IL-10 deficiency increases superoxide and endothelial dysfunction during inflammation

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Gunnnett, Carol A., Donald D. Heistad, Daniel J. Berg, and Frank M. Faraci. IL-10 deficiency increases superoxide and endothelial dysfunction during inflammation. Am J Physiol Heart Circ Physiol 279: H1555–H1562, 2000.—Little is known about the role of interleukin-10 (IL-10), an anti-inflammatory cytokine, in blood vessels. We used IL-10-deficient mice (IL-10−/−) to examine the hypothesis that IL-10 protects endothelial function after lipopolysaccharide (LPS) treatment. The responses of carotid arteries were studied in vitro 6 h after injection of a relatively low dose of LPS (10 μg ip). In IL-10−/− mice, the maximum relaxation to ACh (3 μM) was 56 ± 6% (means ± SE) after LPS injection and 84 ± 4% after vehicle injection (P < 0.05). Thus endothelium-dependent relaxation was impaired in carotid arteries from IL-10−/− mice after LPS injection. In contrast, this dose of LPS did not alter relaxation to ACh in vessels from wild-type (IL-10+/-) mice. Relaxation to nitroprusside and papaverine was similar in arteries from both IL-10−/− and IL-10+/- mice after vehicle or LPS injection. Because inflammation is associated with increased levels of reactive oxygen species, we also tested the hypothesis that superoxide contributes to the impairment of endothelial function by LPS in the absence of IL-10. Results using confocal microscopy and hydroethidine indicated that levels of superoxide are elevated in carotid arteries from IL-10−/− mice compared with IL-10+/- mice after LPS injection. The impaired relaxation of arteries from IL-10−/− mice after LPS injection was restored to normal by polyethylene glycol-suspended superoxide dismutase (50 U/ml) or allopurinol (1 mM), an inhibitor of xanthine oxidase. These data provide direct evidence that IL-10 protects endothelial function after an acute inflammatory stimulus by limiting local increases in superoxide. The source of superoxide in this model may be xanthine oxidase.

nitric oxide; reactive oxygen species; gene-targeted mice; endothelium-dependent relaxation; interleukin-10

LIPOLYSACCHARIDE (LPS) produces changes in gene expression within the vessel wall and alters function of blood vessels. These responses include the expression of the inducible isoform of nitric oxide (NO) synthase (iNOS), impaired vasoconstriction, and impaired endothelium-dependent relaxation (17, 18, 38, 46). We and others (17, 18, 33, 43, 45) have provided evidence that impaired vasoconstriction after LPS administration is due to the expression of iNOS. In the present study, we examined mechanisms that modulate endothelium-dependent relaxation.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that plays a key role in systemic responses to LPS (4, 17, 20). Very little is known, however, about the role of IL-10 in blood vessels. A recent study in IL-10 gene-targeted mice suggested that IL-10 plays an important role by limiting the expression of iNOS and, thus, impairment of vasoconstrictor responses (17). Because IL-10 can attenuate expression and/or production of proinflammatory cytokines (4, 11, 13–15, 30, 35), which impair endothelial function (5, 24, 38), we hypothesized that IL-10 would also protect endothelial function during inflammation. Thus the first goal of this study was to examine the effects of LPS on endothelium-dependent relaxation in wild-type (IL-10+/-) mice and IL-10-deficient (IL-10−/−) mice.

Increased levels of superoxide are known to impair endothelium-dependent relaxation under some pathological conditions (22, 23, 32). There are several potential sources of superoxide in blood vessels, including xanthine oxidase (7, 21, 44). Recent studies suggest that xanthine oxidase is an important source of superoxide in vessels during hypercholesterolemia/atherosclerosis (8, 16, 49) and hypertension (44). LPS increases the expression of xanthine oxidase and superoxide levels in blood vessels from normal animals (7, 19, 26). Thus a second goal of this study was to examine the hypothesis that impaired endothelial function after LPS injection in IL-10−/− mice is due to increased levels of superoxide. We also examined the possibility that the source of superoxide in IL-10−/− mice after LPS treatment is xanthine oxidase.

METHODS

Animals. IL-10−/- mice were generated on a C57Bl/6/129-Ola background at Simonsen Laboratory (Gilroy, CA). Mice in our colony have been backcrossed for more than ten generations onto the C57Bl/6 strain to yield mice with a C57BL/6-defined background. Therefore, we used C57BL/6 mice as wild-type controls (IL-10+/-) in these experiments.

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Male and female mice (8–16 wk old) were randomly assigned to receive either LPS (10 μg ip) or vehicle (saline). This relatively low dose of LPS was chosen because IL-10−/− mice have an increased sensitivity to LPS (4, 17), and although this dose of LPS produced no measurable changes in vascular function in IL-10+/+ mice, this dose produced a significant impairment in the function of vessels from IL-10−/− mice (17).

**Vascular function.** Six hours after treatment with the vehicle or LPS, mice were anesthetized with pentobarbital sodium (75–100 mg/kg ip). The carotid arteries were removed and immediately placed in cold, oxygenated Krebs buffer with the following ionic composition (in mmol/l): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11 glucose. Loose connective tissue covering the adventitia was removed, and each carotid artery was cut into two rings (3–4 mm in length). Each carotid ring was mounted between two stirrup-shaped support hooks and suspended in organ baths containing 25 ml of Krebs solution maintained at 37°C and bubbled with a mixture of 95% O₂-5% CO₂. One stirrup was connected to a stationary bracket, and the other stirrup was connected to a force transducer to measure isometric tension. Optimal resting tension for these vessels was determined by the endothelial isoform of NO synthase (eNOS). Endothelium-independent vasorelaxation was evaluated using sodium nitroprusside (10 nM–1 mM) and papaverine (10 nM–300 µM).

In some experiments, pharmacological agents were added to the organ baths before administration of U-46619 and the subsequent vasodilators. To determine whether superoxide was involved in impaired vasodilation, superoxide dismutase [in suspension with polyethylene glycol (PEG-SOD)] (50 U/ml) or the superoxide scavenger Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid) (1 mM) was added before testing the effects of acetylcholine or nitroprusside. Some experiments were performed in the presence of indomethacin (10 μM) to determine whether the activity of cyclooxygenases contributed to the impaired responses to acetylcholine in vessels from IL-10−/− mice. Because LPS is known to increase the expression or activity of xanthine oxidase and levels of superoxide (7), we also examined the effects of allopurinol (1 mM), a xanthine oxidase inhibitor, in some experiments.

**Detection of superoxide.** Hydroethidine was used to detect superoxide in the vessel wall. In the presence of superoxide, hydroethidine is oxidized to red-fluorescent ethidium bromide, which is trapped in cells by intercalation with DNA (42). Thus, hydroethidine staining is a measure of intracellular superoxide. Oxidation of hydroethidine is specific for superoxide (3, 32) and does not occur in the presence of other reactive oxygen species (6).

Unfixed vessels were frozen, cut into 10-μm-thick sections, and mounted on glass slides. Hydroethidine (10 μM) was applied topically to the sections, and samples were then covered with a coverslip. In some experiments, sections were preincubated with PEG-SOD (30 min) and PEG-SOD was then coapplied with hydroethidine. Slides were incubated for 2 h at 37°C. Images were obtained using a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected using a 585-nm long-pass filter. Ethidium bromide is excited at 488 nm with an emission spectrum of 610 nm. Control and treatment vessels were examined in parallel. Laser settings were maintained constant throughout each experiment. We have used this technique previously (28, 32).

**Drugs.** Lipopolysaccharide (from *Escherichia coli*), acetylcholine, sodium nitroprusside, papaverine, PEG-SOD, Tiron, indomethacin, and allopurinol were obtained from Sigma Chemical (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. Allopurinol was dissolved in 1 N NaOH and then diluted in normal saline. The pH of the allopurinol solution was adjusted by using 1 N HCl. All other drugs were dissolved and diluted in normal saline. All of the concentrations were expressed as the 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. The relaxation responses to acetylcholine and sodium nitroprusside were expressed as the percentage of relaxation from precontraction to U-46619.

**RESULTS**

**Vascular responses in IL-10+/+ mice.** U-46619 produced concentration-dependent contraction in vessels from IL-10+/+ mice (data not shown). Contraction was similar in carotid arteries from IL-10+/+ mice treated with LPS and vehicle. These results are similar to our previous finding (17).

The relaxation produced by acetylcholine was concentration dependent and reached a maximum of >85% in vessels from IL-10+/+ mice injected with either LPS or vehicle (Fig. 1A). The vasorelaxation in response to nitroprusside reached a maximum of almost 100% in arteries from either LPS- or vehicle-injected IL-10+/+ mice (Fig. 1B). Relaxation to papaverine was also similar in vessels from IL-10+/+ mice treated with LPS or vehicle (data not shown). These findings suggest that endothelium-dependent and -independent relaxation in carotid arteries from IL-10+/+ mice were not altered after treatment with a relatively low dose of LPS.

Incubation with PEG-SOD (50 U/ml) or indomethacin (10 μM) for 45 min had no effect on the responses to acetylcholine or nitroprusside in vessels from IL-10+/+ mice (data not shown). Allopurinol had no effect on the responses to U-46619 or nitroprusside in vessels in IL-10+/+ mice.

**Vascular responses in IL-10−/− mice.** Carotid arteries from IL-10−/− mice contracted in a concentration-dependent manner to U-46619 (data not shown). As we (17) reported previously, contraction was less in vessels from IL-10−/− mice than from IL-10+/+ mice after LPS treatment. Despite these differences, we produced a similar level of precontraction with U-46619 in arteries from both IL-10+/+ and IL-10−/− mice in these studies of vasorelaxation.
The impaired relaxation of carotid arteries from IL-10 −/− mice in response to acetylcholine was restored to normal by 45 min of incubation in vitro with PEG-SOD (50 U/ml) (Fig. 3). Similarly, the relaxation of the carotid artery from IL-10 −/− mice in response to acetylcholine was improved by the superoxide scavenger Tiron (1 mM) (data not shown). Tiron and PEG-SOD also normalized the slightly impaired response to the intermediate dose of nitroprusside in carotid arteries from IL-10 −/− mice after LPS injection. These findings suggest that superoxide mediates the impairment of responses to acetylcholine and nitroprusside in vessels from IL-10 −/− mice after LPS treatment.

Incubation of arteries from LPS-treated IL-10 −/− mice with allopurinol (1 mM), an inhibitor of xanthine oxidase, also improved the relaxation in response to acetylcholine (Fig. 4B). Allopurinol produced a modest increase in sensitivity to low concentrations of acetylcholine in vessels from both IL-10 +/+ and IL-10 −/− mice after LPS treatment but improved the responses to higher concentrations of acetylcholine only in vessels from IL-10 −/− mice after LPS injection (Fig. 4B). Allopurinol had no effect on responses to U-46619 or nitroprusside in vessels from IL-10 −/− mice. Although we cannot rule out a potential contribution from other enzymes that produce superoxide, these findings with allopurinol suggest that xanthine oxidase may be an important source of superoxide in vessels from IL-10 −/− mice injected with LPS. The responses to acetylcholine after LPS injection in vessels from IL-10 −/− mice were not improved by indomethacin (10 μM), which suggests that cyclooxygenase enzymes do not contribute to the impaired responses in vessels from IL-10 −/− mice (data not shown).

Confocal microscopy using hydroethidine. Hydroethidine fluorescence was not detectable in vessels from IL-10 +/+ mice injected with the vehicle (Fig. 5, bottom left). Arteries from IL-10 +/+ mice that were injected with LPS appeared to exhibit somewhat more fluorescence than arteries from vehicle-treated mice, but the intensity of fluorescence remained relatively low (Fig. 5, bottom middle). Vessels from vehicle-treated IL-10 −/− mice also displayed low-intensity fluorescence. Fluorescent intensity, however, was markedly increased in vessels from IL-10 −/− mice after LPS treatment (Fig. 5, top middle). The fluorescent signal was distributed fairly uniformly within the vessel wall, which suggests that superoxide may be present in multiple cell types. Similar results were obtained in four of five different experiments. When PEG-SOD (50 U/ml) was coapplied with hydroethidine, fluorescent intensity was reduced to basal levels (data not shown). Coapplication of allopurinol (1 mM) with hydroethidine also quenched the superoxide signal in vessels from IL-10 −/− mice after LPS treatment (Fig. 5, top right).

DISCUSSION

There are three major new findings of this study. First, LPS produces impaired endothelium-dependent
Relaxation in carotid arteries from IL-10−/− mice but not from wild-type controls (IL-10+/+). Previous studies have suggested that IL-10 is a powerful immunosuppressant (1, 4, 10), but to our knowledge, this is the first direct evidence that IL-10 plays a protective role in preserving endothelial function after an inflammatory stimulus. Second, IL-10 protects endothelial function after LPS treatment by attenuating increases in superoxide in the vessel wall. This elevation of superoxide is functionally important because the scavengers of superoxide restored endothelial function to normal. Finally, the effects of allopurinol suggest that the source of superoxide that produces vascular dysfunction in IL-10-deficient animals is xanthine oxidase. These findings support the new concept that endogenous IL-10 protects endothelial function by attenuating superoxide levels during inflammation.

Impaired endothelial function in IL-10−/− mice after LPS treatment. Studies utilizing pharmacological approaches, direct measurements of NO, and gene-targeted mice have shown that acetylcholine produces relaxation of carotid arteries by activating eNOS (9, 12). The impairment of the responses to acetylcholine in vessels from IL-10−/− mice after LPS injection suggests that IL-10 normally protects endothelium-dependent relaxation during acute inflammation. We also observed a very modest impairment of responses to nitroprusside in vessels from IL-10−/− mice after LPS injection, but the effect on the responses to acetylcholine was much greater. Because relaxation to papaverine is normal in arteries from IL-10−/− after LPS treatment, the impairment appears to be specific for the NO-mediated pathway.

Previously, our laboratory and others have shown that vascular function is altered after exposure to LPS. For example, contractile responses of vessels from experimental animals and humans are impaired after...
vasoactivity is impaired in normal animals after LPS treatment (17, 18, 33, 45, 46). Vasorelaxation to acetylcholine also is impaired in normal animals after inflammatory stimuli including LPS (38). The lack of impairment in carotid arteries from IL-10 +/+ mice in these studies is presumably related to our choice of a relatively low dose of LPS that has minimal effects in normal mice (4, 17). We chose this dose of LPS to avoid mortality of IL-10 −/− mice, which have a heightened sensitivity to LPS (4, 17).

Mechanism of impairment. When generated by eNOS, NO is known to produce a relaxation of vascular muscle by activation of soluble guanylate cyclase (12) and cGMP-dependent protein kinase (40). One mechanism that could account for the impairment of eNOS-mediated relaxation is the decreased availability of NO for activation of soluble guanylate cyclase due to scavenging of NO by superoxide. Because superoxide readily binds to NO (2), conditions that produce elevated superoxide levels in blood vessels are associated with impaired eNOS-dependent relaxation (22, 31, 47, 48). For example, impaired endothelium-dependent relaxation in atherosclerosis and diabetes is improved by SOD (31, 32). In the present study, the restoration of endothelium-dependent relaxation by PEG-SOD and Tiron provides strong evidence that the impairment is mediated by superoxide in arteries from LPS-treated IL-10 −/− mice.

In addition to using PEG-SOD and Tiron, we examined the relative levels of superoxide in the vessel wall using confocal microscopy and hydroethidine. We and others (28, 32, 44) have used this method previously to detect superoxide in blood vessels. The findings with hydroethidine staining were consistent with our pharmacological data and indicate that superoxide levels are higher in blood vessels from IL-10 −/− mice after LPS treatment than in vessels from IL-10 −/− mice injected with the vehicle. Several lines of evidence, including the quenching of the fluorescent signal by incubation with PEG-SOD in the present study, have provided evidence that the assay is specific for superoxide (3, 32).

In the present study, it is interesting to note that the hydroethidine signal was distributed throughout the vessel wall rather than being limited to a specific region (such as the endothelium, media, or adventitia). Thus elevation of the local superoxide levels in response to LPS may occur in multiple cell types. In contrast to the responses to acetylcholine, the responses to nitroprusside were nearly normal in vessels from IL-10 −/− mice after LPS treatment. This finding might seem surprising because superoxide appears to be present in smooth muscle as well as in the endothelium. One explanation for this apparent paradox is the possibility that subcellular production of NO in smooth muscle cells from nitroprusside may occur in close proximity to soluble guanylate cyclase (the key molecular target for NO in producing vasorelaxation), precluding the interaction of superoxide with NO. In contrast, NO from eNOS must traverse two plasma membranes and the intracellular space between the endothelium and smooth muscle before activation of guanylate cyclase within smooth muscle. It is noteworthy that there are many examples in which responses to acetylcholine and other endothelium-dependent agonists are impaired, whereas responses to nitroprusside or other NO donors are normal in models in which vascular superoxide levels are elevated (in hypertension, atherosclerosis, and diabetes, for example). In at least some studies, superoxide appears to be elevated in smooth muscle in addition to other components of the vessel wall (28, 32).

Our findings that IL-10 protects eNOS-mediated relaxation by attenuating increases of superoxide in the vessel wall are consistent with previous findings related to reactive oxygen species and IL-10. IL-10 is known to inhibit production of reactive oxygen species in monocytes and neutrophils (25, 27). IL-10 also inhibits production of other cytokines (13, 34) that are known to stimulate production of reactive oxygen species in endothelial cells (36, 41) as well as in leukocytes.

One potentially important source of superoxide in vessels is xanthine oxidase. The production of super-
Oxide by xanthine oxidase appears to contribute to vascular dysfunction after treatment with LPS (7) and during hypertension (44) and atherosclerosis (8, 49). Because proinflammatory cytokines activate xanthine oxidase in tissue culture (37, 39), and because IL-10 attenuates increases in concentrations of proinflammatory cytokines (4), we speculate that the absence of IL-10 could enhance the activation of xanthine oxidase after LPS treatment. Thus we used allopurinol to examine the potential role of xanthine oxidase in impaired endothelium-dependent relaxation in arteries from IL-10−/− mice after LPS treatment.

Incubation of vessels from both LPS-treated IL-10+/+ and IL-10−/− mice with allopurinol, an inhibitor of xanthine oxidase, tended to enhance the responses to low concentrations of acetylcholine. These results suggest that low levels of superoxide may be generated by xanthine oxidase in vessels in IL-10+/+ mice as well as in IL-10−/− mice after LPS treatment. However, the responses to higher concentrations of acetylcholine were improved by allopurinol only in vessels from IL-10−/− mice after LPS treatment. These results suggest that low levels of superoxide in vessels from IL-10+/+ mice after LPS treatment are not sufficient to impair the NO-mediated responses produced by higher concentrations of acetylcholine. The effects of allopurinol appear to be specific for endothelium-dependent relaxation because the inhibitor of xanthine oxidase had no effect on the responses to U-46619 or to nitroprusside.

Cyclooxygenase enzymes are another potential source of superoxide in blood vessels. Data using indomethacin, however, suggest that cyclooxygenase activity does not contribute to the impaired responses to acetylcholine in vessels from IL-10−/− mice after LPS treatment. Although we cannot exclude the possibility that other enzymes produce superoxide in vessels from IL-10−/− mice after LPS treatment, both the functional data and findings with hydroethidine suggest that xanthine oxidase is a major source of superoxide in carotid arteries after treatment with LPS.

Fig. 5. Fluorescent confocal micrographs of sections of carotid artery labeled with the oxidative dye hydroethidine (which fluoresces red when oxidized to ethidium bromide by superoxide). Fluorescent intensity is remarkably elevated in vessel sections from IL-10−/− mice (top) after LPS (top center) vs. vehicle (top left) injection (representative of 5 experiments). Topical application of allopurinol to vessel sections attenuated the fluorescent signal. Fluorescent intensity remained constant in vessel sections from IL-10+/+ mice (bottom) after vehicle (left), LPS (middle), or LPS + allopurinol (right).
In summary, the findings from this study using genetargeted mice provide evidence that IL-10 attenuates responses that produce local increases in superoxide and endothelial dysfunction during inflammation. Results with allopurinol suggest that the source of increased superoxide is xanthine oxidase. The present data provide new insight into the role of IL-10 in blood vessels during acute inflammation. Evidence in a recent study using IL-10−/− mice suggested that IL-10 also plays a critical role in the development of atherosclerotic lesions (29). Our data are consistent with a protective role for IL-10 in blood vessels. Because several disease states such as atherosclerosis, hypertension, and ischemia appear to have an inflammatory component, the current finding may have broader implications for vascular disease. We speculate that IL-10 may have similar protective effects during chronic inflammation.

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VASCULAR FUNCTION IN IL-10-DEFICIENT MICE


