Vascular Effects of Lipopolysaccharide Are Enhanced in Interleukin-10–Deficient Mice

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Background and Purpose—The role in blood vessels of interleukin-10 (IL-10), a potent anti-inflammatory cytokine, is not known. Using mice with targeted deletion of the gene for IL-10 (IL-10⁻/⁻), we examined the hypothesis that IL-10 is a major modulator of the vascular effects of lipopolysaccharide (LPS).

Methods—We examined in vitro responses of carotid arteries obtained from wild-type (129/SvEv or C57BL/6; IL-10⁺/⁺) and IL-10–deficient mice 6 hours after injection of a relatively low dose of LPS (10 μg).

Results—Contraction of the carotid artery in response to U46619 was impaired in IL-10–deficient mice treated with LPS compared with LPS-treated controls. After LPS, U46619 (0.03 and 0.1 μg/mL) contracted the carotid artery by 0.11±0.02 (mean±SEM) and 0.38±0.03 g in wild-type (n=10) and 0.03±0.01 and 0.19±0.03 g in IL-10–deficient (n=8) mice (P<0.05 versus control). Aminoguanidine, an inhibitor of inducible nitric oxide synthase (iNOS), had no significant effect on contraction of the carotid artery from LPS-treated control mice but restored contraction of the carotid artery in response to U46619 in IL-10–deficient mice to levels seen in wild-type mice. Similar findings were obtained when phenylephrine was used as a vasoconstricting agent. These findings indicate that LPS produces much greater impairment of contractile responses of the carotid artery in IL-10–deficient mice than in control mice. Impaired contractile function was eliminated by aminoguanidine, suggesting that expression of iNOS is enhanced in arteries from IL-10–deficient mice. In carotid arteries from animals injected with LPS, reverse transcription–polymerase chain reaction (RT-PCR) products for iNOS were found more frequently in IL-10–deficient mice than in wild-type mice. RT-PCR products for iNOS were not present in arteries from vehicle-treated animals (IL-10–deficient or wild-type mice).

Conclusions—This is the first evidence that endogenous IL-10 is a major determinant of the effects of LPS on vascular tone. The results suggest that impaired constrictor responses of the carotid artery after LPS in IL-10–deficient mice are mediated by enhanced expression of iNOS. (Stroke. 1999;30:2191-2196.)

Key Words: nitric oxide ■ inducible NO synthase ■ acetylcholine ■ endothelium

Interleukin-10 (IL-10) is a potent immunosuppressant (anti-inflammatory cytokine) and thus may be an important modulator of acute and chronic inflammation.¹⁻⁵ Endogenously produced IL-10 may provide a negative feedback mechanism to limit production of proinflammatory cytokines.¹⁻⁵ For example, increases in serum levels of tumor necrosis factor-α (TNF-α) after treatment with lipopolysaccharide (LPS) are much greater in IL-10–deficient mice than in controls.² Although IL-10 is known to exhibit anti-inflammatory properties, the role of IL-10 in blood vessels is not known.

The recent generation of mice with targeted disruption of the IL-10 gene (IL-10⁻/⁻)¹⁶ provides the opportunity for a novel approach to examine the role of IL-10 in vascular biology. On the basis of previous studies, we hypothesized that IL-10 may be a key modulator of the vascular effects of LPS. Thus, the first goal of this study was to examine the effect of LPS on vascular function in IL-10–deficient mice and wild-type controls. Our hypothesis was that impairment of vascular function after treatment with LPS would be enhanced in IL-10–deficient mice.

Impaired vasoconstriction after treatment with LPS in arteries from normal animals is mediated by expression of the inducible isoform of nitric oxide synthase (iNOS).⁷⁻¹⁰ This alteration in vascular function can be restored toward normal with inhibitors of iNOS, including aminoguanidine,⁸,¹⁰ and is absent in iNOS-deficient mice.⁸ Exogenous IL-10 can inhibit expression of iNOS in cells in culture.³ Thus, the second goal of the present study was to examine the hypothesis that impaired contraction of the carotid artery after treatment with LPS in mice lacking the gene for IL-10 can be restored toward normal with aminoguanidine.

Materials and Methods

Animals
IL-10–deficient mice were produced as described previously.²,⁶ These mice have been backcrossed >10 times onto both 129/SvEv-
and C57BL/6-defined backgrounds. Both strains of mice were used in these experiments, and similar results were obtained with both strains. C57BL/6 and 129/SvEv mice were used as wild-type controls for experiments on C57BL/6 IL-10−deficient and 129/SvEv IL-10−deficient mice, respectively.

Mice (14 to 24 g) were randomly assigned to receive either vehicle or LPS (10 μg IV or IP). This dose of LPS was used because previous studies have shown that IL-10−deficient mice exhibit enhanced sensitivity to LPS.2

Six hours after injection of LPS or vehicle, mice were anesthetized with pentobarbital (75 to 100 mg/kg IP), and both carotid arteries were quickly removed. A 6-hour time point was chosen on the basis of the time course of known effects of LPS in IL-10−deficient mice.2 After removal, arteries were placed in Krebs buffer with the following ionic composition (mmol/L): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and glucose 11. Loose connective tissue in the adventitia was removed, and each carotid artery was cut into 2 rings 3 to 4 mm in length. Vascular rings were suspended in an organ bath containing 25 mL Krebs solution maintained at 37°C. The rings were connected to a force transducer to measure isometric tension (contraction and relaxation). Resting tension was increased stepwise to reach the final tension of 0.2 to 0.25 g, and the rings were allowed to equilibrate for at least 60 minutes. Preliminary studies indicated that this amount of resting tension was optimal for contraction in these arteries. We have used these methods previously to study responses in aorta and carotid arteries from mice in vitro.8,11,12,13

Protocols

We examined contractile responses of carotid arteries from wild-type and IL-10−deficient mice to the thromboxane analogue U46619 (9,11-dideoxy-11-carbomethoxy-11α,9α-epoxy-prostaglandin F2α). U46619 produced greater maximal contraction of mouse carotid arteries and aorta than high concentrations of KCl. In other experiments, we examined contractile responses of carotid arteries to phenylephrine.

To provide pharmacological evidence that iNOS may contribute to impaired contraction of the carotid artery after treatment with LPS, some vessel rings were treated with aminoguanidine (1 mmol/L). Aminoguanidine is a relatively selective inhibitor of iNOS and has frequently been used for this purpose.9,9,14–16

To determine whether targeted disruption of the IL-10 gene alters vascular function in the absence of treatment with LPS, carotid arteries were harvested from additional wild-type and IL-10 mice. In these arteries, we examined contraction to U46619 and relaxation in response to acetylcholine (an endothelium-dependent agonist). We have shown previously that relaxation of the carotid artery in response to acetylcholine is mediated by the endothelial isoform of NO synthase (eNOS).13 At the end of each experiment, maximum constrictor responses to U46619 were obtained.

Reverse Transcription Coupled With Polymerase Chain Reaction

Our functional studies (results described below) were performed with IL-10−deficient and wild-type mice on both C57BL/6 and 129/SvEv backgrounds. Similar results were obtained in both strains of mice. Because of better availability of animals, the following experiments using reverse transcription–polymerase chain reaction (RT-PCR) were performed with IL-10−deficient and wild-type mice on a C57BL/6J background.

Total RNA was extracted from carotid arteries from 28 mice (IL-10−deficient or wild-type mice injected with vehicle or LPS) according to the method described in detail previously.8 RNA (0.5 to 1 μg) was reverse-transcribed to produce cDNA with random hexamers used as primers. Four microliters of RT product was used for the PCR reaction. As a positive control for mRNA, all samples were run with primers for a housekeeping gene, β-actin. A plasmid containing cDNA for mouse iNOS was used as a positive control for PCR.

The forward primer for iNOS was 5′-TGGAGAGGGCGAGCTAC-TGGG-3′ (No. 2587-2606, M84373 in Genbank). The reverse primer for iNOS was 5′-TGTGTCCTGGGAGGACT-3′ (No. 2897-2915). The expected length of the amplification product was 330 bp. The 5′ primer for β-actin was 5′-GAGAAGATGACCCAGATCATG-3′ and the 3′ primer was 5′-GGGCGACGTCGAAGTCT-3′, as modified previously.8 The expected length of the amplification product was 350 bp.

Statistical Analysis

All data are expressed as mean±SEM. Comparisons were made by either an unpaired t test or ANOVA, as appropriate. Statistical significance was accepted at P<0.05. Relaxation responses to acetylcholine were expressed as the percent relaxation from the amount of precontraction produced by PGF2α.

Results

Vascular Responses in Wild-Type (IL-10+/+) Mice

In vessels from wild-type mice treated with vehicle, U46619 produced concentration-dependent contraction. Contraction of the carotid artery was similar in vehicle-treated and LPS-treated wild-type mice. For example, in wild-type mice, U46619 (0.1 μg/mL) contracted the carotid artery by 0.36±0.06 g (n=7) after vehicle and 0.38±0.03 g (n=10) after LPS. Maximum contraction of the carotid artery in response to U46619 (0.3 μg/mL) was similar in vehicle- and LPS-injected mice (0.57±0.06 versus 0.51±0.04 g, respectively). Similar to the findings with U46619, treatment with LPS did not inhibit contraction of the carotid artery in response to phenylephrine in wild-type mice. For example, 10 μmol/L phenylephrine contracted the artery by 0.11±0.05 and 0.14±0.02 g in vehicle- and LPS-treated wild-type mice, respectively.

Contractile responses of the carotid artery to U46619 after treatment with LPS in wild-type mice was similar in the absence and presence of aminoguanidine [0.38±0.03 (n=10) versus 0.41±0.03 g (n=9)]. Aminoguanidine also did not alter vasoconstriction in response to U46619 in wild-type mice treated with vehicle. For example, 0.3 μg/mL U46619 contracted the carotid artery by 0.57±0.06 and 0.58±0.05 g in the absence and presence of aminoguanidine, respectively.

Vascular Responses in IL-10−Deficient (IL-10−/−) Mice

Contraction of the carotid artery in vehicle-treated IL-10−deficient mice (Figure 1) was similar to that observed in vehicle-treated wild-type mice. Thus, the absence of the IL-10 gene per se does not alter contraction of the carotid artery under normal conditions. In contrast to findings in wild-type mice, treatment with LPS produced marked impairment of contraction of the carotid artery from IL-10−deficient mice (Figure 1). Maximum contraction elicited by 0.3 μg/mL U46619 was 0.56±0.10 and 0.38±0.05 g in arteries from vehicle- and LPS-injected mice, respectively (P<0.05 versus vehicle). Contractile responses of the carotid artery in response to phenylephrine were also impaired in IL-10−deficient mice treated with LPS. For example, 10 μmol/L phenylephrine contracted the artery by 0.14±0.02 and 0.09±0.02 g in vehicle- and LPS-treated IL-10−deficient mice, respectively.

Impaired contractile responses of carotid artery from IL-10−deficient mice treated with LPS were restored essentially to normal by treatment with aminoguanidine. Results for
U46619 are shown in Figure 2. In addition, aminoguanidine also restored responses to phenylephrine to normal. For example, in carotid arteries from LPS-treated IL-10–deficient mice, phenylephrine (10 μmol/L) increased tension by 0.09 ± 0.02 and 0.14 ± 0.05 g in the absence and presence of aminoguanidine, respectively.

Reverse Transcription–Polymerase Chain Reaction

In carotid arteries from both wild-type (n = 4) and IL-10–deficient (n = 4) mice injected with vehicle, no PCR products corresponding to iNOS mRNA were detected. PCR products for β-actin were observed in all samples from vehicle-treated mice. In contrast to vehicle-treated mice, a clear PCR product corresponding to iNOS mRNA was observed in 9 of 10 LPS-treated, IL-10–deficient mice (see Figure 3 for examples from 2 mice).

In wild-type mice injected with LPS, 5 (of 10) had no detectable band corresponding to iNOS mRNA, and 4 had a very faint iNOS band (see Figure 3 for examples from 2 mice). One wild-type mouse injected with LPS had a clear iNOS PCR product. All samples from LPS-treated mice had positive β-actin bands (Figure 3).

Vasorelaxation in Response to Acetylcholine

After precontraction (to 40% to 50% of maximum in both strains of mice), acetylcholine produced concentration-dependent vasorelaxation that was similar in wild-type and IL-10–deficient mice (Figure 4). Maximum contraction of carotid artery rings to U46619 was similar in wild-type (0.65 ± 0.07 g; n = 7) and IL-10–deficient (0.64 ± 0.07 g; n = 7) mice.

Discussion

There are several major new findings in the present study. First, LPS produces greater impairment of vascular function in IL-10–deficient mice than in wild-type controls. This finding provides direct evidence that IL-10 is a key determinant of the vascular effects of LPS. Second, impaired contraction of the carotid artery after treatment with LPS in IL-10–deficient mice could be restored to normal with aminoguanidine. PCR products for iNOS were not present in arteries from animals injected with vehicle (wild-type or IL-10–deficient mice). mRNA for iNOS was detected more
Vascular Effects of LPS

Exposure to LPS is known to alter vascular function. For example, in arteries from both experimental animals and humans, LPS impairs contractile responses. Although the mechanisms that mediate this impairment are not completely defined, several lines of evidence suggest that expression of iNOS is important. iNOS is not expressed in vessels under normal conditions, but mRNA for iNOS can be detected in vessels after treatment with LPS. Impaired vasoconstrictor responses after treatment with LPS can be restored toward normal with inhibitors of iNOS, including aminoguanidine. Recent studies have shown that LPS produces impaired contraction of carotid arteries from wild-type but not iNOS-deficient mice. These findings provide direct evidence that iNOS plays an essential role in mechanisms that mediate impairment of vasoconstrictor responses after LPS treatment.

In the present study, arteries from wild-type mice treated with a relatively low dose of LPS (10 μg) had normal vasoconstrictor responses. Thus, the dose of LPS used was not sufficient to impair vascular function in wild-type mice. The finding that aminoguanidine did not alter vascular responses to U46619 in wild-type mice treated with vehicle and after treatment with LPS provides additional evidence that the dose of LPS did not alter normal vasomotor function. We chose this dose of LPS on the basis of a previous study that demonstrated that IL-10-deficient mice have greatly increased sensitivity to LPS.

Role of IL-10

In contrast to proinflammatory cytokines, which are produced in response to LPS, IL-10 is a potent anti-inflammatory cytokine. For example, increases in serum levels of TNF-α and interferon-γ after treatment with LPS are much greater in IL-10-deficient mice than in controls. In relation to vascular function, it is noteworthy that exogenous IL-10 can inhibit expression of iNOS in cells in culture, and mRNA for iNOS is increased in IL-10-deficient mice after cardiac transplantation. Serum levels of nitrate (a metabolite of NO) after LPS treatment are much greater in IL-10-deficient mice than in wild-type, consistent with increased expression of iNOS. In the present study, bands for iNOS PCR products were not detected in vehicle-treated mice and were absent or very faint in wild-type mice injected with LPS. In contrast, clear PCR products for iNOS could be detected in 90% of IL-10-deficient mice treated with LPS. These findings are consistent with the functional data that suggest that after treatment with LPS, expression of iNOS is greater in IL-10-deficient mice than in wild-type.

On the basis of these known properties, we hypothesized that IL-10 is a major determinant of the vascular effects of LPS. Contraction of the carotid artery in response to U46619 and phenylephrine was markedly impaired in LPS-treated IL-10-deficient mice compared with LPS-treated wild-type mice. Aminoguanidine had no significant effect on contraction of the carotid artery from LPS-treated wild-type mice but completely restored contraction of the carotid artery in IL-10-deficient mice to levels seen in wild-type mice.

Aminoguanidine has frequently been used as a relatively selective inhibitor of iNOS. Although we used aminoguanidine for the same purpose, we recognize that it may not be completely selective for iNOS. The finding that aminoguanidine did not augment contractile responses in the absence of LPS provides some evidence against nonspecific effects. We cannot, however, exclude the possibility that other mechanisms (in addition to expression of iNOS) may contribute to impaired vascular function after treatment with LPS in IL-10-deficient mice.

The known cellular sources of IL-10 include macrophages, monocytes, and T and B cells, and astrocytes. The limited available evidence suggests that IL-10 may also be produced within blood vessels under pathophysiological conditions, although the cell type responsible for producing IL-10 is not known. For example, mRNA for IL-10 was not detected by RT-PCR in normal arteries but was present in atherosclerotic arteries. IL-10 may also be produced by arteries after administration of LPS. Local production of IL-10 may represent an important negative feedback mechanism to limit production of proinflammatory cytokotyly, such as TNF-α and iNOS, during such conditions as atherosclerosis or in response to LPS.

While this article was in preparation, Hickey et al reported that LPS produced more hypotension and greater leukocyte-endothelial interactions in IL-10-deficient mice than in wild-type controls. Our finding that carotid arteries from IL-10-deficient mice have greater impairment of vasoconstrictor responses after treatment with LPS is consistent with the previous report of augmented hypotensive effects in response to the same stimuli.

Endothelium-Dependent Relaxation

Relaxation of the carotid artery in response to acetylcholine is known to be mediated by NO produced by eNOS. Vasorelaxation in response to acetylcholine was similar in IL-10-deficient mice and wild-type controls. Vasoconstrictor responses were also similar in the 2 groups of mice. These findings suggest that the IL-10 does not influence eNOS or endothelial function, the ability of vascular muscle to respond to NO, or contractile responses under normal conditions (in the absence of LPS).

In summary, these findings obtained by use of carotid arteries from IL-10-deficient mice provide direct evidence that IL-10 does not influence vascular tone under basal conditions but that IL-10 is a key determinant of the vascular effects of LPS. Although the carotid artery probably does not consistently in IL-10-deficient mice injected with LPS than in corresponding wild-type mice. Both these findings suggest that vascular expression of iNOS after treatment with LPS is greater in IL-10-deficient mice than in controls. Third, vasorelaxation in response to acetylcholine and vasoconstrictor responses were not altered in IL-10-deficient mice in the absence of LPS. These findings indicate that the absence of IL-10 per se does not affect endothelium-dependent relaxation or the ability of blood vessels to contract. The present study is the first direct evidence that IL-10 is a critical determinant of vascular hypocoactivity that occurs after exposure to bacterial LPS.

In summary, these findings obtained by use of carotid arteries from IL-10-deficient mice provided direct evidence that IL-10 does not influence vascular tone under basal conditions but that IL-10 is a key determinant of the vascular effects of LPS. Although the carotid artery probably does not...
contribute to regulation of cerebral blood flow, studies of effects of inflammatory stimuli on this blood vessel are relevant, because they may relate to the pathophysiology of carotid artery disease. Several pathophysiological conditions have an inflammatory component (atherosclerosis, hypertension, and diabetes, for example) and are associated with vascular dysfunction.

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**Editorial Comment**

The article by Gunnett et al explores vasomotor responses of carotid arteries to a proinflammatory xenobiotic, lipopolysaccharide endotoxin (LPS). The message of this study is that in the absence of the cytokine IL-10, carotid arteries of LPS-treated mice had diminished constriction when exposed to 2 different constrictors (TXA2 mimetic or phenylephrine); in contrast, “wild-type” vessels showed robust constrictive response by both agonists under the same condition. The authors then attempted to associate the deficiency in the contractile responses in IL-10 KO mice to the upregulation of iNOS, and hence NO, a vasodilator that probably counteracted the constrictors action. The variances between wild-type and IL-10 KO vessels were brought to bear only in the condition of exposure to LPS. So, what does this all mean in the context of stroke? First, IL-10 has been shown to reduce infarct volume in rat focal (permanent) MCAO model when administered intracerebroventricularly or intravenously after ischemia, which suggests a salutary effect of IL-10 in brain ischemia. In view of the data presented by Gunnett et al, IL-10 might act to downregulate NOS and excessive production of NO, which was reported to have deleterious effects in brain ischemia. Second, the anti-inflammatory properties of IL-10, elegantly summarized by the authors, implicate IL-10 as a risk factor for stroke from both the vascular perspective and the in situ inflammatory reaction following brain ischemia. In particular, the role of inflammation in vascular lesions (atherosclerosis, plaques) progression has been extensively studied. Thus, it is plausible that in the absence of an anti-inflammatory mediator/mechanism, accelerated vascular pathology ultimately results in increased risk of strokes. The need to “trigger” the phenomenon by LPS may represent a prevalent priming effect of infectious agents (e.g., cytomegalovirus, chlamydia, and others) that have been reported as risk factors for atherosclerotic vascular diseases and cardiovascular events. Thus, immune and infectious agents/antigens could well be key precipitators and enhancers of vascular pathology—from plaque progression to impaired vasoemotion, rupture, and thromboembolism. What is missing? A
connection of the fundamental observation in IL-10 KO (−/−) mice to humans. There is no evidence that deficiency of IL-10 exists in humans (either expression or function). There is no evidence for allele polymorphism or mutations in the human IL-10 gene in individuals who have had strokes or are at higher risk for strokes or other arteriosclerosis-based cardiovascular diseases. It would be of interest to explore IL-10 polymorphism in humans and its role in atherosclerosis and stroke.

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