production, averaged 3.6 ng/ml in supernatants derived from LPS-activated macrophages. Additional evidence for the necessity of macrophage activation by LPS was provided by our experiments showing that activation of macrophages, by incubation for 18 h with LPS and their subsequent infusion into the artery without LPS in the medium, did not depress PE-induced contraction. This might be due to fast macrophage deactivation, such as that previously reported by Gifford et al. (14) who observed that the removal of LPS resulted in the sudden disappearance of TNF production.

Effect of LPS-activated macrophage-derived supernatants. Supernatants derived from LPS-activated macrophages depressed the contractility of the rabbit carotid in response to PE and the depression increased with time. This is in agreement with the previous report by McKenna et al. (13) that the contraction of the rat aorta in response to norepinephrine diminished when the vessel was incubated for 6 h in medium derived from LPS-activated macrophages. In their experiments, gel filtration of this medium yielded suppressive activity by substances at a molecular mass equivalent to the weights of IL-1/TNF: 15,000–17,000 D. It is therefore likely that the depression we observed with LPS-activated macrophages and their supernatants was mediated by the monokines IL-1 and TNF. Other authors reported that monokines have depressive effects on vascular functions. For instance, recent evidence indicates that IL-1 and/or TNF can induce, in rabbits and other mammals, a shocklike state associated with low vascular resistance index and hyporesponsiveness to contractile agonists (15–17). Elsewhere, monokines have been identified in vitro as potent inhibitors of vascular contraction via an endothelium-independent mechanism: thus, IL-1 was found to inhibit the PE-induced contraction of the rat aorta (18, 19), and TNF, that of the rabbit carotid (20). The fact that IL-1 and TNF have many overlapping biological properties and act synergistically to produce hemodynamic shock in rabbits (16) led us to conclude that, in our experiments, simultaneous release of these two monokines would produce a synergistic response on the vascular wall of the carotid and greatly reduce its contractile response.

Role of LPS-activated macrophages. A striking finding in the present study was that carotid reactivity was significantly depressed by ~ 25%, after as little as 1 h of exposure to LPS-activated macrophages, whereas carotid exposure to their supernatants for the same period induced no significant depression. Furthermore, at 2 h, the depressive effect of LPS-activated macrophages remained significantly greater than that of their supernatants. It is noteworthy that Salari and Walker (21), using a rat model of Langendorff perfused heart, also observed that LPS-activated macrophages depressed myocardial contractility more than their supernatants.

One possible mechanism indicative of cell-dependent contractility suppression might have been the presence of cell forms of IL-1 and TNF. In this connection, the existence of cell-associated IL-1 and TNF was demonstrated using their respective bioassays (22, 23). Here, however, paraformaldehyde (PFA)-fixed macrophages had no significant effect on vascular contraction. Nevertheless, we cannot rule out the possibility that the activities of other membrane bound molecules might have been inactivated by the PFA fixation technique, especially adhesion molecules.

The cell-dependent contractility depression might have resulted from differences between the cell concentration of the medium recovered after 18 h of incubation and that of the solutions containing macrophages infused in the carotids. This, however, seems unlikely in the case of the intraluminal solution, since the latter was tested at a concentration of 2×10^6 cells/ml, whereas the supernatants were derived from activated macrophages incubated at a concentration of 5×10^6 cells/ml.

Since the carotids were perfused with LPS-activated macrophages during a second exposure to LPS for 3 h, after their 18-h stimulation with LPS, these activated macrophages could have developed LPS hyporesponsiveness as a result of endotoxin tolerance. To elucidate whether such tolerance was present during the 3 h of our experimental protocol, the monokines were assayed in the supernatants from LPS-activated macrophages reincubated with LPS and from activated macrophages exposed to a challenge dose of LPS in the carotid lumen. We found that IL-1 release continued under both sets of conditions. However, TNF release dropped markedly when macrophages were reincubated in flasks (unpublished data), in agreement with previous results reported by Mathison et al. (24). These authors showed that treatment of elicited peritoneal rabbit macrophages with two doses of LPS given 9 h apart markedly reduced TNF production after the second dose, in relation to macrophage LPS-adaptation, but they did not establish whether or not the production of other LPS-induced monokines was also affected. From our results, it seems that the early phase of LPS tolerance did not affect IL-1 production, contrarily to that of TNF. This agrees with recent findings reported by Takasuka et al. (25). It appears thus that IL-1 and TNF production are differentially regulated in cells of the monocyte/macrophage lineage (26). Whatever the regulation of cytokine release in cultured macrophages, we found an excessive concentration of both IL-1 and TNF (unpublished data), in the intraluminal medium of LPS-activated macrophages, as the IL-1 concentration in this medium was 12 times higher than in the supernatants derived from macrophages incubated 18 h with LPS. These results are not surprising because Mathison et al. (24) showed that LPS hyporesponsiveness can be partly reversed by raising, for instance, the LPS concentration of the challenge dose. Moreover, this hyporesponsiveness seems to be specific for LPS and does not result from overall inhibition of TNF, irrespective of the stimulus (24, 25). LPS-tolerant macrophages display TNF production in response to heat-killed *Staphylococcus aureus* that is at least as good as that observed in control elicited rabbit macrophages. In the present work, activated macrophages were resuspended with a challenge dose of LPS and then exposed to the luminal surface of the rabbit carotid, in an intraluminal medium that was not rigorously aseptic, where they might have come into contact with secretory products of the vascular wall, especially adhesion molecules, as well as with fragments of extracellular matrix when the endothelium was removed. All these new afferent signals might have lead to a different state of macrophage activation. In addition, monokine concentrations might have risen in the cellular intraluminal solution, as a result of autocrine

and/or paracrine interactions between LPS-activated macrophages and vascular cells, since vascular cells have been shown to be not only special targets for monokines, but also potent sources of IL-1 and TNF production (27, 28). Moreover, heterologous secretion is regulated by positive autocrine mechanisms, so that IL-1 and TNF are capable of amplifying the production of their respective molecules as well as of each other. It is interesting that the target cells concerned by this amplification network include macrophages, smooth muscle cells and endothelial cells. In support for such amplification, McKenna and his co-worker (19, 29) reported that the release of endogenous IL-1 from the rat aorta was elicited by its exposure to IL-1 and LPS. Here, therefore, the intraluminal solution containing LPS-activated macrophages left for 3 h in the carotids obviously contained high concentrations of potentially harmful monokines. However, we did not determine specifically whether these monokines were released by the intraluminal macrophages owing to their highly activated state, or produced by the stimulated carotid wall. Nevertheless, these observations strongly support the possibility that monokines contribute to the vascular impairment induced by LPS-activated macrophages, and highlight the critical role of cell-to-cell interactions.

An additional explanation of the stronger depressive effects induced by LPS-activated macrophages than by their supernatants might be the presence, in the intraluminal solution containing the activated macrophages, of labile products secreted in situ and not recovered when the supernatant was tested separately. In this connection, labile products possessing vasodilatory activity such as prostacyclin, platelet-activating-factor, and β-endorphin have been reported to be released by LPS-activated macrophages (3). Nevertheless, two types of labile product secreted by these macrophages are known to be highly reactive: reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), and these were the focus of specific experiments whose results are discussed below.

Role of ROI. In response to activation by LPS, elicited macrophages undergo a respiratory burst owing to the NADPH-oxidase catalysis of oxygen to superoxide anion, a reactive oxygen radical subject to further reactions leading to the production of other toxic derivatives such as hydrogen peroxide and hydroxyl radical (30). These reactive molecules have been found to be involved in different mechanisms of injury, including bacterial killing and tissue injury. Oxidative injury to the myocardium and vascular endothelium were prevented to a significant extent by ROI scavengers (31), especially by SOD and catalase. In addition, recent studies seem to link the intervention of monokines in the reperfusion injury with that of ROI (32). In the present work, carotid pretreatment with SOD provided significant protection of the vascular contractile response after one hour of exposure to LPS-activated macrophages, but not after longer periods, suggesting that ROI were partly involved in the contractility depression by macrophages. This is in agreement with the results reported by Salari et al. (21) showing that free radical scavengers reduced the harmful effect of LPS-activated macrophages on the myocardial function of the rat heart.

The protection afforded by SOD cannot be ascribed solely to the preservation of the endothelium, since it also protected deendothelialized carotids.

No damage to the vascular wall was observed in our histological studies. Cellular necrosis was not detected by light microscopy examination, at least not during the 3 h of our experiments, and did not seem to be involved in the observed contractile depression. However, other metabolic activities of the ROI secreted by LPS-activated macrophages and/or by stimulated vascular cells might be involved in this depression.

ROI have been reported to influence vasomotor tone. Thus, O_2^-, which inactivates NO, has been proposed to be responsible for the vasoconstriction induced when vessels were exposed to polymorphonuclear leucocytes (PMN) (33). However, this is unlikely since we found that SOD partly reduced the vascular contractile depression induced by LPS-activated macrophages, instead of enhancing it. In addition, O_2^- might have activated cGMP directly in the smooth muscle cells of rabbit carotids, and prostaglandin compounds could have been produced by free radical-catalyzed mechanisms leading to activation of the cAMP vasodilatory pathway (34).

ROI were recently shown to be involved in leukocyte adhesion mechanisms, and this might be of primary importance in our model. Suzuki et al. (35) showed, in cat ischemic intestinal tissues, that O_2^- mediates reperfusion-induced leukocyte adherence, which was significantly prevented by SOD treatment. Little is known about the effect of ROI on monocyte/macrophage adherence. Bath et al. (36) reported that SOD pretreatment inhibited FMLP-stimulated human monocyte adhesion to porcine aortic endothelial cells in culture. These authors proposed that NO inhibits the adhesion whereas O_2^- promotes it. It is therefore possible that here, O_2^- promoted the adhesion of LPS-activated macrophages to the luminal vascular surface of rabbit carotids. As a result, it might have induced a more harmful state of macrophage activation for the carotid.

Role of reactive nitrogen intermediates. Nitric oxide is a labile product reacting readily with molecular oxygen to form nitrite/nitrate (NO_2^-, NO_3^-) and, in biological systems, the distance over which NO travels is probably limited by its reaction with dissolved O_2. In septic subjects, several authors found enhanced release of NO_3^- (37, 38). NO, or a closely related molecule, has now been identified as the endothelium-derived relaxing factor (EDRF) required for the acetylcholine-induced relaxation of vascular smooth muscle (39). In this relaxation process, NO activates the soluble guanylate cyclase to enhance the intracellular cGMP levels and induce cGMP-dependent vasodilation (40), by inhibiting vasoconstriction at different molecular steps. Accordingly, vascular hyporesponsiveness might be caused by stimulation of the vasodilation pathway. In vitro, an enhanced cGMP-dependent pathway was found to impair the α-agonist–induced contraction with a shift to the right of the dose-response curve. This is believed to play a major role in the vascular hyporesponsiveness in sepsis (41). In quite unrelated experiments, LPS-activated macrophages were shown to release RNI, which resembled EDRF as regard their effect on the vascular wall (42, 43). Therefore, RNI might be released by LPS-activated macrophages close to the vascular wall during the early phase of the depression of the carotid contraction induced by intraluminal infusion of LPS-activated macrophages.

However, this is unlikely to have occurred with the present LPS-activated rabbit macrophages for the following reasons: No detectable nitrite was found in the supernatants of these macrophages, either in the efflux from the carotids exposed to them, or intracellularly after the assay of macrophages lysates. This could be due to poor sensitivity of the Griess colorimetric assay, if rabbit macrophages were less productive than murine macrophages, but HPLC analysis of amino acid metabolites did not allow us either to detect the existence of an L-arginine oxidation pathway in the rabbit macrophage. As in human

monocytes/macrophages, rabbit macrophages might have different metabolic pathways from those of rodents (44), for reasons that are not yet clear. Nevertheless, as NO is a potent vasodilator acting at nanomolar concentrations, it cannot be excluded that NO was released at such low levels that neither nitrite nor aminoacid metabolites were detectable by presently available methods.

Role of the L-arginine pathway. As indicated by the protection afforded by LNMMA, the L-arginine pathway was involved in the mechanisms that depressed the contractility of carotids exposed to LPS-activated macrophages, even though this metabolic effector seemed to be activated in the vascular smooth muscle rather than in the endothelium or in the macrophages.

Recent advances have been made in clarifying the complex L-arginine-NO-cGMP metabolic pathway that leads to vascular smooth muscle relaxation. At least, two types of NO synthase responsible for NO formation from L-arginine have been identified (45). One of them is constitutive, calcium-calmodulin dependent and predominant in brain and endothelial cells. The other is inducible by inflammatory mediators and its prototype is the NO synthase of the macrophage. It is noteworthy that NO synthase is induced by a discrete set of immunostimulants including LPS, TNF, IL-1, and interferon-γ (IFNγ), and has been found in vascular smooth muscle cells (46). Inhibitors of these enzymes are now available and their use has made it possible to show that the contractile depression of arteries taken from septic animals (47) or arteries treated in vitro for long periods with LPS (48) involves the L-arginine pathway in smooth muscle cells. Moreover, the monokines IL-1 and TNF induce an L-arginine dependent effector system in vascular smooth muscle, as shown in vascular cells in culture (49, 50). In the present study, addition of LNMMA to the extraluminal solution when the rabbit carotid was exposed intraluminally to LPS-activated macrophages provided protection that was complete at 1 and 2 h but only partial at 3 h. This suggests that monokines present in the supernatants derived from LPS-activated macrophages and in the intraluminal solution containing these macrophages depress the contractile function by activating the L-arginine/NO pathway of the vascular smooth muscle. The endothelium also contains NO synthase (51), which was probably inhibited when LNMMA was added extraluminally. However, as we found that the endothelium played a protective role and was not the primary mediator of the harmful effect of LPS-activated macrophages, the L-arginine pathway of the endothelium was unlikely to have been involved in contractility depression. This is corroborated by in vitro studies showing that LPS and monokines reduce EDRF release (47, 52, 53). In the present work, similar protection against LPS-activated macrophages was observed in both deendothelialized and intact arteries exposed extraluminally to LNMMA. Julou-Schaeffer et al. (47) and Rees et al. (48) also observed such protection by LNMMA on vascular rings without endothelium, either obtained from septic animals or treated in vitro with LPS. On the other hand, it might be assumed that extraluminal LNMMA administration did not alter the metabolism of LPS macrophages tested intraluminally, firstly, because of limited diffusion of LNMMA within the lumen, and secondly, because care was taken to perfuse the carotids discontinuously (every 15 min) with new LPS macrophage solution.

The protection afforded by LNMMA occurred early in our experiments whereas immunologically inducible NO synthase takes time to be effective as it requires de novo protein synthesis. Thus, Kilbourn and Belloni (51) showed, in in vitro experiments, that endothelial cells released NO only after 8 h of exposure to monokines in the presence of LPS, and Beasley et al. (49) reported that, in cultured vascular smooth muscle cells, an L-arginine–dependent increase in cGMP only occurred after 6 h of exposure to IL-1. By contrast, hypotension induced by TNF injection was found to begin 20 min after its administration, and complete reversion was provided by LNMMA injection (54), suggesting that TNF stimulates an early effective L-arginine pathway. Our model seems to mimic in vivo conditions closely and differ from those of the above in vitro cell culture experiments. Synergy between the different inflammatory mediators released by interacting cells might account for this discrepancy. In addition, neither carotid excision, nor our extraluminal and intraluminal solutions were strictly aseptic, so that there might have been a priming contact with exogenous uncontrolled bacterial products. Rees et al. (48) observed decreased contractile responses by rat arterial rings as soon as 3–4 h after their incubation in physiological solution. They proposed that minimal amounts of LPS present in their medium bath accounted for this decrease. Moreover, according to Wood et al. (46), smooth muscle cells possess both the inducible and constitutive NO synthases, the latter being involved in the immediate formation of NO, and the former induced subsequently. Therefore, we cannot rule out the possibility that, in vascular smooth muscle cells, several types of NO synthases are present which have different activation kinetics and respond to different types of agonist, at least one of which is an immunoinflammatory mediator of LPS-activated macrophages.

The failure of extraluminal pretreatment with LNMMA to provide complete protection against the depression at 3 h indicates that other metabolic effectors were probably involved in mediating the macrophages' action. Desensitization to LNMMA has been reported, as a result of the transformation of LNMMA to L-arginine via L-citrulline (55). However, this is unlikely to have occurred in our model, as the extraluminal solution was changed every 30 min.

Lastly, we observed that protection against contractile depression induced by LPS-activated macrophages was effective 1 hour after carotid treatment with either SOD or LNMMA. This might be due to the fast reaction that occurs between superoxide anion and nitric oxide to form peroxynitrite anion, which under certain conditions decomposes to generate a strong oxidant whose reactivity is similar to that of hydroxyl radical. Beckman et al. (56) proposed that, under pathological conditions in which both O_2^- and NO are produced, SOD might protect vascular tissue, by preventing the formation of peroxynitrite. In that case, ROI and RNI might synergize to depress vascular contractility.

Role of the endothelium. After removal of the endothelium, inhibition by intraluminal LPS-activated macrophages of vascular contraction increased markedly, but inhibition by their supernatants only slightly increased. In this respect, the endothelium might constitute a physicochemical barrier, although we noted with interest that in the presence of SOD, the effect of LPS-activated macrophages on deendothelialized arteries, was not significantly different from their effect on intact vessels (Table V). This implies that the endothelium might act as a scavenger of free radicals. Likewise, Parker et al. (57) demonstrated that EDRF-NO can be considered as a scavenger of the superoxide anion released by FMLP-activated human PMN, while Mehta et al. (58) found that the activity of PMN-derived smooth muscle relaxant, which is closely related to NO, was

more pronounced in deendothelialized vascular segments than in intact segments, suggesting that the endothelial lining provides a physiological barrier to the relaxant effects of PMN. Other mechanisms of protection afforded by the endothelium can also be suggested: for instance, it might act as a diffusional barrier against damaging mediators especially monokines, whose molecular weight of ~ 17 kD allows them to be transported through the endothelium by transcytosis (59). The endothelium might also be an active chemical opponent of diffusion, as specific receptors for monokines are present on endothelial cells (60). The trapping of macrophage products other than monokines, with endothelium-independent vasodilator properties, is another possibility. In addition, the endothelium might act as a functional inhibitor, by producing monokines inhibitors. Thus, inhibitory activities against IL-1 (61) and TNF (62) have been described in the supernatants of a variety of cells, especially those secreting monokines. However, endothelial cells have not been so far reported to produce monokine inhibitors. It might also be suggested that the protective role of the endothelium is mediated by endothelium-derived constricting factors, as IL-1 has been shown to induce endothelium-derived constricting factor production in cultured endothelial cells (63).

Role of the subendothelium. The enhancement of contractility suppression by LPS-activated macrophages after endothelial removal suggest the occurrence of intercellular events between the subendothelium and LPS-activated macrophages.

If the endothelium is a barrier of diffusion to monokines, its removal might elicit a major monokine self-amplification network linking macrophages and smooth muscle cells in that these cells are known to have a greater capacity for the autocrine production of monokines than endothelial cells (64). Thus, stimulated smooth muscle cells elaborate more IL-1 and IL-6 than endothelial cells.

A more convincing possibility is that components of the vascular extracellular matrix might synergize with LPS to act as cofactors of macrophage activation. Kauffman et al. (65) demonstrated that monocytes adhering to smooth muscle cell–derived matrix develop a functional phenotype change leading to an activated state. Furthermore, Pacifi et al. (66) showed that bone matrix constituents including collagen fragments stimulate IL-1 release from human blood monocytes in a dose-dependent manner requiring physical contact.

Conclusion. We have shown that macrophages play a major role in regulating the smooth muscle tone. The present findings suggest that several of the mechanisms controlling the cellular interactions between macrophages and vascular cells might be involved in the inflammatory responses of vascular tissue and might thus account for the defective state of the circulation during sepsis.

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