IL-6 Deficiency Protects Against Angiotensin II–Induced Endothelial Dysfunction and Hypertrophy

Laura I. Schrader, Dale A. Kinzenbaw, Andrew W. Johnson, Frank M. Faraci, Sean P. Didion

Objective—The goal of this study was to test the hypothesis that IL-6 mediates the increases in superoxide, vascular hypertrophy, and endothelial dysfunction in response to angiotensin II (Ang II).

Methods and Results—Responses of carotid arteries from control and IL-6–deficient mice were examined after acute (22-hour) incubation with Ang II (10 nmol/L) or chronic infusion of Ang II (1.4 mg/kg/d for 14 days). The hypertrophic response and endothelial dysfunction produced by Ang II infusion was markedly less in carotid arteries from IL-6–deficient mice than that in control mice. IL-6 deficiency also protected against endothelial dysfunction in response to acute (local) Ang II treatment (eg, 100 μmol/L acetylcholine produced 100 ± 4 and 98 ± 4% relaxation in vehicle-treated and 51 ± 4 and 99 ± 4% relaxation in Ang II–treated, control, and IL-6–deficient vessels, respectively). Endothelial dysfunction could be reproduced in vessels from IL-6–deficient mice with combined Ang II plus IL-6 (0.1 nmol/L) treatment. Increases in vascular superoxide and IL-6, as well as reductions in endothelial nitric oxide synthase mRNA expression, produced by Ang II were absent in IL-6–deficient mice.

Conclusions—These data demonstrate that IL-6 is essential for Ang II–induced increases in superoxide, endothelial dysfunction, and vascular hypertrophy. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: genetically-altered mice ■ inflammation ■ oxidative stress ■ endothelium-dependent responses
for 5 days, after which blood pressure was measured 1 day before (Day 0) and on Day 14 of vehicle or Ang II infusion.

After the last day of blood pressure measurement mice were euthanized with pentobarbital (100 mg/kg, ip) followed by removal of both common carotid arteries (for vascular studies as well as for measurement of medial cross-sectional area [CSA]; see supplemental materials, available online at http://atvb.ahajournals.org, for methods) and aorta (for real-time RT-PCR experiments). Arteries were placed in buffer, loose connective tissue was removed, and vessels were cut into rings for studies of vascular function. Relaxation of carotid arteries in response to acetylcholine and nitroprusside was measured after submaximal precontraction (50% to 60%) using the thromboxone analog, U46619 (9,11-dideoxy-11a,9a-epoxy-methanoprostoglandin-F2α) as described previously. To confirm that the effect of Ang II on endothelial function is mediated by superoxide, some responses were examined in the absence and presence of Tempol (a superoxide scavenger; 1 mmol/L). We have shown previously that this concentration of Tempol is very efficacious in improving endothelium-dependent responses.

Ang II Incubation Protocol
Methods used for overnight incubation of arteries with Ang II have been described previously. Briefly, carotid arteries and thoracic aorta were incubated in DMEM with either vehicle (ddH2O) or Ang II (10 nmol/L) for 22 hours at 37°C. Vascular responses were then examined (as described above) and superoxide was measured (in aorta, see below). To determine whether the effect of Ang II on superoxide levels and endothelial function in mouse carotid artery is mediated by NAD(P)H oxidase (a key potential source of superoxide in blood vessels), effects of Ang II were also examined in vessels from mice deficient in Nox2, a major component of the vascular NAD(P)H oxidase.

In reconstitution experiments, vessels from IL-6- and Nox2-deficient mice were coincubated with either vehicle or Ang II, with or without recombinant mouse IL-6 (0.1 nmol/L). To determine whether 0.1 nmol/L IL-6 is capable of producing endothelial dysfunction in the absence of Ang II, separate experiments examined responses of arteries from control mice incubated with IL-6 alone. This concentration of IL-6 was chosen based on preliminary experiments as well as reports in the literature. A concentration of 0.1 nmol/L of IL-6 is within the range observed in plasma of hypertensive individuals.

Measurement of IL-6 Levels
Levels of IL-6 in conditioned DMEM (from overnight incubation experiments of carotid artery and aorta) were determined using a mouse IL-6 ELISA kit (ALPCO Diagnostics) according to the manufacturer’s instructions.

Measurement of Vascular Superoxide
Relative superoxide levels were measured in aorta from control, Nox2-, and IL-6–deficient mice incubated overnight with either vehicle or Ang II using lucigenin (5 μmol/L)-enhanced chemiluminescence as described.

Real-Time RT-PCR
Expression of Nox2, Nox4, endothelial nitric oxide synthase (eNOS) and β-actin was assessed using RT-PCR using total aortic RNA from control and IL-6–deficient mice infused with vehicle or angiotensin II (see online supplement for details).

Drugs and Reagents
Acetylcholine, Ang II (human, acetate salt), lucigenin, nitroprusside, and Tempol were obtained from Sigma and all were dissolved in saline. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol with subsequent dilution being made with saline. Recombinant IL-6 (mouse; R&D Systems) was dissolved in sterile PBS containing 0.1% BSA.

**Figure 1.**
A, Ang II infusion increased systolic blood pressure to a similar extent in control and IL-6–deficient mice. B, Vascular responses to acetylcholine in vehicle- and Ang II–infused control and IL-6–deficient mice. Means±SE; n=4 to 6; *P<0.05 vs respective vehicle.

**Statistical Analysis**
All data are expressed as means±SE. Relaxation to acetylcholine and nitroprusside is expressed as a percent relaxation to U46619-induced contraction. Comparisons of relaxation and contraction as well as vessel cross-sectional area were made using ANOVA for repeated measures followed by Student-Newman-Keuls post-hoc test. Comparisons of IL-6 and superoxide levels, medial hypertrophy, as well as vascular expression of Nox2, Nox4, and eNOS, were made using unpaired t test, paired t test, or 1-way ANOVA followed by Dunnett post-hoc test. Statistical significance was accepted at P<0.05.

**Results**

**Effect of Chronic Ang II Infusion on Blood Pressure**
Baseline blood pressure was similar (P>0.05) in control and IL-6–deficient mice, and infusion of vehicle had no significant effect (P>0.05) in either group of mice (Figure 1A). In contrast, infusion of Ang II produced a marked increase in blood pressure that was similar (P>0.05) in control and IL-6–deficient mice (Figure 1A).

**Effect of Chronic Ang II Infusion on Vascular Responses and CSA**
Acetylcholine produced relaxation in carotid artery that was similar in vehicle-infused control and IL-6–deficient mice (Figure 1B), suggesting that IL-6 deficiency does not alter endothelium-dependent responses under baseline conditions. In contrast to mice treated with vehicle, relaxation of arteries to acetylcholine was markedly impaired in Ang II-infused
control mice and was much less in IL-6–deficient mice as compared with that in controls (Figure 1B). Ang II produced a marked increase in medial CSA \( (\mu\text{m}^2) \) in carotid arteries from control mice (vehicle \( 3.1 \pm 0.2 \) and Ang II \( 21.1 \pm 6.6 \times 10^3 \mu\text{m}^2 \) [means \( \pm \) SE; \( P<0.05 \]), consistent with previous studies.\(^{20-22} \) This change was absent in carotid arteries from IL-6–deficient mice infused with Ang II (vehicle \( 5.3 \pm 1.1 \) [means \( \pm \) SE; \( P>0.05 \)] and supplemental Figure I). Thus, the hypertrophic response to Ang II appears to be dependent on IL-6 expression. Taken together, the present findings provide strong evidence that IL-6 plays a major role in vivo in Ang II–induced vascular hypertrophy and endothelial dysfunction.

Effect of Acute (Local) Ang II on Vascular Responses

Acetylcholine produced relaxation that was similar \( (P>0.05) \) in carotid arteries from control and IL-6–deficient mice incubated overnight with vehicle (Figure 2A). In contrast, 10 nmol/L Ang II produced marked impairment of acetylcholine-induced relaxation in arteries from control mice. Similar to results obtained from chronic (systemic) treatment with Ang II, relaxation of carotid arteries to acetylcholine in IL-6–deficient mice was not affected by incubation with Ang II (Figure 2A). Relaxation to nitroprusside was similar in carotid arteries from control and IL-6–deficient mice and was not affected by incubation with Ang II (supplemental Figure II). Consistent with these functional findings, IL-6 levels (in incubation media) were increased in vessels from control mice treated with Ang II, however this increase was absent in IL-6 deficient mice (supplemental Table I). Taken together, these data support the concept that IL-6 mediates endothelial dysfunction in response to Ang II. These findings are also important as they suggest that effects of IL-6 within the vessel wall (ie, local IL-6) are essential for Ang II–induced impairment of endothelium-dependent responses.

**Effect of Treatment With IL-6 on Vascular Responses in IL-6–Deficient Mice**

In reconstitution experiments, acetylcholine produced normal relaxation in carotid arteries from control mice incubated with 0.1 nmol/L IL-6 in the absence of Ang II (Figure 2B). Similarly, IL-6 had no effect \( (P>0.05) \) on responses to nitroprusside in this group of mice (data not shown). These findings suggest that at this concentration, IL-6 alone is not sufficient to impair endothelial function. In contrast to the above, IL-6 produced impairment of endothelial function in carotid arteries from IL-6–deficient mice coincubated with Ang II (Figure 2B). IL-6 plus Ang II had no effect \( (P>0.05) \) on responses to nitroprusside in this group of IL-6–deficient mice (data not shown). These findings further implicate IL-6 as an essential factor in mediating endothelial dysfunction produced by Ang II.

**Endothelial Dysfunction Produced By Ang II Is Dependent on NAD(P)H Oxidase**

Consistent with many previous findings,\(^{8,21,23-26} \) the effect of Ang II on endothelial function in the present study was mediated by superoxide, as responses to acetylcholine in Ang II–infused control mice were normalized by Tempol (Figure 1B). Similarly, endothelial dysfunction produced by acute Ang II incubation involved NAD(P)H oxidase as Ang II had no effect on responses to acetylcholine or nitroprusside in carotid arteries from Nox2-deficient mice (Figure 3) consistent with previous studies in aorta and the cerebral circulation.\(^{23,27} \) Moreover, incubation of carotid arteries from Nox2-deficient mice with Ang II plus IL-6 had no effect on vascular responses to either acetylcholine or nitroprusside (Figure 3).
suggesting that the combined effect of Ang II and IL-6 on endothelial function in IL-6–deficient mice is dependent on Nox2 expression.

Effect of IL-6- and Nox2-Deficiency on Ang II–Induced Increases in Superoxide
Basal superoxide levels tended to be lower in aorta from IL-6–deficient mice incubated with vehicle, but these values were not significantly different from that in vehicle-treated controls (Figure 4). Importantly, Ang II treatment increased superoxide in aorta from control mice but not in IL-6–deficient or Nox2-deficient mice (Figure 4). These findings are supportive of our functional data and clearly implicate a role for IL-6 and NAD(P)H oxidase in response to Ang II–induced increases in vascular superoxide.

Effect of Ang II on Expression Nox and eNOS
Aortic expression of Nox2, Nox4 (supplemental Figure III), and eNOS were similar in control and IL-6–deficient mice infused with vehicle (Figure 5 and supplemental Table II), suggesting that deficiency of IL-6 per se does not alter expression of NAD(P)H oxidase or eNOS. In contrast, Ang II infusion produced a marked increase in Nox2 expression in both control and IL-6–deficient mice, suggesting that IL-6 is not necessary for Ang II to increase expression of Nox2. Ang II decreased eNOS expression in control, but not IL-6–deficient mice, suggesting that at least one protective mechanism of IL-6 deficiency involves maintenance of normal eNOS expression during Ang II–dependent hypertension.

Discussion
The present study has several major findings. First, IL-6 deficiency protects against vascular hypertrophy and endothelial dysfunction produced by systemic administration of Ang II. At least one protective effect of IL-6 deficiency appears to be related to maintenance of normal eNOS expression in response to Ang II. Second, in vitro studies revealed that protective effects of IL-6 deficiency can occur within the vessel wall. Both the in vivo and in vitro effects of Ang II appear selective, as responses to nitroprusside were unaffected by Ang II or genotype. In addition, the effect of Ang II in the mouse carotid was mediated by NAD(P)H oxidase–derived superoxide as endothelial dysfunction could be inhibited with Tempol and was absent in Nox2-deficient mice. Third, treatment of vessels from IL-6–deficient mice with Ang II and IL-6 reproduced endothelial dysfunction observed in Ang II–treated control mice. Fourth, Ang II–induced increases in vascular superoxide were absent in IL-6– and Nox2-deficient mice. In addition, Ang II–induced increases in IL-6 from carotid artery and aorta were absent in IL-6–deficient mice, supporting the concept that IL-6 and NAD(P)H oxidase–derived superoxide contribute to Ang II–induced vascular dysfunction. Collectively, these findings provide very strong evidence that IL-6 is a major mediator of Ang II–induced vascular hypertrophy and endothelial dysfunction.

Ang II–Induced Hypertension and Endothelial Dysfunction
In the present study, infusion of Ang II produced marked hypertension and endothelial dysfunction in carotid arteries from control mice. The increase in blood pressure produced by this dose of Ang II is consistent with many previous studies.17,20,21,22 In addition, Tempol was very effective in restoring the impaired endothelial responses in control mice implicating a role for superoxide in Ang II–induced endothelial dysfunction. This finding is consistent with studies where scavenging of superoxide or overexpression of CuZn-superoxide dismutase (CuZnSOD) was very effective in limiting Ang II–induced increases in superoxide and endothelial dysfunction.8,10,24,25

Although studies involving systemic infusion of Ang II have been very important in elucidating the effects of Ang II on blood vessels and arterial pressure, these studies have limitations in that administration of Ang II has multiple
potential effects in vivo. Unless additional controls are used,\(^8,24,25\) it is difficult to distinguish between the effects of hypertension per se and nonvascular effects versus the direct effect of Ang II within the vessel wall in such studies. To directly examine the effects of local Ang II (independent of changes in blood pressure), we also performed studies using isolated vessels incubated with Ang II. Consistent with previous results, a relatively low concentration of Ang II produced endothelial dysfunction in carotid artery in control mice.\(^8\)

IL-6 Deficiency Largely Prevents Ang II–Induced Endothelial Dysfunction

Previous studies have shown that IL-6 deficiency does not alter blood pressure under normal conditions.\(^28,29\) Consistent with this finding, we found that blood pressure was similar in control and IL-6–deficient mice. Ang II infusion produced similar levels of hypertension (as measured using tail-cuff) in control and IL-6–deficient mice in the present study. This conclusion is consistent with previous data where Ang II–dependent hypertension (as measured using telemetry) is not affected by IL-6 deficiency.\(^26\) Taken together, IL-6 does not appear to be a major contributor to the pressor response in a commonly used model of hypertension (ie, infusion of Ang II in mice). In contrast, while our manuscript was in revision, a study was published which found that IL-6 deficiency improved endothelial function, we did not observe significant change in blood pressure, but in the absence of hypertension.\(^32,33\)

Exogenous IL-6 has been found to impair endothelium-dependent responses to acetylcholine via reductions in NO-cyclic GMP signaling.\(^9\) Consistent with this, we found that the impairment of endothelial function in response to Ang II in our study was associated with increases in IL-6 from carotid artery and aorta. Moreover, Ang II reduced eNOS expression in control, but not IL-6–deficient, mice. This finding is consistent with a study which reported that IL-6 decreases eNOS expression in human aortic endothelial cells.\(^32\) To our knowledge, the present study provides the first examination of endothelial function in IL-6–deficient mice. We found that IL-6 deficiency did not produce alterations of endothelial function, suggesting that IL-6 is not necessary for maintenance of normal vascular function. This result is not surprising as we would be predict that IL-6 levels would be relatively low in normal vessels. In addition, we also found that Ang II–induced hypertrophy was absent in IL-6–deficient mice. Taken together, these findings suggest an important role for IL-6 in limiting endothelial dysfunction and vascular hypertrophy in response to Ang II.

A major finding of this study is that IL-6 deficiency protects against endothelial dysfunction produced by Ang II both in vivo and in vitro. Moreover, the effect of IL-6 appears to occur independent of increases in blood pressure and to occur locally within the vessel wall. The magnitude of the protection produced by IL-6 deficiency was very large in both models and suggests that IL-6 within the vascular wall mediates vascular dysfunction produced by Ang II. Although IL-6 deficiency improved endothelial function, we did not detect any major effect on the pressor response to Ang II. Several possibilities could explain these findings. First, we examined endothelial dysfunction in carotid artery, which may or may not be representative of resistance blood vessels. Second, blood pressure is regulated at multiple levels, including central and renal mechanisms, in addition to vascular mechanisms.\(^33,34,35\) Thus, although IL-6 deficiency may limit endothelial dysfunction, it is possible that other mechanisms are the key determinants of blood pressure in this model. Third, endothelial dysfunction may be a consequence, as opposed to a cause of hypertension.\(^36\) Thus, it may be possible to improve endothelial function independent of reductions in blood pressure. Finally, there are many examples where endothelial dysfunction is present but in the absence of hypertension.\(^37,38\)

Because Ang II increases oxidative stress, we considered the possibility that IL-6 was the mediator of increased vascular superoxide in response to Ang II. We found that a scavenger of superoxide restored endothelial responses to normal in arteries treated with Ang II. These findings are consistent with many previous studies and suggest that the effect of Ang II on endothelial function is attributable to superoxide-mediated inactivation of NO.\(^8,24,27,39\) In this study, Ang II increased vascular superoxide levels in control mice and this increase was absent in mice deficient in IL-6 or Nox2 and suggest that NAD(P)H oxidase is a major source of superoxide and mediator of endothelial dysfunction in our model. Thus, IL-6 may be a critical link in NAD(P)H-derived superoxide-mediated impairment in NO-mediated vascular signaling. Whether activation of NAD(P)H oxidase by Ang II occurs upstream or downstream of IL-6 expression is not yet known. However, our data in Nox2-deficient mice suggest that expression of Nox2 is necessary for Ang II plus IL-6 to produce endothelial dysfunction.

In conclusion, the present findings clearly demonstrate an essential role of IL-6, most likely occurring within the vascular wall, in mediating effects of Ang II on vascular function. IL-6 expression may be an important link between Ang II–induced increases in NAD(P)H oxidase activity, thereby limiting the bioavailability of NO for normal vascular responses. The present findings have important implication in terms of our understanding of IL-6 in mediating the effects of Ang II, particularly in disease states associated with increased Ang II–mediated signaling.

Acknowledgments

The authors thank Mary L. Modrick, Darrin W. Kinzenbaw, and the Central Microscopy Core for excellent technical assistance.

Sources of Funding

This work was supported by National Institutes of Health grants NS-24621, HL-38901, HL-62984, and by a Bugher Award (0575092N), a National Scientist Development Grant (0230327N), and a Heartland Affiliate Beginning Grant-in-Aid (0565486Z) from the American Heart Association.

Disclosures

None.
References


