Interleukin-6 Induces Oxidative Stress and Endothelial Dysfunction by Overexpression of the Angiotensin II Type 1 Receptor

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Abstract—Angiotensin II type 1 (AT1) receptor activation as well as proinflammatory cytokines such as interleukin-6 (IL-6) are involved in the development and progression of atherosclerosis. The detailed underlying mechanisms including interactions between inflammatory agonists and the renin-angiotensin system are poorly understood. Stimulation of cultured rat aortic vascular smooth muscle cells (VSMCs) with IL-6 led to upregulation of AT1 receptor mRNA and protein expression, as assessed by Northern and Western blot experiments. Nuclear run-on and transcription blockade experiments showed that IL-6 increases AT1 receptor mRNA de novo synthesis but not mRNA stability. Preincubation of VSMCs with IL-6 resulted in an enhanced angiotensin II–induced production of reactive oxygen species, as assessed by DCF fluorescence laser microscopy. Treatment of C57BL/6J mice with IL-6 for 18 days increased vascular AT1 receptor expression (real-time RT-PCR) and angiotensin II–induced vasoconstriction, enhanced vascular superoxide production (L-012 chemiluminescence, DHE fluorescence), and impaired endothelium-dependent vasodilatation. These effects were completely omitted in AT1 receptor knockout mice (AT1A−/− mice). Upregulation of vascular AT1 receptor expression in vitro and in vivo is decisively involved in IL-6–induced propagation of oxidative stress and endothelial dysfunction. This interaction of the proinflammatory cytokine IL-6 with the renin-angiotensin system may represent an important pathogenetic mechanism in the atherosclerotic process. (Circ Res. 2004;94:534-541.)

Key Words: endothelial dysfunction ■ AT1 receptor ■ interleukin-6 ■ inflammation ■ oxidative stress

Basic science as well as clinical studies have recently demonstrated that inflammatory events play an important role at virtually all stages of atherosclerosis.1,2 Exposure to risk factors such as hyperlipidemia or smoking triggers early vascular inflammation, which includes recruitment of leukocytes to the diseased endothelium, penetration of these inflammatory cells into the vessel wall, and initiation of atherosclerotic lesions.1,3 This so-called endothelial dysfunction resembles not only an early state of atherosclerosis but also predicts a high cardiovascular event rate.4–7 Ultimately, rupture of atherosclerotic lesions marks the event of acute coronary syndromes associated with high morbidity and mortality rates.1,2 Inflammatory events decisively contribute to the underlying destabilization of these vulnerable plaques.1,2 Cellular events such as adhesion, migration, and invasion of leukocytes into vascular cells occur concomitantly with the production of proinflammatory cytokines such as interleukin-6 (IL-6), which are thought to play a major role in the pathogenesis of atherosclerosis.5,9 Besides their potential role in local vascular disease progression, the circulating concentrations of these cytokines serve as markers for adverse prognosis.10

On the other hand, the renin-angiotensin system (RAS) and especially activation of the angiotensin II type 1 (AT1) receptor participates in all stages of atherosclerosis.11,12 This is particularly evident for endothelial dysfunction and plaque destabilization, which is in part explained by the induction of oxidative stress by AT1 receptor overexpression and activation.11,12 In addition, angiotensin II is known to increase the production of proinflammatory cytokines such as IL-6.13–16 However, the detailed mechanisms underlying the interaction of the RAS and inflammatory events remain poorly understood.

We reasoned that IL-6 could possibly influence the RAS on the level of AT1 receptor expression, which could in turn be important for the development of vascular dysfunction. Therefore, we examined the effect of IL-6 on AT1 receptor

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expression in vitro and in vivo and the potential relevance for the onset of endothelial dysfunction.

Materials and Methods

Materials

Recombinant mouse IL-6 was obtained from Calbiochem (Bad Soden, Germany). Angiotensin II, 5,6-dichlorobenzimidazole, Taq DNA polymerase, nucleotides, salts, and other chemicals were purchased from Sigma Chemical. MMLV reverse transcriptase, antibiotics, calf serum, and cell culture medium were obtained from Invitrogen. SYBR Green PCR master mix was purchased from Applied Biosystems. H2 DCF-DA was obtained from Molecular Probes. [3H]-dCTP and Hybond N-nylon membranes were purchased from Amersham. RNA-clean was obtained from AGS. L-012 was kindly provided by Dr Sohn, Institute of Physiology, Ludwig Maximilians University, Munich, Germany.

Cell Culture

Vascular smooth muscle cells (VSMCs) were isolated from rat thoracic aorta and cultured over several passages, as described previously.17 Experiments were performed with cells from passage 5 to 10.

Animals

Male 12-week-old wild-type C57BL/6J mice (WT; Charles River, Salzfeld, Germany) and male, age-matched AT1 receptor knockout mice (AT1A−/) with the identical genetic background (C57BL/6J; kindly provided by Dr Coffman, Department of Medicine, University of North Carolina, Chapel Hill, NC) were used for this study.18 The knockout animals were additionally backcrossed five times with C57BL/6J mice, which were used as control animals. Genotypes were determined by polymerase chain reaction (PCR) amplification of tail DNA. Animals were maintained in a 22°C room with a 12-hour light/dark cycle and received standard chow and drinking water ad libitum. The mice were treated for 18 days twice daily with increasing doses of recombinant mouse IL-6 (40 ng/day, days 1 to 4; 20 ng/day, days 5 to 12; 160 ng/day; days 13 to 18) or vehicle intraperitoneally according to the protocol of Wallenius et al.19 In the latter study, intraperitoneal injection of 80 ng IL-6 led to a 2-fold increase of IL-6 serum levels. These serum levels were about 10- to 20-fold lower than after endotoxin challenge.19 Body weights were assessed over 15 minutes in a scintillation counter (Lumat LB 9501, Berthold) in 1-minute intervals. The vessel segments were then dried, and dry weight was determined. Superoxide release was assessed by second approach, dihydroethidium (DHE) fluorescence of aortic tissue sections was determined.

Western Blotting

After treatment, VSMCs were homogenized in ice-cold lysis buffer containing additional leupeptin and aprotinin. Membrane proteins were isolated, and 30 μg-aquitos were separated on SDS/PAGE, as described previously.20 Blot membranes were stained with Ponceau S to verify protein transfer and equal loading for each lane. Immunoblotting was performed with an AT1 receptor rabbit polyclonal IgG antibody (1:250 dilution, sc 1173, AT1 (N-10), Santa Cruz Biotechnology). Immunodetection was accomplished using a goat anti-rabbit secondary antibody (1:5000 dilution, Sigma Chemical) and the enhanced chemiluminescence kit (Amersham).

Measurement of Reactive Oxygen Species

Intracellular production of reactive oxygen species in VSMCs was measured with 2'-7'-dichlorofluorescein (DCF; 10 μmol/L) fluorescein-AM, using confocal laser-scanning microscopy techniques (Zeiss Axiosvert 135 microscope, Bio-Rad MRC-600 confocal attachment, laser excitation 488 nm, emission longpass LP515-nm filter set) as described previously.17 Images were collected by single rapid scans selected from the image, and fluorescent intensity was taken. The relative fluorescence intensities are average values of all experiments.

Superoxide release in intact aortic segments was determined by L-012 chemiluminescence according to Sohn et al.21 L-012 is a luminal derivative with a high sensitivity for reactive oxygen species, which does not exert redox cycling itself.21 Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (pH 7.4; in mmol/L: NaCl 99.01, KCl 4.69, CaCl2 1.87, MgSO4 1.20, NaH2PO4 20.0, K2HPO4 1.03, NaHCO3 25.0, and D(+)-Glucose 11.1). Connective tissue was removed, and aortas were cut into 3-mm segments. The aortic segments were transferred into scintillation vials containing Krebs-HEPES buffer with 100 μmol/L L-012 and were incubated for 5 minutes. Chemiluminescence was then assessed over 15 minutes in a scintillation counter (Lumat LB 9501, Berthold) in 1-minute intervals. The vessel segments were then dried, and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue.

To assess vascular superoxide production by a second approach, dihydroethidium (DHE) fluorescence of aortic tissue sections was
used. Aortas were prepared as described above and 4-mm segments were embedded in Tissue Tek OCT embedding medium (Miles Laboratories), snap-frozen, and stored at -80°C. Samples were sectioned on a Leica cryostat (20 μm) and placed on glass slides. Krebs-HEPES buffer containing 2 μmol/L DHE was topically applied to each tissue section and sections were incubated in a dark, humidified chamber at 37°C for 30 minutes. In situ production of superoxide was visualized as fluorescence by confocal laser-scanning microscopy, as described above (laser excitation 514 nm, emission longpass LP550-nm filter). Paired aortas from vehicle-treated and IL-6-treated animals were processed in parallel, and images were acquired with identical acquisition parameters and were stored digitally.

### Aortic Ring Preparations and Tension Recording

After excision of the descending aorta, the vessel was immersed in chilled buffer containing (in mmol/L) NaCl 118.0, CaCl2 2.5, KCl 4.73, MgCl2 1.2, KH2PO4 1.2, NaHCO3 25.0, NaEDTA 0.026, and D(+)-Glucose 5.5; pH 7.4. Adventitial tissue was carefully removed. Three-millimeter rings were mounted in organ baths filled with the described buffer (37°C; continuously aerated with 95% O2 and 5% CO2), were attached to a force transducer, and isometric tension was recorded. The vessel segments were gradually stretched over 60 minutes to a resting tension of 10 mN (1 gram), which was maintained throughout the experiment, and were allowed to equilibrate for further 30 minutes. Drugs were added in increasing concentrations in order to obtain cumulative concentration-response curves: KCl 20 and 40 mmol/L, angiotensin II 1 nmol/L to 1 μmol/L, phenylephrine 1 nmol/L to 10 μmol/L, carbachol 10 nmol/L to 100 μmol/L, and nitroglycerin 1 nmol/L to 10 μmol/L. The drug concentration was increased when vasoconstriction or relaxation was completed. Drugs were washed out before the next substance was added.

### Statistical Analysis

Data are presented as mean±SEM obtained in at least three separate experiments. Statistical analysis was performed using the ANOVA test. A value of P<0.05 indicates statistical significance.

### Results

#### IL-6 Upregulates AT1 Receptor Gene Expression in VSMCs

VSMCs were incubated for 0, 2, 4, 12, and 24 hours with 1 nmol/L IL-6 or vehicle, followed by quantification of AT1 receptor mRNA expression by Northern analysis. Figures 1A and 1B show a representative autoradiogram and the densitometric analysis of four separate experiments and reveal a time-dependent upregulation of AT1 receptor mRNA expression to a maximum of 166±25% of control after 12 hours (P<0.05 versus control). The IL-6 effect was already detected at a concentration of 0.1 nmol/L (139±15% of control; P<0.05 versus control; n=3), as demonstrated in Figure 1C.

AT1 receptor protein expression was assessed by Western blotting in VSMCs stimulated for 24 hours with 1 nmol/L IL-6 or vehicle. Figures 2A and 2B show that IL-6 also induced an enhanced AT1 receptor protein level (165±20% of control; P<0.05 versus control; n=6). Expression of β-actin, which was used as internal control, was not altered by IL-6 (115±24% of control).

#### IL-6 Increases AT1 Receptor mRNA Transcription Rate

To explore the gene regulation mechanisms, VSMCs were incubated for 12 hours with 1 nmol/L IL-6 or vehicle, before nuclear mRNA de novo synthesis was assessed by nuclear run-on assays. Figure 3A demonstrates that IL-6 induced an increase of AT1 receptor mRNA transcription rate to 129±3% of control (P<0.05 versus control; n=3). GAPDH mRNA expression was not significantly altered (data not shown).

In addition, mRNA stability was assessed in VSMCs stimulated for 12 hours with 1 nmol/L IL-6 or vehicle, followed by transcriptional blockade with 50 μg/mL of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB), which blocks de novo synthesis of mRNA. 22 Figure 3B shows the quantification of AT1 receptor mRNA decay and reveals that IL-6 had no influence on AT1 receptor mRNA stability (n=4).

#### IL-6–Induced AT1 Receptor Overexpression Enhances Reactive Oxygen Species Production in VSMCs

VSMCs were preincubated for 24 hours with 1 nmol/L IL-6 or vehicle, before 1 μmol/L angiotensin II was added for an
additional 3 hours. Production of reactive oxygen species was assessed by DCF fluorescence confocal laser microscopy. Figures 4A and 4B depict a representative microscopic scan and the data analysis of four separate experiments. Preincubation with IL-6 significantly increased angiotensin II–mediated free radical production in VSMCs (angiotensin II, 181 ± 14% of control; IL-6 + angiotensin II, 225 ± 14% of control; \( P < 0.05 \) versus control and versus angiotensin II alone, \( n = 4 \)). IL-6 had no significant effect on basal production of reactive oxygen species (114 ± 9% of control).

**Figure 2.** Effect of IL-6 on AT₁ receptor protein expression in VSMCs. VSMCs were stimulated for 24 hours with 1 nmol/L IL-6 or vehicle and AT₁ receptor protein expression was assessed by Western blotting. A, Representative immunoblot. B, Densitometric analysis. Mean ± SEM, \( n = 6 \), *\( P < 0.05 \) vs control.

**Figure 3.** Effect of IL-6 on AT₁ receptor mRNA transcription rate and mRNA stability. A, VSMCs were incubated for 12 hours with 1 nmol/L IL-6 or vehicle, before nuclear mRNA de novo synthesis was assessed by nuclear run-on assays using real-time RT-PCR. AT₁ receptor mRNA synthesis was normalized to the expressed housekeeping gene GAPDH. Data analysis of 3 separate experiments. Mean ± SEM, *\( P < 0.05 \) vs control. B, mRNA stability was assessed in VSMCs stimulated for 12 hours with 1 nmol/L IL-6 or vehicle, followed by transcriptional blockade with 50 μg/mL DRB. AT₁ receptor mRNA expression was assessed by Northern analysis 0 to 8 hours after addition of DRB to the culture medium. Densitometric analysis. Mean ± SEM, \( n = 4 \).

**Figure 4.** Effect of IL-6 on reactive oxygen species production in VSMCs. VSMCs were preincubated for 24 hours with 1 nmol/L IL-6 or vehicle (Con), followed by a 3-hour incubation with 1 μmol/L angiotensin II (Ang II). Reactive oxygen species production was visualized by DCF fluorescence confocal laser microscopy. A, Representative microscopic scan. B, Quantification of reactive oxygen species production. Data analysis of 4 separate experiments, expressed as relative fluorescence. Mean ± SEM, *\( P < 0.05 \) vs control; #\( P < 0.05 \) vs angiotensin II alone.

**IL-6 Enhances Vascular AT₁ Receptor Expression and Oxidative Stress In Vivo**

Male wild-type C57BL/6J mice were treated intraperitoneally with increasing doses of murine IL-6 or vehicle for 18 days (40 ng/day, days 1 to 4; 80 ng/day, days 5 to 12; 160 ng/day; days 13 to 18). Aortas were excised after treatment and AT₁ receptor mRNA expression was measured by real-time quantitative RT-PCR. Figure 5A shows that IL-6 treatment enhanced vascular AT₁ receptor mRNA expression to 163 ± 35% of the control animals (\( P < 0.05 \) versus control; \( n = 5 \) per group). GAPDH mRNA expression was not significantly altered (data not shown).

Concomitantly, superoxide production in aortic segments was enhanced after treatment with IL-6, as assessed by DHE fluorescence of aortic tissue sections (\( n = 4 \) per group) (Figure 5B). This increase in superoxide production was confirmed by L-012 chemiluminescence (163 ± 32% of control; \( P < 0.05 \) versus control; \( n = 6 \) per group), as shown in Figure 5C.

In addition, vascular function of isolated, intact aortic ring preparations was assessed in organ chamber experiments
Treatment with IL-6 induced a marked impairment of endothelium-dependent vasodilation, as assessed by stimulation with carbachol (\(P<0.05\) versus control group; 10 and 100 \(\mu\)mol/L carbachol) (Figure 6A). In contrast, endothelium-independent vasorelaxation induced by nitroglycerin was not altered (Figure 6B). Moreover, angiotensin II–induced vasoconstriction was significantly enhanced after IL-6 treatment compared with vehicle-treated mice (\(P<0.05\) versus control group; 1 \(\mu\)mol/L angiotensin II), whereas vasoconstriction induced by phenylephrine or KCl was not affected by IL-6 treatment (data not shown).

**Discussion**

The data of the presented study demonstrate that the proinflammatory cytokine IL-6 induces an upregulation of AT\(_1\) receptor gene expression in cultured VSMCs and in vascular tissue of mice. In consequence, this leads to increased angiotensin II–mediated vasoconstriction, enhanced free ox-
Proinflammatory cytokines such as IL-6 have been implicated in the pathogenesis of atherosclerosis. Activation of the latter enhances IL-6 production which suggests important interactions between IL-6 and the AT1 receptor. Second, a great body of evidence derived from experimental studies indicates the decisive involvement of AT1 receptor activation in the development and progression of endothelial dysfunction and atherosclerosis. Interventional trials have revealed that prevention of AT1 receptor activation by ACE inhibition or AT1 receptor antagonism may inhibit onset and progression of endothelial dysfunction, atherosclerosis and related organ damage.

AT1 receptor expression seems to be limiting for the effect of angiotensin II. Upregulation of AT1 receptor expression by, for example, LDL cholesterol enhances the action of angiotensin II in vitro as well as in vivo. AT1 receptors are downregulated by growth factors, estrogen, and angiotensin II, which diminishes the effects of angiotensin II on intracellular signaling. In the present study, upregulation of AT1 receptor expression by IL-6 in VSMCs was associated with an increased angiotensin II–mediated production of reactive oxygen species, indicating that the AT1 receptor overexpression led to an enhancement of AT1 receptor–mediated effects and to increased oxidative stress in VSMCs. Angiotensin II is abundantly circulating in the blood of mice and locally expressed in vascular cells and evokes more pronounced effects in the case of enhanced vascular AT1 receptor expression. Therefore, the increase in oxidative stress in association with AT1 receptor upregulation after treatment with IL-6 is in good agreement with the in vitro data and confirms the

Figure 7. Oxidative stress and endothelial function in AT1 receptor knockout mice. Male AT1A receptor knockout mice (AT1A /–) with the same genetic background as the wild-type group. A, Aortic superoxide production was assessed by DHE fluorescence of aortic tissue sections (n=4 per group). Representative microscopic scans. B, Aortic superoxide production measured by L-012 chemiluminescence, expressed as relative chemiluminescence per milligram of aortic tissue. Mean±SEM, n=7 per group. C, Endothelium-dependent vasodilation assessed in aortic ring preparations, expressed in percent of maximal phenylephrine-induced vasoconstriction. Mean±SEM, n=10 rings per group.

This assumption is mainly based on two pieces of evidence. On one hand, experimental data suggest that cytokines are important for the recruitment, adhesion, invasion, and functioning of leukocytes within the diseased vessel wall through propagated migration, proliferation, activity, and phagocytosis of leukocytes. In a murine model of atherosclerosis, IL-6 was shown to increase atherosclerotic lesion size. On the other hand, circulating levels of cytokines such as IL-6 have been shown to correlate with an adverse prognosis of patients suffering from coronary artery disease. However, the detailed mechanisms of how cytokines may accelerate the development of atherosclerosis remain poorly understood. Finding these mechanisms could be essential to establish antiatherosclerotic treatment strategies depending on circulating levels of IL-6.

The presented data demonstrate that IL-6 upregulates vascular AT1 receptor expression. This is of potential importance for the following reasons. First, IL-6 has been detected within stable and unstable plaques together with components of the RAS such as the AT1 receptor. Activation of the latter enhances IL-6 production which suggests important interactions between IL-6 and the AT1 receptor. Second, a great body of evidence derived from experimental studies indicates the decisive involvement of AT1 receptor activation in the development and progression of endothelial dysfunction and atherosclerosis. Interventional trials have revealed that prevention of AT1 receptor activation by ACE inhibition or AT1 receptor antagonism may inhibit onset and progression of endothelial dysfunction, atherosclerosis and related organ damage.
relevance of these data in vivo. Moreover, the effect on vascular oxidative stress in the absence of exogenously administered angiotensin II underlines the pathophysiological relevance of the presented findings, because it occurs without a pharmacologically induced increase of angiotensin II concentrations.

In the present study, IL-6 concentrations were chosen for the cell culture experiments that were used in other in vitro studies as well.41 However, these concentrations exceed the plasma levels found in humans. Mice display 10- to 20-fold higher IL-6 plasma levels than humans.19 Importantly, the treatment of mice with IL-6 according to the protocol that was used in the present study leads to a 2-fold increase in IL-6 plasma concentrations.19 In human studies, different basal IL-6 levels were detected; however, in patients with acute coronary syndromes, a 2-fold increase in IL-6 concentrations was found.42,43 Therefore, the findings of the presented in vivo study may be relevant for the in vivo situation in humans.

By upregulating vascular AT1 receptor expression, the harmful effects of AT1 receptor activation are enhanced by IL-6. Overexpression of AT1 receptors leads to increased oxidative stress in vitro and in vivo, which in turn causes the development of endothelial dysfunction, the prerequisite of atherosclerosis, which is associated with a poor prognosis.4,7,11,23 It could be argued that the diverse effects of IL-6 may cause a variety of harmful events, ultimately leading to vascular damage. However, the presented data derived from AT1A−/− mice strongly suggest that the demonstrated AT1 receptor upregulation is a major event leading to the adverse vascular effects of IL-6. Thus, the herein presented data combined with previous findings could be summarized in a proinflammatory vicious circle: AT1 receptor activation by angiotensin II increases the production of IL-6,13–16 which in turn enhances AT1 receptor expression, leading to increased vasoconstriction, enhanced oxidative stress, inflammatory events, endothelial dysfunction, and possibly to the subsequent development of vascular lesions.

If confirmed in humans, these results could be of potential importance for antiatherosclerotic treatment strategies. It may be speculated that elevated IL-6 plasma concentrations mark patients with an activated RAS and increased expression of AT1 receptors. This could not only predict a poor prognosis but also putatively identify patients who especially benefit from a medical treatment that diminishes AT1 receptor activation.

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References


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