Vascular interleukin-10 protects against LPS-induced vasomotor dysfunction

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Gunnett, Carol A., Donald D. Lund, Frank M. Faraci, and Donald D. Heistad. Vascular interleukin-10 protects against LPS-induced vasomotor dysfunction. Am J Physiol Heart Circ Physiol 289: H624–H630, 2005; doi:10.1152/ajpheart.01234.2004.—We tested the hypotheses that 1) systemic IL-10, after adenoviral gene transfer, protects arteries from impaired relaxation produced by LPS; 2) local expression of IL-10 within the arterial wall protects against vasomotor dysfunction after LPS; and 3) IL-10 protects against vascular dysfunction mediated by inducible NO synthase (iNOS) after LPS. In IL-10-deficient (IL-10−/−) and wild-type (WT, IL-10+/+) mice, LPS in vivo impaired relaxation of arteries to acetylcholine and gene transfer of IL-10 improved responses to acetylcholine. Superoxide levels were elevated in arteries after LPS, and increased levels of superoxide were prevented by gene transfer of IL-10. In arteries incubated with a low concentration of LPS in vitro to eliminate systemic effects of LPS and IL-10 from nonvascular sources, responses to acetylcholine were impaired in IL-10-deficient mice and impairment was largely prevented by gene transfer in vitro of IL-10. In arteries from WT mice in vitro, the low concentration of LPS did not impair responses to acetylcholine. Thus IL-10 within the vessel wall protects against LPS-induced dysfunction. In IL-10-deficient mice, aminoguanidine, which inhibits iNOS, protected against vasomotor dysfunction after LPS. In arteries from iNOS-deficient mice, LPS did not impair responses to acetylcholine. These findings suggest that both systemic and local effects of IL-10 provide important protection of arteries against an inflammatory stimulus and that IL-10 decreases iNOS-mediated impairment of vasorelaxation after LPS. IL-10 is secreted by leukocytes and possibly other cells, and plasma concentrations of the peptide are elevated after systemic LPS (3). A second goal was to test the hypothesis that IL-10 expressed in blood vessels, distinct from systemic effects, protects against endothelial dysfunction after LPS. We compared vasomotor responses of arteries from WT and IL-10-deficient mice after incubation with LPS in vitro. By incubating arteries with LPS in vitro, we eliminated the potential for systemic effects of LPS, including nonvascular production of IL-10. We also expressed IL-10 in arteries from IL-10-deficient mice, using adenoviral gene transfer before exposure to LPS, to determine whether reconstitution of IL-10 within vascular cells protects vascular function. The third goal of this study was to examine the interaction of IL-10 with iNOS, a mediator of vascular dysfunction during inflammation (4, 21, 28, 33). Among its anti-inflammatory functions, IL-10 inhibits activation of NF-κB, thereby inhibiting expression of iNOS during inflammation (31, 34). We tested the hypothesis that iNOS contributes to impaired relaxation in arteries from IL-10-deficient mice during inflammation produced by LPS.

METHODS

Animals. We used WT (n = 57), IL-10-deficient (n = 78), and iNOS-deficient (iNOS−/−, n = 6) mice. IL-10-deficient mice in our colony have been back-crossed >10 generations onto the C57BL/6 strain to yield mice with a C57BL/6-defined background. Therefore, we used C57BL/6 mice as WT controls in these experiments. Mice with targeted disruption of the gene for iNOS were obtained initially from Drs. John Mudgett and Carl Nathan (Cornell University, Ithaca, NY) (30). Most of the WT mice used in these studies with iNOS-deficient mice were littermate controls. A few C57BL/6 mice, which are the background strain for the iNOS-deficient strain, were used as controls. We have found no differences in responses of arteries from WT littermates and C57BL/6 mice in this or previous studies (17, 21). Thus, in the present studies, data from WT littermates of iNOS-
deficient mice and C57BL/6 mice were combined for comparison with iNOS-deficient mice. All protocols were approved by the Animal Use Committee at the University of Iowa.

Vascular function. Vasomotor function of carotid arteries was evaluated in vitro by measurement of isometric tension as described previously (16, 21, 23, 24). Briefly, mice were anesthetized with Nembutal (75–100 mg/kg ip), and carotid arteries were removed and immediately placed in oxygenated Krebs buffer. Vessels were then suspended between two triangular hooks in an organ bath and attached to a force transducer for measurement of isometric tension.

We examined contraction of carotid rings to the thromboxane A2 analog U-46619 and relaxation in response to acetylcholine (1 nM–3 μM) or nitroprusside (1 nM–100 μM) after submaximal precontraction with U-46619. Responses of the carotid artery to acetylcholine, an endothelium-dependent agonist, are mediated by endothelial NO synthase (eNOS). Nitroprusside was used to examine endothelium-independent vasorelaxation.

**Effects of LPS and IL-10 in vivo.** To study the effects of gene transfer in vivo, male mice were randomly assigned to groups for treatment with vehicle (PBS), LPS + control virus (AdLacZ), or LPS + IL-10 virus (AdIL-10). AdIL-10 contains the gene for human (h)IL-10 with a cytomegalovirus promoter, produced by the Vector Core at the University of Iowa. Recombinant viruses [100 μl, 1 × 10^{12} plaque-forming units (PFU)] were injected into a tail vein 72 h before LPS. LPS (Escherichia coli serotype 026:B6) or vehicle (sterile Krebs plus penicillin-streptomycin) was administered at a dose of 1 mg/kg (ip) in IL-10-deficient mice and 5 mg/kg in WT mice. A higher dose was needed in WT mice to produce impaired responses to acetylcholine because IL-10-deficient mice are more sensitive to inflammatory stimuli (3, 16, 20). Vasomotor function was examined in the aorta in vitro 20–24 h after LPS. We found previously (1, 20–24) that responses to acetylcholine, nitroprusside, and U-46619 are similar between the aorta and the carotid artery.

**Effects of LPS and IL-10 ex vivo.** Carotid arteries from IL-10-deficient and WT mice were used for incubation ex vivo in LPS (E. coli serotype 026:B6) or vehicle (sterile Krebs plus penicillin-streptomycin). For studies of IL-10-deficient vs. WT mice, a concentration of LPS was chosen (10–30 μg/ml) that produced little or no impairment of endothelium-dependent relaxation in arteries from WT mice. We chose lower concentrations for IL-10-deficient mice because this inflammatory stimulus was more susceptible to this inflammatory stimulus. Concentrations of LPS were titrated to produce similar levels of impairment in each series of experiments. The same concentration of LPS was always used on IL-10-deficient and WT vessels that were being compared simultaneously.

In some experiments, aminoguanidine (300 μM), an inhibitor of iNOS, was used during studies of vasomotor function. In other studies, both aminoguanidine and LPS were added simultaneously to incubation media. To provide sufficient aminoguanidine for the extended time period of overnight incubation, we used a concentration of 1 mM in the incubation medium. When the vessels were placed in organ baths for studies of vasomotor function, the concentration of aminoguanidine was reduced to 300 μM to avoid nonspecific inhibition of eNOS.

For studies of iNOS-deficient mice, we chose higher concentrations of LPS (250–500 μg/ml in culture medium) that produced impairment of endothelium-dependent relaxation in arteries from WT mice. Our goal was to determine whether arteries from iNOS-deficient mice are protected from LPS. Thus we used sufficient LPS to produce impairment in WT mice to provide a basis for comparison with iNOS-deficient mice.

To study the effects of gene transfer of IL-10 ex vivo, we incubated carotid arteries from IL-10-deficient mice with AdIL-10 or AdLacZ (3 × 10^{10} PFU) for 3 h before addition of LPS to the incubation media. Arteries were then incubated for an additional 18–20 h before evaluation of vasomotor function. In previous studies, we found (18, 19) vascular function to be normal after incubation for 24 h in normal medium.

**Detection of superoxide.** Superoxide levels were measured by lucigenin-enhanced chemiluminescence as described previously (16). Rings of aorta were placed in 0.5 ml of PBS and lucigenin 5 μM, and relative light units (RLUs) were measured for 5 min. Background counts were determined and subtracted, and RLUs were normalized to surface area.

**Detection of IL-10 and TNF-α.** IL-10 and TNF-α were measured in plasma after LPS and gene transfer of IL-10 with immunoassays kits from R&D Systems and Biosource, respectively.

**RT-PCR.** We used RT-PCR to verify expression of hIL-10 in carotid arteries after adenoviral gene transfer. Arteries were snap-frozen in liquid nitrogen and ground with a glass pestle, and RNA was extracted with TRI reagent. To verify the quality of RNA in samples that produced no expression bands for hIL-10, we examined side-by-side samples from all arteries, using primers for hIL-10 and for the ubiquitous protein β-actin as modified from Cheng et al. (6). The following primers were used for hIL-10 PCR: sense 5′−CTC TTG TTT TCA CAG GGA AG-3′ and antisense 5′−AGT CTG AGA ACA GCT GCA C-3′.

**Adhesion of leukocytes.** Rings of carotid arteries were pinned out flat, fixed in formalin, and stained with Wright stain. Vessel segments were examined with a dissecting light microscope. Six cross-sectional fields were counted from each tissue sample. One average value was calculated per animal and reported as the number of leukocytes per square millimeter of vessel.

**Drugs.** LPS, acetylcholine, sodium nitroprusside, and aminoguanidine were obtained from Sigma (St. Louis, MO), U-46619 was obtained from Cayman Chemical (Ann Arbor, MI), U-46619 was dissolved in ethanol and then diluted with normal saline. All other drugs were dissolved and diluted in normal saline. All concentrations are expressed as final concentration of each drug in the organ bath.

**Statistical analysis.** All data are expressed as means ± SE. Group differences were determined by ANOVA to evaluate significant differences between means, followed by Tukey’s post hoc test. P < 0.05 was considered to be statistically significant. Relaxation to acetylcholine and sodium nitroprusside is expressed as percent relaxation from precontraction to U-46619.

**RESULTS**

**Effects of LPS in vivo.** After LPS treatment in vivo, responses to acetylcholine were impaired in aortas from IL-10-deficient and WT mice (Fig. 1A). A higher dose of LPS was required in WT mice to impair responses to acetylcholine (Fig. 1B). Gene transfer with AdIL-10, but not AdLacZ, improved relaxation in arteries from both IL-10-deficient and WT mice to control (vehicle) levels (Fig. 1).

Plasma from control mice and mice after treatment with LPS + AdLacZ contained no measurable hIL-10. In mice treated with LPS + AdIL-10, plasma levels of hIL-10 were 6.1 ± 1.5 ng/ml in IL-10-deficient mice (n = 8, P < 0.05 vs. control) and 9.4 ± 1.0 ng/ml in WT mice (n = 8, P < 0.05 vs. control, not significant (NS) vs. IL-10-deficient mice).

We measured plasma levels of the proinflammatory cytokine TNF-α and hIL-10. In IL-10-deficient mice, levels of TNF-α were below the level of detection after vehicle (n = 8), 9.7 ± 3.2 pg/ml after LPS + AdLacZ (n = 8, P < 0.05 vs. vehicle), and 0.3 ± 0.2 pg/ml after LPS + AdIL-10 (n = 8, P < 0.05 vs. LPS + AdLacZ, NS vs. vehicle). In WT mice, levels of TNF-α were below the levels of detection in all treatment groups.

Levels of superoxide were elevated in aortas from IL-10-deficient (n = 10) and WT (n = 5) mice after LPS (Fig. 2A, LPS + AdLacZ). Gene transfer of AdIL-10 prevented increases in vascular superoxide in IL-10-deficient mice (Fig. 2).
A) and in WT mice (Fig. 2B). Levels of superoxide produced by LPS tended to be higher in IL-10-deficient than in WT mice, but there was no significant difference between the groups (Fig. 2).

Effects of LPS in vitro. After incubation overnight in vehicle in vitro, relaxation to acetylcholine was similar in carotid arteries from WT and IL-10-deficient mice (Fig. 3A). After incubation in low concentrations of LPS, responses to acetylcholine were impaired in arteries from IL-10-deficient mice (Fig. 3B) but were not impaired in arteries from WT mice. Responses to nitroprusside were normal after treatment with LPS in arteries from both WT mice (data not shown) and IL-10-deficient mice (Fig. 3C).

Aminoguanidine (300 μM for 30 min) in the organ bath did not improve responses to acetylcholine in arteries from IL-10-deficient mice after incubation of arteries in vitro with LPS (data not shown). We showed previously (20, 21) that this concentration of aminoguanidine in the organ bath improves iNOS-mediated impairment of relaxation in mouse carotid arteries after acute exposure to LPS in vivo. Treatment with aminoguanidine throughout the overnight incubation with LPS, however, did improve endothelium-dependent relaxation (Fig. 4).

Expression of hIL-10 by gene transfer in vitro to arteries from IL-10-deficient mice improved relaxation after LPS (Fig. 5). Maximal responses to acetylcholine were similar in arteries treated with AdIL-10 + LPS and in arteries without LPS (Fig. 5)

Higher concentrations of LPS produced substantial impairment of responses to acetylcholine in arteries from WT mice (Fig. 6B). Responses to nitroprusside were normal in arteries from WT mice after LPS (data not shown). These higher concentrations of LPS produced no impairment of responses to acetylcholine in arteries from iNOS-deficient mice (Fig. 6A).

Using RT-PCR, we found expression of hIL-10 in carotid arteries from IL-10-deficient mice after gene transfer in vitro with AdIL-10 (Fig. 6). No PCR products for hIL-10 were observed in AdLacZ-treated arteries from IL-10-deficient mice (Fig. 7).

Leukocytes on arteries. The absence of IL-10 in IL-10-deficient mice generates an environment with proinflammatory potential (3, 20). We considered the possibility that activation of leukocytes might lead to adhesion to endothelium in arteries in IL-10-deficient mice under basal conditions, and thereby contribute to impairment of vasomotor function. The number of leukocytes adherent to endothelium of carotid artery, however, was small, and there was no difference in basal numbers of adherent leukocytes in WT and IL-10-deficient mice (data not shown).

DISCUSSION

The first major new finding of these studies is that intravenous gene transfer of IL-10 in mice protects endothelium-dependent relaxation from impairment produced by systemic
inflammation (LPS). To our knowledge, the finding that vascular function can be improved during inflammation by an increase in plasma levels of IL-10 is novel. A second major finding is that endogenous IL-10 within the vessel wall reduces vascular dysfunction produced by LPS. IL-10 has emerged as a potentially important protective molecule for blood vessels, and this study is the first to define a role for local IL-10 produced in arteries. We further demonstrated that restoration of IL-10 by gene transfer to carotid arteries from IL-10-deficient mice protects against impaired relaxation produced by LPS. These results provide additional evidence that IL-10 expressed locally in blood vessels provides important protection against vascular dysfunction. Finally, our results suggest that the protective effects of vascular IL-10 result, at least in part, from the ability of the cytokine to limit iNOS-mediated vascular dysfunction.

Previous studies suggest that IL-10, a cytokine produced systemically by leukocytes, especially T lymphocytes, inhibits inflammation (11). Activation of the inflammatory response (or its components) impairs vasomotor function, including endothelium-dependent relaxation (20, 29). In previous studies, we reported (16, 20, 23) that endogenous IL-10 limits increases in superoxide and protects against endothelial dysfunction following LPS in vivo or during diabetes.

Effects of IL-10 in vivo. IL-10-deficient mice are extremely sensitive to proinflammatory stimuli such as LPS (3, 16, 20). LPS had proinflammatory effects in IL-10-deficient mice, with increased levels of circulating TNF-α. Levels of superoxide were elevated in arteries after LPS, which is consistent with inflammation and associated oxidative stress (16, 20). Responses to acetylcholine were impaired after a low dose of LPS in IL-10-deficient mice and a higher dose of LPS in WT mice.

Gene transfer of IL-10 increased levels of circulating hIL-10 in IL-10-deficient and WT mice, with concomitant preservation of endothelium-dependent relaxation after LPS and protection against vascular dysfunction during systemic inflammation. Previous studies from our laboratory (7) using adenoviral gene transfer of extracellular SOD demonstrate substantial transgene expression in the liver, with release of protein into blood after intravenous injection of adenovirus. It is likely that elevated levels of plasma IL-10 after intravenous injections of AdIL-10 also are the result of synthesis of IL-10 in the liver.

Effects of endogenous and transgene IL-10 ex vivo. Because IL-10 is a cytokine, which may be produced by many cell types, and circulates in plasma, it was not clear from previous studies and our initial studies in vivo whether the protective effects of IL-10 were a result of systemic or local production of the peptide. In our experiments performed in vitro, the results were not dependent upon systemic inflammatory responses, such as activation of leukocytes, because arteries were exposed to LPS only in culture. Thus responses to LPS in arteries
studied ex vivo are limited to cells normally present within the arterial wall. The importance of cytokines produced locally in vascular cells, and their effects on vascular dysfunction during inflammation, has not been defined previously.

To determine whether IL-10 of vascular origin plays an important role in vasomotor function during inflammation, we incubated carotid arteries with LPS ex vivo. Impairment of responses to acetylcholine in arteries from mice deficient in expression of IL-10, but not in arteries from WT mice, provides strong evidence that IL-10 in normal arteries protects against inflammation-induced dysfunction. Normal responses to nitroprusside after LPS are consistent with previous studies (16, 17) and suggest that dysfunction after LPS is specific for endothelium-dependent relaxation. To our knowledge, the present study is the first to demonstrate a role for local IL-10 within the vessel wall as an important regulator of vascular function.

To further explore the hypothesis that local IL-10 in arteries protects vascular function during inflammation, we examined the effects of gene transfer of IL-10 to carotid arteries from IL-10-deficient mice. After incubation with LPS, responses to acetylcholine in arteries from IL-10-deficient mice incubated with AdIL-10 were not impaired. In contrast, responses to acetylcholine in arteries incubated with a control virus were impaired after LPS, similar to responses of IL-10-deficient vessels without gene transfer. Thus reconstitution of IL-10 via gene transfer to arteries ex vivo protected against impairment of endothelium-dependent relaxation by LPS in arteries from IL-10-deficient mice.

IL-10 and iNOS in inflammation. Adenovirus produces inflammation, but we showed in previous studies (32) that vasomotor function was the same in blood vessels incubated with and without control adenoviruses. The similar results in experiments using both in vitro and in vivo techniques minimize the likelihood that results are affected by inflammatory effects of adenovirus.

In cells in culture and in vessels after systemic treatment with LPS, IL-10 appears to inhibit expression of iNOS (12, 20, 34). Recent evidence suggests that iNOS is a major mediator of vascular dysfunction during inflammation (9, 14, 17, 21, 26, 28, 33). Although it is clear from previous studies that iNOS impairs NO-dependent relaxation (4, 13, 19, 21, 28, 33), mechanisms that mediate this effect are not defined. We used iNOS-deficient mice and aminoguanidine, which is a relatively selective inhibitor of iNOS (15), to examine the role of iNOS in these studies.

A higher concentration of LPS ex vivo was required to impair vasomotor function in arteries from WT mice than in IL-10-deficient mice. Consistent with previous studies, when sufficient LPS was used, vascular dysfunction coincided with expression of iNOS in arteries from WT mice (16). In contrast to arteries from WT mice, arteries from iNOS-deficient mice did not develop endothelial dysfunction after high-dose LPS. These data are consistent with effects of LPS in vivo (5) and provide direct evidence that iNOS is required for LPS to impair endothelium-dependent relaxation.

To determine whether iNOS contributes to impaired relaxation in arteries from IL-10-deficient mice after LPS, we examined effects of aminoguanidine. In previous experiments, we found (20, 21) that responses of normal carotid arteries to acetylcholine were not inhibited by aminoguanidine, which implies that aminoguanidine at the concentration used did not inhibit eNOS. In the present study, aminoguanidine in the organ bath did not reverse endothelial dysfunction, which suggests that continuous iNOS activity is not responsible for impairment. To determine whether iNOS might be involved in

![Figure 6](http://ajpheart.physiology.org/)

Fig. 6. Responses to acetylcholine were impaired in arteries from wild-type mice (B) after high dose of LPS (250–400 μg/ml; n = 6, *P < 0.05 vs. vehicle). Responses were not impaired by LPS in arteries from inducible NO synthase (iNOS)-deficient mice (A; n = 6, P > 0.05).

![Figure 7](http://ajpheart.physiology.org/)

Fig. 7. RT-PCR demonstrates expression of IL-10 in carotid arteries from IL-10-deficient mice after adenoviral gene transfer with AdIL-10, but not AdLacZ. Expression of β-actin in all samples confirmed integrity of RNA recovery in samples that were negative for IL-10. Left lane in each panel is a negative reagent control.
the pathway that leads to impaired relaxation after LPS, we studied vessels that were incubated overnight in LPS with aminoguanidine. Our data suggest that the presence of aminoguanidine during incubation (i.e., during early responses to LPS) protected against development of endothelial dysfunction but did not correct endothelial dysfunction once it was present. The present results examining relaxation are concordant with results from an earlier study, in which NO appeared to be important for initiating impaired contractile responses after LPS, but not for maintaining impairment (8). Thus findings in this and a previous study (8) suggest that iNOS may contribute primarily to early inflammatory responses in vessels, but perhaps not to later responses. These data also support the hypothesis that vascular IL-10 protects vasomotor function during inflammation, at least in part by inhibiting effects of iNOS.

In summary, intravenous adenosine-mediated gene transfer of IL-10 protects against impairment of endothelium-dependent relaxation produced by systemic inflammation (LPS). Perhaps the most important finding in this study is the new concept that expression of IL-10 within the wall of blood vessels protects endothelium-dependent relaxation during inflammation. In addition, pharmacological inhibition of iNOS in arteries from IL-10-deficient mice and the absence of iNOS in iNOS-deficient mice protect arteries from impaired relaxation produced by LPS. Thus we conclude that one mechanism by which IL-10 within vascular cells preserves vascular function during inflammation is by decreasing impairment produced by iNOS, an important mediator of vascular dysfunction. Because inflammation is emerging as an important common denominator of vascular dysfunction in several cardiovascular diseases, including atherosclerosis and diabetes, mechanisms by which inflammation can be modified in blood vessels are of great interest and importance.

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