

Interleukin-10 Protects Nitric Oxide–Dependent Relaxation During Diabetes

Role of Superoxide

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Interleukin (IL)-10, an anti-inflammatory cytokine, preserves endothelial function during acute inflammation. We tested the hypotheses that IL-10 plays a protective role in blood vessels during diabetes by suppressing impairment of endothelium-dependent relaxation and that protection by IL-10 is mediated by effects on superoxide (O_2^-). Streptozotocin (150 mg/kg i.p.) or citrate buffer was injected into IL-10-deficient (IL-10^{-/-}) mice and wild-type controls (IL-10^{+/+}). In IL-10^{+/+} and IL-10^{-/-} mice, blood glucose levels were ~120 mg/dl after citrate administration and ~400 mg/dl after streptozotocin administration. Vasorelaxation was examined in arteries in vitro 12–16 weeks later. Maximum relaxation to acetylcholine (30 μ mol/l) was $88 \pm 3\%$ (means \pm SE) in nondiabetic mice and $84 \pm 3\%$ in diabetic IL-10^{+/+} mice ($P > 0.05$). Thus, at this time point, diabetes did not impair endothelium-dependent relaxation in vessels in wild-type mice. In contrast, maximum relaxation in vessels from diabetic IL-10^{-/-} mice was significantly decreased ($74 \pm 5\%$) compared with nondiabetic IL-10^{-/-} mice ($93 \pm 2\%$, $P < 0.05$). Superoxide dismutase with polyethylene glycol (PEG-SOD) restored impaired responses to acetylcholine to levels seen in controls. Responses to acetylcholine also were improved by allopurinol (an inhibitor of xanthine oxidase) in vessels from diabetic IL-10^{-/-} mice. Thus, diabetes produces greater impairment of relaxation to acetylcholine in IL-10^{-/-} mice than in IL-10^{+/+} mice. These findings provide direct evidence that IL-10 impedes mechanisms of endothelial dysfunction during diabetes. Restoration of vasorelaxation with PEG-SOD or allopurinol suggests that the mechanism(s) by which IL-10 preserves endothelium-dependent vasorelaxation involves O_2^- , perhaps by reducing production of O_2^- by xanthine oxidase. *Diabetes* 51:1931–1937, 2002

Endothelium-dependent vasorelaxation is impaired by proinflammatory cytokines during inflammation (1,2). Interleukin (IL)-10 attenuates expression and/or production of proinflammatory cytokines (3). IL-10 preserves vascular function, including endothelium-dependent relaxation, during acute inflammation produced by endotoxin (4,5). We hypothesized that IL-10 is also important for preservation of endothelium-dependent relaxation during diabetes.

Endothelium-dependent relaxation is impaired in humans with type 1 or type 2 diabetes (6,7) and also in animal models of diabetes (8,9). Diabetes has several indexes of inflammation. Circulating levels of proinflammatory cytokines are elevated in hyperglycemia or diabetes (10,11). Nuclear factor- κ B (NF κ B), a key transcription factor that regulates expression of many inflammation-related genes, is activated by hyperglycemia in smooth muscle and endothelial cells in culture (12,13) and during diabetes in both endothelium and vascular muscle (14). Thus, the first goal of this study was to examine the anti-inflammatory effects of IL-10 on endothelium-dependent relaxation during diabetes using wild-type (IL-10^{+/+}) and IL-10-deficient (IL-10^{-/-}) mice.

Inflammatory conditions and proinflammatory cytokines are also known to stimulate production of reactive oxygen species in endothelial cells and blood vessels (15,16). Xanthine oxidase, which produces superoxide (O_2^-) in response to proinflammatory cytokines in cells in culture (17), appears to be an important source of O_2^- in vessels during hypercholesterolemia and atherosclerosis (18), during hypertension (19), and in response to lipopolysaccharide (LPS) (20). Because increased levels of O_2^- impair endothelium-dependent relaxation during diabetes (21,22), a second goal of these studies was to determine whether xanthine oxidase contributes to impaired endothelium-dependent relaxation during diabetes in IL-10^{-/-} mice.

RESEARCH DESIGN AND METHODS

Animals. IL-10-deficient mice (IL-10^{-/-}) were generated originally on a C57BL/6J129-Ola background at Simonsen Laboratory (Gilroy, CA). 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 wild-type controls (IL-10^{+/+}) in these experiments.

Experimental procedures. Male and female mice (8–16 weeks old) were randomly assigned to receive either streptozotocin (150 mg/kg i.p.) or vehicle (citrate buffer). Although repeated low doses of streptozotocin are sometimes

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eNOS, endothelial isoform of nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; O_2^- , superoxide; PEG-SOD, superoxide dismutase with polyethylene glycol; sGC, soluble guanylate cyclase.

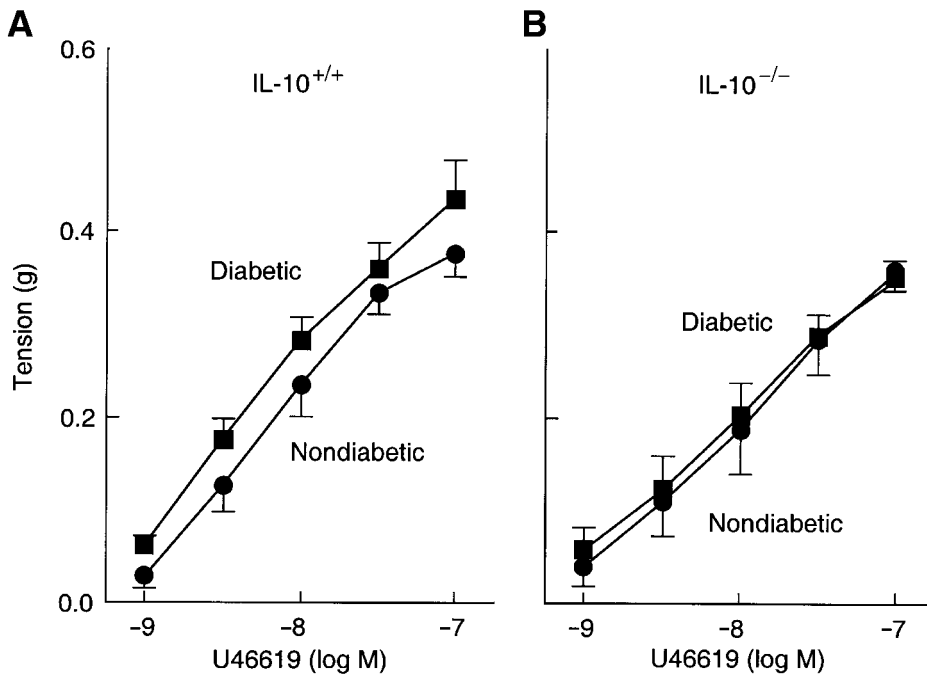


FIG. 1. Responses of the carotid artery to U46619 in IL-10^{+/+} (A) and IL-10^{-/-} (B) mice after vehicle (nondiabetic) or streptozotocin (diabetic) administration. Responses between treatments were not different; $P > 0.05$ ($n = 20$).

used to produce diabetes in mice, our preliminary experiments indicated that a single dose of 150 mg/kg was effective in producing hyperglycemia in ~70% of the mice. Thus, we used the single high-dose regimen throughout these studies. Mice that did not become diabetic after streptozotocin administration were used as nondiabetic controls. No differences were found between responses of vessels from mice that were normoglycemic after administration of streptozotocin or citrate buffer.

Vascular function. Vasomotor function of carotid arteries was examined in vitro 12–16 weeks after vehicle or streptozotocin administration. Based on preliminary data, we intentionally chose a duration of diabetes that had modest effects on vascular function in wild-type mice because we anticipated that dysfunction would be augmented in IL-10^{-/-} mice.

Vasomotor function of carotid arteries was evaluated in vitro by measurement of isometric tension as described previously (4,23,24). Briefly, carotid arteries from mice were removed and immediately placed in oxygenated Krebs buffer. We examined relaxation of carotid rings in response to acetylcholine (1 nmol/l to 3 μ mol/l) after submaximal precontraction using the thromboxane-A₂ analog (U46619). We have previously shown, using pharmacological approaches and gene-targeted mice, that responses of the carotid artery to acetylcholine are mediated by the endothelial isoform of nitric oxide synthase (eNOS) (25). Endothelium-independent vasorelaxation was evaluated using sodium nitroprusside (10 nmol/l to 1 μ mol/l).

In some experiments, pharmacological agents were added to the organ baths before administration of U46619 and subsequent vasodilators. To determine whether O₂⁻ was involved in impaired vasodilation, superoxide dismutase with polyethylene glycol (PEG-SOD) (50 units/ml) was added before testing effects of acetylcholine or nitroprusside. We also examined effects of allopurinol (1 mmol/l), an inhibitor of xanthine oxidase.

Detection of superoxide. Superoxide was detected in vessels in situ with laser confocal microscopy. Hydroethidine was used to detect O₂⁻ in the vessel wall, as described previously (5,24,26).

Drugs. Streptozotocin, acetylcholine, sodium nitroprusside, PEG-SOD, and allopurinol were obtained from Sigma (St. Louis, MO). U46619 was obtained from Cayman Chemical (Ann Arbor, MI). U46619 was dissolved in ethanol and then diluted with 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 saline. Concentrations were expressed as the final concentration of each drug in the organ bath.

Statistical analysis. All data are expressed as means \pm 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 responses to acetylcholine and sodium nitroprusside were expressed as percent relaxation from precontraction to U46619.

RESULTS

Hyperglycemia in IL-10^{+/+} and IL-10^{-/-} mice. Blood glucose concentrations were evaluated 2 weeks after treatment with vehicle or streptozotocin and at the termination of the study. Glucose levels were not different at 2 vs. 12–16 weeks (data not shown). Blood glucose concentrations were 372 ± 14 mg/dl in mice that received streptozotocin compared with 134 ± 8.6 mg/dl in mice that were treated with vehicle. Importantly, plasma glucose levels were similar in IL-10^{+/+} and IL-10^{-/-} mice.

Vascular responses. U46619 produced concentration-dependent contraction that was similar in vessels from IL-10^{+/+} (Fig. 1A) and IL-10^{-/-} mice (Fig. 1B) treated with vehicle (nondiabetic) or streptozotocin (diabetic). Thus, a similar level of precontraction with U46619 was used to test relaxation in vessels from all groups of mice.

Relaxation of the carotid artery produced by acetylcholine was similar in nondiabetic and diabetic IL-10^{+/+} mice (Fig. 2A). Likewise, in vessels from nondiabetic IL-10^{-/-} mice, maximum relaxation to acetylcholine was >90% (Fig. 2B). In contrast to results in IL-10^{+/+} mice, however, relaxation in response to acetylcholine was impaired in arteries from diabetic IL-10^{-/-} mice (Fig. 2B).

Vasorelaxation in response to nitroprusside, an endothelium-independent agonist, was similar in carotid arteries from nondiabetic and diabetic mice in both IL-10^{+/+} and IL-10^{-/-} groups (Fig. 3). The finding that all vessels responded similarly to nitroprusside suggests that effects of diabetes in this study were selective for endothelium-dependent relaxation in IL-10^{-/-} mice after 12–16 weeks of diabetes.

Effects of superoxide dismutase and xanthine oxidase. Incubation with PEG-SOD (50 units/ml) for 45 min had no effect on contraction to U46619, or relaxation to nitroprusside, in vessels from IL-10^{+/+} mice (data not shown). There was, however, a modest decrease in sensitivity to acetylcholine in vessels from diabetic IL-10^{+/+}

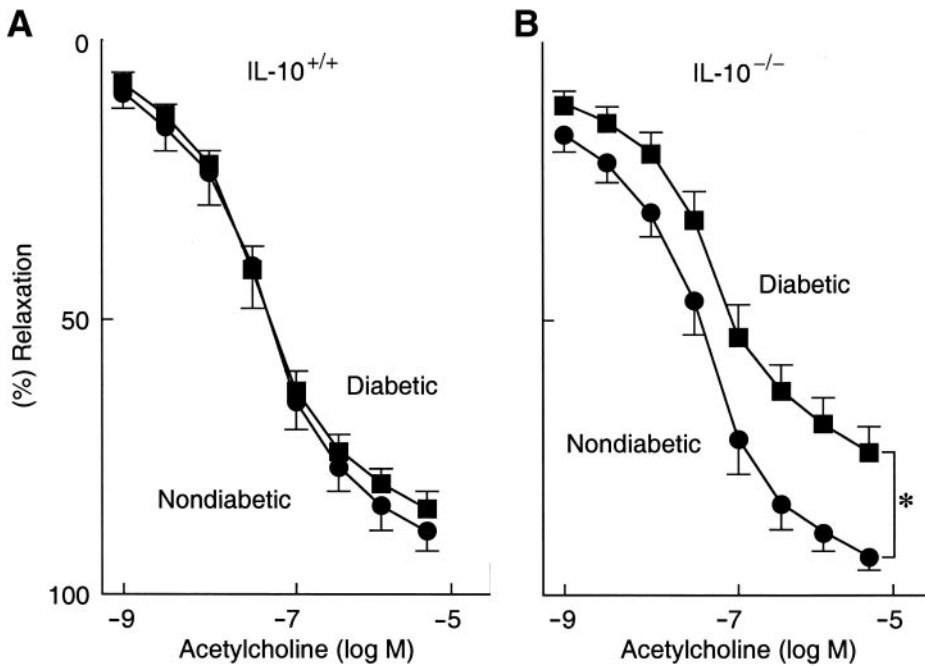


FIG. 2. Responses of the carotid artery to acetylcholine in wild-type (IL-10^{+/+}) (A) and IL-10-deficient (IL-10^{-/-}) (B) mice after vehicle (nondiabetic) or streptozotocin (diabetic) administration. Relaxation to acetylcholine was significantly impaired in arteries from diabetic IL-10^{-/-} mice. **P* < 0.05 vs. nondiabetic (*n* = 20).

mice (Fig. 4A). This effect of PEG-SOD in vessels from diabetic IL-10^{+/+} mice is somewhat surprising but, importantly, is opposite from the effect of PEG-SOD in vessels from diabetic IL-10^{-/-} mice (see below). Allopurinol (1 mmol/l) had no effect on responses to acetylcholine (Fig. 5A), U46619, or nitroprusside (data not shown) in vessels in IL-10^{+/+} mice.

Impaired relaxation of carotid arteries from diabetic IL-10^{-/-} mice in response to acetylcholine was restored to normal by incubation *in vitro* with PEG-SOD (50 units/ml) (Fig. 4B). These findings suggest that O₂⁻ mediates impairment of responses to acetylcholine in vessels from IL-10^{-/-} mice during diabetes.

Incubation of arteries from diabetic IL-10^{-/-} mice with

allopurinol (1 mmol/l), an inhibitor of xanthine oxidase, also improved relaxation in response to acetylcholine (Fig. 5B). Allopurinol produced a modest increase in sensitivity to low concentrations of acetylcholine in vessels from both IL-10^{+/+} and IL-10^{-/-} mice during diabetes but improved responses to higher concentrations of acetylcholine only in vessels from IL-10^{-/-} mice during diabetes (Fig. 5). Allopurinol had no effect on responses to U46619 or nitroprusside in arteries from nondiabetic or diabetic IL-10^{-/-} mice.

Detection of O₂⁻ with hydroethidine. To directly determine whether O₂⁻ levels were elevated in arteries from IL-10^{-/-} mice during diabetes, we used hydroethidine and confocal fluorescent microscopy. In two separate experiments, hydroethidine produced low-level fluorescence in

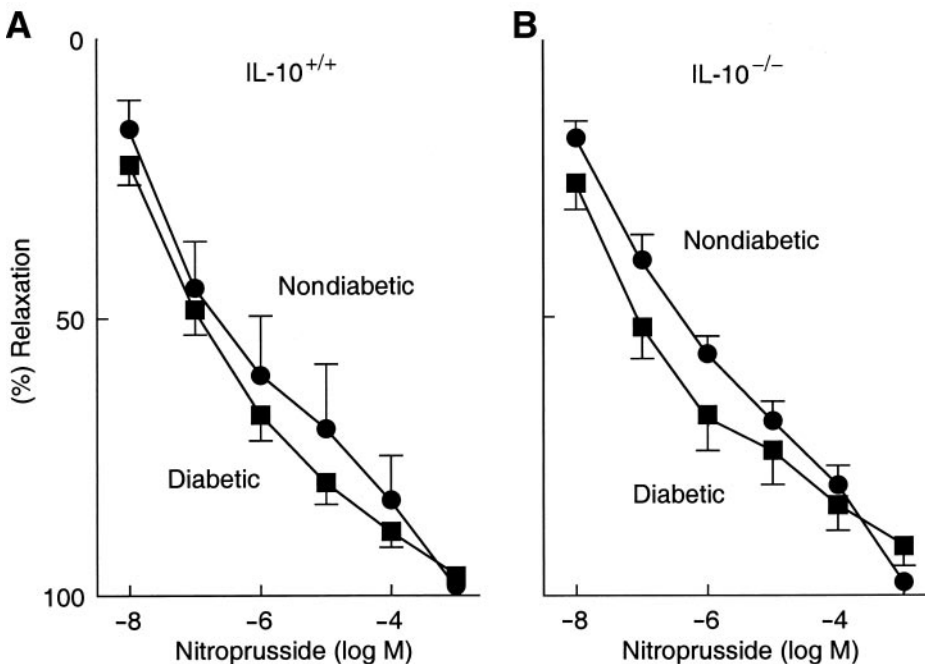


FIG. 3. Responses of the carotid artery to nitroprusside in wild-type (IL-10^{+/+}) (A) and IL-10-deficient (IL-10^{-/-}) (B) mice after vehicle (nondiabetic) or streptozotocin (diabetic) administration. Responses to nitroprusside were not affected by streptozotocin; *P* > 0.05 (*n* = 20).

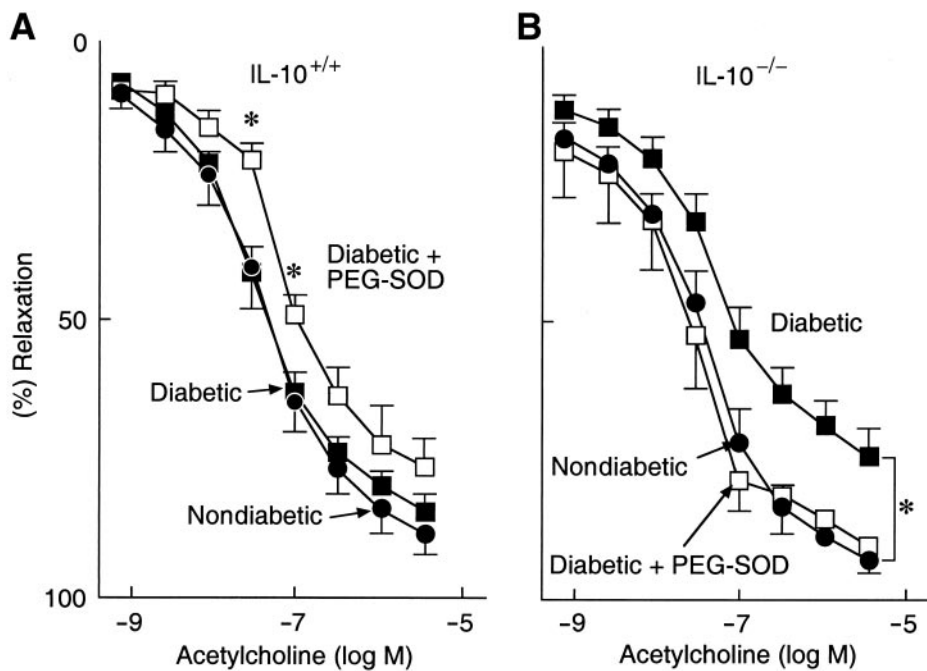


FIG. 4. Effects of PEG-SOD (50 units/ml) on responses to acetylcholine in IL-10^{+/+} (A) and IL-10^{-/-} (B) mice. Responses to acetylcholine were modestly attenuated by PEG-SOD in vessels from diabetic IL-10^{+/+} mice (n = 5), but PEG-SOD improved responses to acetylcholine in vessels from diabetic IL-10^{-/-} mice. *P < 0.05, diabetic + PEG-SOD vs. diabetic (n = 8).

arteries from nondiabetic and diabetic IL-10^{+/+} mice as well as from nondiabetic IL-10^{-/-} mice (Fig. 6). In marked contrast, vessels from IL-10^{-/-} mice with diabetes expressed high fluorescent intensity when treated with hydroethidine (Fig. 6).

DISCUSSION

There are three major new findings in this study. First, streptozotocin-induced diabetes (12–14 weeks' duration) produces impaired endothelium-dependent relaxation in carotid arteries from IL-10^{-/-} mice but not in wild-type controls (IL-10^{+/+}). Second, IL-10 impedes the development of endothelial dysfunction during diabetes by attenuating increases in O₂⁻ in the vessel wall. This elevation of O₂⁻ is functionally important because a scavenger of O₂⁻

(PEG-SOD) restores endothelium-dependent relaxation to normal. Third, effects of allopurinol suggest that xanthine oxidase may be an important source of O₂⁻ that produces vascular dysfunction in diabetic IL-10^{-/-} mice. These findings support the new concept that, by attenuating O₂⁻ levels, endogenous IL-10 is an important counterbalance to mechanisms that produce endothelial dysfunction during diabetes, a disease in which inflammatory mechanisms are activated within the wall of blood vessels.

We have shown previously that IL-10 limits expression of inducible nitric oxide (NO) synthase and preserves endothelium-dependent relaxation during acute inflammation produced by endotoxin in mice (5,23). IL-10 reduces inflammatory responses in cultures of human leukocytes (27). In other animal models, IL-10 reduces brain injury in

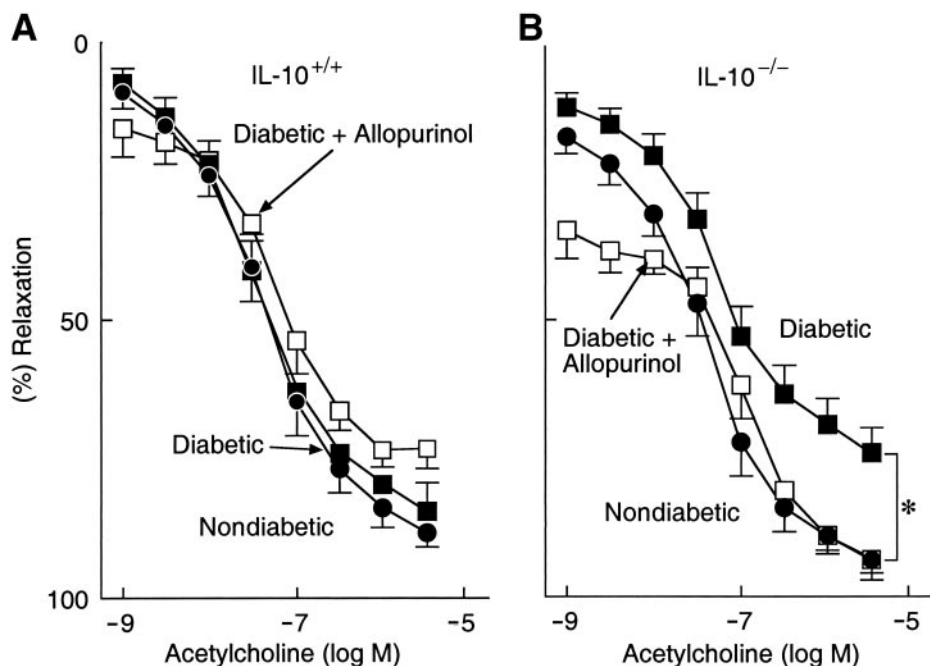


FIG. 5. Effects of allopurinol (1 mmol/l) on responses of the carotid artery to acetylcholine in IL-10^{+/+} (A) and IL-10^{-/-} (B) mice. Allopurinol improved responses to acetylcholine in vessels from diabetic IL-10^{-/-} mice. *P < 0.05 diabetic + allopurinol vs. diabetic (n = 6).

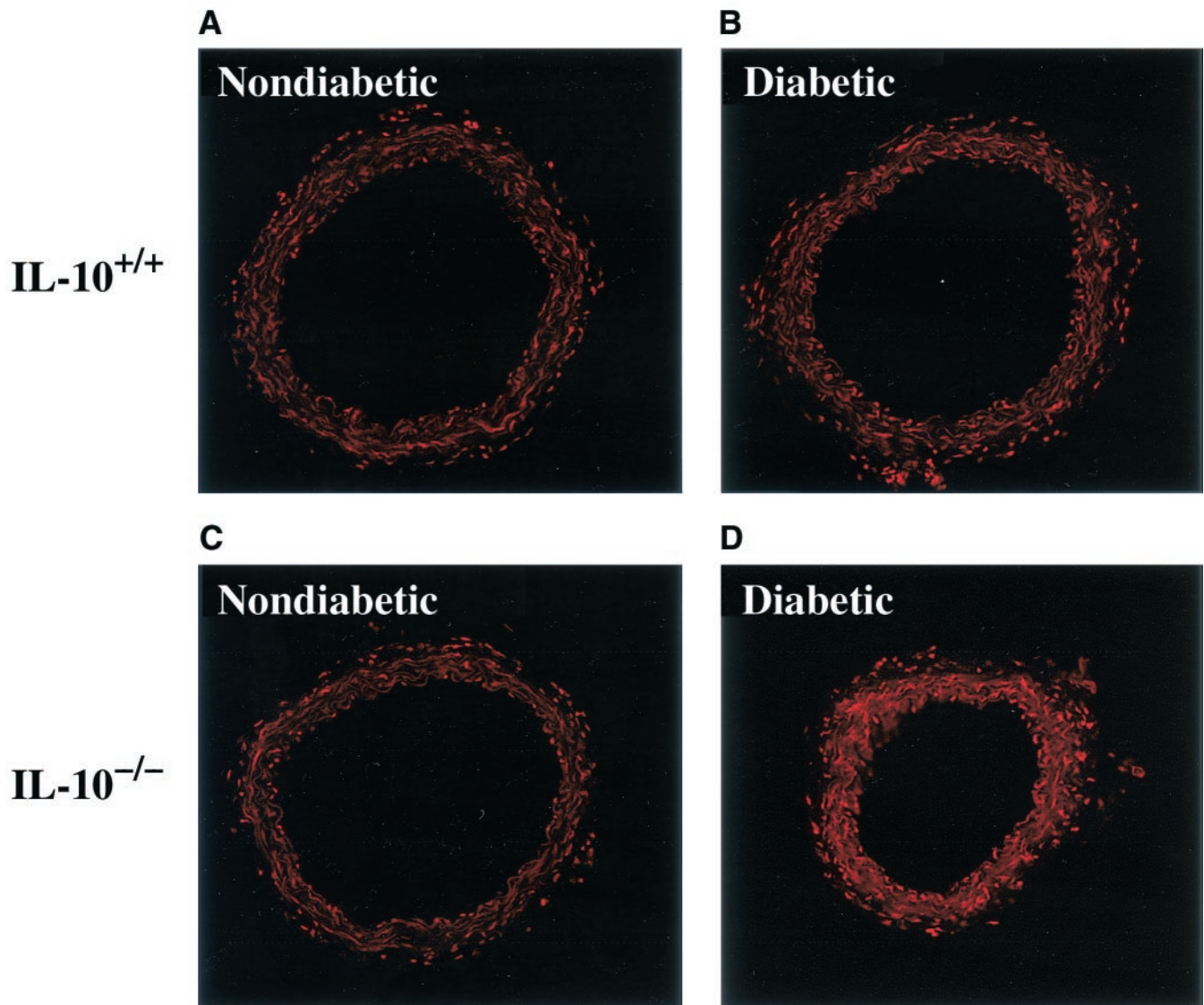


FIG. 6. Confocal micrographs of carotid artery labeled with hydroethidine (fluoresces red when oxidized by O_2^-). Low-level fluorescent intensity was similar in vessel sections from nondiabetic and diabetic $IL-10^{+/+}$ mice (A and B) and from nondiabetic $IL-10^{-/-}$ mice (C). In contrast, fluorescent intensity is remarkably elevated in vessel sections from diabetic $IL-10^{-/-}$ mice (D).

stroke (28), protects against atherosclerosis (29,30), and reduces venous thrombosis (31). In humans, IL-10 reduces inflammation during endotoxemia (32), is present in atherosclerotic plaques and may reduce cell death within the plaque (33), and reduces rheumatoid arthritis (34). To our knowledge, however, the present study provides the first direct evidence that IL-10 plays a protective role in vascular biology during diabetes.

Impaired endothelial function in $IL-10^{-/-}$ mice during diabetes. Studies using pharmacological approaches, direct measurements of NO, and gene-targeted mice have demonstrated that acetylcholine produces relaxation of carotid arteries by activating eNOS (25). Normal responses to nitroprusside with impaired responses to acetylcholine in vessels from diabetic $IL-10^{-/-}$ mice suggest that the observed impairment is selective for endothelium-dependent relaxation, rather than a generalized inhibition of vasorelaxation. Impairment of responses to acetylcholine in vessels from $IL-10^{-/-}$ mice but not $IL-10^{+/+}$ mice

suggests that IL-10 protects endothelium-dependent relaxation during diabetes.

Mechanisms of impairment. Previously, we and others have shown that vascular function, including endothelium-dependent relaxation, is altered during diabetes (8,9,35,36). Effects of diabetes on endothelium-dependent relaxation vary with the duration of the disease (36). In the current study, we chose a time point at which we found minimal impairment in arteries from wild-type mice to test the hypothesis that IL-10 deficiency produced greater dysfunction. We observed impairment of endothelium-dependent relaxation in carotid arteries from wild-type mice after 20 weeks of diabetes (C.A.G., unpublished data).

Superoxide. One mechanism that could account for impairment of eNOS-mediated relaxation is decreased availability of NO to produce relaxation, due to scavenging of NO by O_2^- . Conditions that produce elevated O_2^- in blood vessels are associated with impaired eNOS-dependent relaxation (21,24,26,37). For example, impaired

endothelium-dependent relaxation in inflammation, atherosclerosis, and diabetes is improved by SOD (5,21,26). In the present study, restoration of endothelium-dependent relaxation by PEG-SOD provides strong evidence that the impairment during diabetes is mediated by O_2^- in arteries from IL-10^{-/-} mice.

In addition to using PEG-SOD in studies of vasomotor function, we examined relative levels of O_2^- in the vessel wall using confocal microscopy and hydroethidine. We used this method previously to detect O_2^- in blood vessels (5,9,26). Findings with hydroethidine staining were consistent with effects of PEG-SOD on function and suggest that O_2^- levels are higher in blood vessels from diabetic IL-10^{-/-} mice than in vessels from nondiabetic IL-10^{-/-} mice. The hydroethidine signal was distributed throughout the vessel wall rather than being limited to a specific region (endothelium, media, or adventitia). These results suggest that elevation of O_2^- levels after streptozotocin administration may occur in multiple cell types.

In contrast to impaired responses to acetylcholine, responses to nitroprusside were normal in vessels from diabetic IL-10^{-/-} mice. Our results are consistent with other studies that have demonstrated normal responses to nitroprusside and impaired responses to acetylcholine in vessels with elevated O_2^- (38). A possible explanation is that the subcellular localization of endogenously produced NO (by eNOS) may differ from NO produced by nitroprusside. Nitroprusside may release NO in close proximity to soluble guanylate cyclase (sGC) within vascular muscle cells, whereas NO from eNOS must traverse endothelial cell membranes and extracellular space as it diffuses to sGC in vascular muscle. Thus, there may be more opportunity for NO from eNOS to react with O_2^- before reaching sGC.

IL-10 and xanthine oxidase. Our findings that IL-10 protects eNOS-mediated relaxation by attenuating increases of O_2^- in the vessel wall are consistent with our previous study using LPS (24) and with findings by others related to reactive oxygen species and IL-10. IL-10 inhibits production of proinflammatory cytokines (39), which are known to stimulate production of reactive oxygen species in endothelial cells (15,16) as well as in leukocytes. IL-10 also inhibits production of reactive oxygen species in monocytes and neutrophils (40).

One potentially important source of O_2^- in blood vessels is xanthine oxidase. Results from previous studies suggest that production of O_2^- by xanthine oxidase is associated with vascular dysfunction after treatment with LPS (20) as well as during hypertension (19) and atherosclerosis (41). Because proinflammatory cytokines activate xanthine oxidase in tissue culture (17) and IL-10 inhibits production of proinflammatory cytokines (3), the absence of IL-10 could increase activation of inflammatory mechanisms in the vessel wall including conversion of xanthine reductase to xanthine oxidase.

We used allopurinol to examine the role of xanthine oxidase in vascular function in diabetic IL-10^{-/-} mice. Incubation of vessels from diabetic IL-10^{+/+} and diabetic IL-10^{-/-} mice with allopurinol tended to modestly enhance responses to low concentrations of acetylcholine. These results suggest that levels of O_2^- generated by xanthine oxidase in vessels in both IL-10^{+/+} and IL-10^{-/-}

mice during diabetes may be sufficient to inhibit NO-mediated relaxation to some extent, but inhibition is clearly more pronounced in vessels from IL-10^{-/-} mice. Because the inhibitor had no effect on responses to U46619 or nitroprusside, effects of allopurinol appear to be specific for endothelium-dependent relaxation. We cannot exclude the possibility that other enzymes produce O_2^- in vessels from IL-10^{-/-} mice during diabetes, but effects of allopurinol on relaxation suggest that xanthine oxidase may be a major source of O_2^- in carotid arteries during diabetes.

Summary. In summary, findings from this study using gene-targeted mice provide direct evidence that IL-10 provides some protection against endothelial dysfunction during diabetes and that this effect is mediated by inhibition of increases in O_2^- in blood vessels. Effects of allopurinol suggest that a source of O_2^- may be xanthine oxidase. Thus, although the number of studies is still relatively small, the present study supports the emerging concept that IL-10 is an important protective molecule in diabetes as well as in other areas of vascular biology.

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