Interleukin-10 counteracts impaired endothelium-dependent relaxation induced by ANG II in murine aortic rings

Saiprasad M. Zemse, Rob H. P. Hilgers, and R. Clinton Webb

Department of Physiology, Medical College of Georgia, Augusta, Georgia

Submitted 4 May 2006; accepted in final form 14 February 2007

Zemse SM, Hilgers RH, Webb RC. Interleukin-10 counteracts impaired endothelium-dependent relaxation induced by ANG II in murine aortic rings. Am J Physiol Heart Circ Physiol 292: H3103–H3108, 2007. First published February 23, 2007; doi:10.1152/ajpheart.00456.2006.—ANG II stimulates the production of reactive oxygen species and activates proinflammatory cytokines leading to endothelial dysfunction. We hypothesized that the anti-inflammatory cytokine IL-10 counteracts the impairment in endothelium-dependent ACH relaxation caused by ANG II. Aortic rings of C57BL/6 mice were incubated in DMEM in the presence of vehicle (deionized H2O), ANG II (100 nmol/l), recombinant mouse IL-10 (300 ng/ml), or both ANG II and IL-10 for 22 h at 37°C. After incubation, rings were mounted in a wire myograph to assess endothelium-dependent vasorelaxation to cumulative concentrations of ACh. Overnight exposure of aortic rings to ANG II resulted in blunted ACh-induced vasorelaxation compared with that shown in untreated rings (maximal response = 44 ± 3% vs. 64 ± 3%, respectively; P < 0.05). IL-10 treatment significantly restored this impairment in relaxation (63 ± 2%). In addition, the NADPH oxidase inhibitor apocynin restored the impairment in relaxation (maximal response = 76 ± 3%)

Western blotting showed increased gp91<sub>phox</sub> expression (a subunit of NADPH oxidase) in response to ANG II. Vessels treated with a combination of ANG II and IL-10 showed decreased expression of gp91<sub>phox</sub>. Immunohistochemical analysis showed increased gp91<sub>phox</sub> expression in ANG II-treated vessels compared with those treated with combined ANG II and IL-10. We found that the anti-inflammatory cytokine IL-10 prevents impairment in endothelium-dependent vasorelaxation in response to long-term incubation with ANG II via decreasing NADPH oxidase expression.

NADPH oxidase

ENDOTHELIAL DYSFUNCTION, OCCURRING in a number of vascular disease states, has generally been attributed to a reduced nitric oxide (NO) bioavailability due to enhanced NO scavenging by superoxide anions and reduced generation of NO from endothelial NO synthase (eNOS) (15, 28). Decreased bioavailability of NO is mainly due to formation of peroxynitrite from NO and superoxide anions (10). Levels of ANG II are increased in a number of cardiovascular diseases, such as atherosclerosis and hypertension, and therapeutic doses of ANG II receptor antagonists provide vascular protection (39). ANG II has numerous actions on the arterial wall; it modulates vasomotor tone (1), cell growth and apoptosis (2, 32), cell migration, and extracellular matrix deposition (10, 24).

ANG II causes increased production of superoxide anion via a membrane-bound NADPH oxidase (13). The activity of the vascular NADPH oxidase is regulated by cytokines, hormones, and mechanical forces that are known to be involved in the pathogenesis of vascular diseases (32). Cytokines, important regulators of inflammatory reactions, are secreted by white blood cells (20). Proinflammatory cytokines like TNF-α, IL-6, and IL-1 are elevated in aged vessels and in patients with congestive heart failure (20). Anti-inflammatory cytokines like transforming growth factor-β, IL-10, and the IL-1 receptor antagonist exert inhibitory effects on injured vascular cells (20). Anti-inflammatory properties of IL-10 include inhibition of NF-κB (leading to suppressed cytokine production), inhibition of matrix degrading metalloproteinases, and reduction of tissue factor expression (27). In the vasculature, IL-10 preserves vascular function by inhibiting the production of proinflammatory cytokines that stimulate production of reactive oxygen species in endothelial cells (27). IL-10 protects eNOS-mediated relaxation of vessels by attenuating increases of superoxide in the vessel wall (16). In these experiments, the increased superoxide production is mainly due to uncoupling of eNOS (10).

We used isolated aortic rings of wild-type (C57BL/6) mice and incubated them for 22 h with ANG II in the absence or presence of recombinant mouse IL-10. Endothelium-dependent vasorelaxation was assessed with standard myograph techniques. Immunohistochemical and Western blot analyses were performed to detect gp91<sub>phox</sub> expression in the aortic wall.

Thus, our primary goal was to test the hypothesis that IL-10 counteracts the impairment in endothelium-dependent vasorelaxation induced by ANG II on murine aortic rings. We tried to achieve this goal in our functional (myograph) studies performed on murine aortic rings. Our secondary goal was to verify whether NADPH oxidase is the primary cause for impairment in vasorelaxation. Because NADPH oxidase is the key mediator of oxidative stress in the ANG II-incubated vessels, we assessed whether IL-10 neutralizes the inflammatory effects of ANG II via inhibition of NADPH oxidase.

MATERIALS AND METHODS

Animals. Experiments were conducted in 8-wk-old male C57BL/6 mice (Harlan, Indianapolis, IN). All procedures were approved by the institutional animal care committee.

Isolation of aortic rings and experimental protocols. Mice were euthanized with pentobarbital sodium (50 mg/kg iv; Abbott Laboratories, Abbott Park, IL), after which the abdominal-thoracic aorta was excised, placed in ice-cold physiological saline solution, and cleaned of adhering connective and adipose tissue. Aorta from each mouse was divided into four rings of 2-mm length. Each of the aortic rings was incubated in 2 ml of DMEM containing 120 U/ml penicillin and 120 µg/ml streptomycin. The incubation medium contained either vehicle (deionized H<sub>2</sub>O), ANG II (100 nmol/l), recombinant mouse ANG II, and/or recombinant mouse IL-10.

Acknowledgments

This work was supported by a pilot grant from the American Heart Association to R. C. Webb.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: S. M. Zemse, Dept. of Physiology, Medical College of Georgia, Augusta, GA 30912 (e-mail: szemse@students.mcg.edu).
IL-10 (300 ng/ml), or a combination of both ANG II and IL-10 for 22 h at 37°C. Pilot studies were performed before the actual concentration of IL-10 was selected; 30 ng/ml IL-10 showed no effect on the improvement of the endothelium-dependent ACh-induced relaxation. Hence, supraphysiological levels of IL-10 (300 ng/ml) were selected for this study, although previous studies have suggested that the plasma level of IL-10 is ~4–6 ng/ml (41).

After incubation, the aortic rings were removed and immediately placed in oxygenated physiological salt solution with the following ionic composition (in mmol/l): 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.18 MgSO4, 14.9 NaHCO3, 5.6 dextrose, 1.56 CaCl2·2H2O, and 0.026 EDTA. Aortic rings were mounted in a wire myograph (Danish Myotech) filled with 5 ml of physiological salt solution maintained at 37°C and continuously gassed with a mixture of 95% O2-5% CO2. A resting tension of 5 mN was applied to the aortic rings, and they were allowed to equilibrate for at least 30 min. Endothelium-dependent relaxation was performed on phenylephrine (PE)-contracted rings followed by a cumulative concentration response curve to ACh (0.001–10 μmol/l). Endothelium-independent relaxation was tested with sodium nitroprusside (SNP; 0.001–100 μmol/l). Some vessels were incubated with apocynin (100 μM) for 0.5 h, and again cumulative concentration-response curves to ACh were performed on PE-contracted vessels.

**Immunohistochemistry.** Four aortas isolated from four mice were treated with either vehicle (deionized H2O), ANG II (100 nmol/l) or a combination of ANG II and IL-10 overnight in DMEM as described above. After incubation, aortas were fixed in 4% paraformaldehyde and subsequently washed with PBS. Aortic rings were frozen in Tissue-Tek OCT medium, cut on a cryostat (Leica Cryostat CM3000) into 8-μm-thick sections, and mounted on glass slides. Slides were protected from light and incubated in a blocking buffer (100 ml PBS, 1 ml goat serum, 500 μl of 20% Triton X-100) and then treated with primary antibody to gp91phox (a subunit of NADPH oxidase; BD Biosciences) overnight at 4°C. Slides were washed with PBS and incubated in Alexa fluor 488 anti-goat, anti-mouse antibody (Invitrogen) for 1 h. After the washing procedure, the slides were allowed to dry and then covered with antifade mounting agent and coverslips. Images were obtained with a Zeiss LSM 510 META confocal microscope (Thornwood, NY), with an excitation of 488 nm and emission of 574–595 nm or a 560-nm long-pass filter.

Time series experiments were performed to quantify gp91phox expression. Time series experiments measure the intensity of the gp91phox expression in terms of figures. We selected eight different areas on the endothelial surface of slides of vehicle-treated vessels, ANG II-treated vessels, and vessels treated in the combined presence of ANG II and IL-10.

**Western blot analysis.** Four aortas were isolated from four mice and incubated overnight as described above. After incubation, aortas were used for Western blot analysis. Aortic tissue was lysed by RIPA lysis buffer, in the presence of 1 mmol/l PMSF, 1 mmol/l sodium or-
those treated alone with IL-10 showed similar responses to rings treated with apocynin (Emax was not different from control values, as depicted in Fig. 1

**RESULTS**

**Effects of ANG II on endothelium-dependent relaxation.**

There was no significant change in the maximal tension generated by 10 μmol/l PE in vessels treated with vehicle (20 ± 0.4 mN), ANG II (21 ± 0.6 mN), IL-10 (18 ± 0.3 mN), or the combination of ANG II and IL-10 (19 ± 0.4 mN) (Fig. 1A). Maximal relaxation with 10 μmol/l ACh was 64 ± 3% (Fig. 1B). Overnight exposure to ANG II (100 nmol/l) produced impairment of ACh-induced relaxation [maximal response (Emax) = 44 ± 3%; P < 0.05; Fig. 1B]. This effect of ANG II appears to be endothelium dependent because relaxation to the NO donor SNP was similar in aortic rings treated with vehicle or ANG II (Fig. 1C).

We also examined the effect of IL-10 on ACh-induced relaxations in aortic rings treated with and without ANG II. Figure 1B shows that aortic rings treated with ANG II (100 nmol/l) together with recombinant IL-10 (300 ng/ml) completely restored responses to ACh (Emax = 63 ± 2%), whereas those treated alone with IL-10 showed similar responses to control vessels (Emax = 61 ± 3%). Relaxation to SNP in aortic rings treated with IL-10 or ANG II in combination with IL-10 was not different from control values, as depicted in Fig. 1C.

**NADPH oxidase inhibition improves the endothelium-dependent relaxation impaired by ANG II.**

Aortic rings incubated with the NADPH oxidase inhibitor apocynin (100 μmol/l) showed nearly similar relaxation patterns to those of control rings. Figure 2 shows that apocynin improved the relaxation impaired by ANG II in aortic rings compared with aortic rings treated with ANG II alone (Emax = 44 ± 3% vs. 76 ± 3%, respectively; P < 0.05). This was similar to control rings treated with apocynin (Emax = 77 ± 3%).

**DISCUSSION**

In the present study, we demonstrate that overnight incubation of pressor doses of ANG II leads to impairment in the ACh-induced endothelium-dependent relaxation in murine aortic rings, caused by an increased contribution and protein expression of NADPH oxidase. We further show that the anti-inflammatory cytokine IL-10 restores this endothelial dysfunction by normalizing NADPH oxidase protein expression.
Endothelial dysfunction is commonly characterized by a reduced NO bioavailability (15, 28). Endothelium-dependent relaxation is attenuated by increased levels of endogenous superoxide anions (8). Superoxide anions can convert NO into peroxynitrite, thereby impairing the eNOS-dependent relaxation (16, 17). ANG II has been shown to increase the formation of superoxide in cultured vascular smooth muscle cells (42, 43), endothelial cells (33), and within the wall of intact arteries (3, 23, 35). It is well established that ANG II induces production of superoxide anions via membrane NADPH oxidase (35). Increased superoxide production leading to endothelial dysfunction within vessels has also been observed in mice made hypertensive by ANG II delivered by osmotic minipumps (37). In this study, we hypothesized that isolated aortic rings placed in culture medium and incubated with ANG II for 22 h would develop endothelial dysfunction. This endothelial dysfunction within vessels has also been observed in mice made hypertensive by ANG II delivered by osmotic minipumps (37). In this study, we hypothesized that isolated aortic rings placed in culture medium and incubated with ANG II for 22 h would develop endothelial dysfunction. This endothelial dysfunction was characterized by a reduced ability of these ANG II-treated and PE-contracted murine aortic rings to relax in response to cumulative concentrations of ACh. We observed a reduced relaxation to ACh in aortic rings treated with ANG II compared with that shown in aortic rings treated with deionized H2O, suggesting that the endothelium-dependent impairment in relaxation is most likely due to increased production of superoxide in the vessel wall of these ANG II-treated vessels.

Vascular inflammation is evident within the vessel wall during many cardiovascular diseases, including atherosclerosis, diabetes, and hypertension. ANG II can activate several inflammatory mechanisms within vascular cells, such as activation of NF-κB (34, 40), production of proinflammatory cytokines, such as IL-6 (46), and expression of adhesion molecules, monocyte chemoattractant protein-1 (19), inducible NO synthase, and matrix metalloproteinases (38, 45). The effect of increased superoxide formation in the vascular wall induced by ANG II is most likely due in part to activation of NAD(P)H oxidases (5, 14). To investigate whether reactive oxygen species derived from NAD(P)H oxidases were responsible for the observed impairment in ACh-induced relaxation, we studied the acute inhibitory effect of the NAD(P)H oxidase blocker apocynin on ACh-mediated relaxations. We found that in ANG II-treated aortic rings endothelium-dependent relaxations to ACh were completely restored by short-term incubation with apocynin, whereas in vehicle-treated aortic rings apocynin had no effect, suggesting that enhanced superoxide formation via NAD(P)H oxidases gives rise to the endothelial dysfunction in aortic rings treated overnight with ANG II.

Upregulation of subunits of NADPH oxidase, including p22phox and gp91phox, has been demonstrated in rat aortic rings during ANG II-induced hypertension (11, 26). In the present study, we showed that overnight exposure of aortic rings with

Fig. 3. Immunohistochemical staining of the subunit of NADPH oxidase (gp91phox) on cross sections (8-μm-thick slices) of aortic rings treated with either vehicle (A), 100 nmol/l ANG II (B), or 100 nmol/l ANG II + 300 ng/ml IL-10 (C) for 22 h at 37°C. D: fluorescence intensity of immunohistochemical staining for gp91phox in aortic rings that were vehicle treated (control), ANG II treated, or treated with a combination of ANG II and IL-10. Values are arbitrary units (n = 8). #P < 0.001.
ANG II causes upregulation of protein levels of the gp91phox subunit of NAD(P)H oxidases. In addition to the endothelial layer, gp91phox expression is also evident in the medium (Fig. 3). In accordance with Gorlach et al. (12), we observed expression of gp91phox in the endothelium of aortic rings. Other studies demonstrated the presence of gp91phox expression in the medium (17, 44), whereas some reported no expression in the smooth muscle cell layers of the aorta (7, 12). Nox2, which is identical to gp91phox, is homologous to Nox1 and Nox4 proteins. Lassegue et al. (22) demonstrated increased Nox1 and Nox4 expression in proliferating vascular smooth muscle cells. Interestingly, ANG II activation of a gp91phox-based NAD(P)H oxidase in the adventitia impaired endothelium-dependent relaxation (36), indicating that fibroblasts in the adventitia are the source of reactive oxygen species (7, 9). We cannot exclude the possibility of migrated fibroblasts from the adventitia to the underlying smooth muscle cells and endothelial cells.

Several studies suggest that the anti-inflammatory cytokine IL-10 may be an important player in vascular protection during atherosclerosis (25, 31) and following exposure to LPS (16, 17, 20). Furthermore, serum levels of IL-10 have been shown to be elevated in patients with acute coronary syndromes (4) and in preeclampsia (29). Besides IL-10, antioxidant vitamins and superoxide dismutase attenuate this inflammation (18). The inhibitory effect of IL-10 on the production of NO by mouse macrophages occurs by an indirect mechanism involving inhibition of cytokine (TNF, IFN-γ) synthesis (27). However, little is known about the significance of the anti-inflammatory mechanisms of IL-10 in hypertension. Therefore, our second hypothesis of this study was that IL-10 would improve the endothelial dysfunction and suppress the expression of gp91phox caused by ANG II in aortic rings. To test this hypothesis, we incubated murine aortic rings with recombinant IL-10 alone or in combination with ANG II. We found that IL-10 restored the impairment in endothelium-dependent ACh-induced relaxations and normalized the protein expression of gp91phox in ANG II-treated aortic rings, suggesting that IL-10 suppresses the formation of superoxide anions via inhibition of the expression of NAD(P)H oxidase. In nonvascular cells, IL-10 has been shown to inhibit the expression of NAD(P)H oxidase (21). Treatment of aortic rings with overnight exposure of IL-10 alone had no effect on endothelium-dependent ACh-mediated relaxations and the expression of gp91phox, indicating that IL-10 does not improve endothelial function in healthy control vessels. Further studies are needed to investigate the precise mechanisms of IL-10 action, but they may involve inhibition of activation of NF-κB (34) and/or increasing the expression of eNOS through a STAT-3-dependent mechanism (6).

In conclusion, our data demonstrate that IL-10 prevents impairment in endothelium-dependent relaxation induced by ANG II by suppressing the expression of NAD(P)H oxidases in the aortic wall, thereby reducing the production of reactive oxygen species. These findings will aid in the unraveling of the molecular mechanisms of anti-inflammatory actions of cytokines on the vascular wall during pathophysiological conditions such as hypertension, diabetes, preeclampsia, and atherosclerosis.

REFERENCES

Gryglewski RJ, Palmer RMJ, Moncada S.


