

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

Sven Wassmann, Michaela Stumpf, Kerstin Strehlow, Andreas Schmid, Bernhard Schieffer, Michael Böhm, Georg Nickenig

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.^{8,9} 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

Original received September 26, 2003; revision received November 20, 2003; accepted December 5, 2003.

From the Medizinische Klinik und Poliklinik (S.W., M.S., K.S., M.B., G.N.), Innere Medizin III, and Physiologisches Institut (A.S.), Universitätskliniken des Saarlandes, Homburg/Saar, Germany; and Department of Cardiology and Angiology (B.S.), Medizinische Hochschule Hannover, Hannover, Germany.

Correspondence to Dr Sven Wassmann, Medizinische Klinik und Poliklinik, Innere Medizin III, Universitätskliniken des Saarlandes, D-66421 Homburg/Saar, Germany. E-mail wassmann@med-in.uni-saarland.de

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DOI: 10.1161/01.RES.0000115557.25127.8D

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. H₂DCF-DA was obtained from Molecular Probes. [³²P]-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, Sulzfeld, 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; 80 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 similar in both mice groups (WT, 22.2±0.6 grams; AT1A^{-/-}, 21.0±0.5 grams) and were not significantly altered by IL-6 treatment (WT, 22.8±0.4 grams; AT1A^{-/-}, 21.5±0.4 grams). Food intake and behavior of the animals were not influenced by IL-6 treatment. The mice were killed after the treatment period and tissue samples were collected immediately. There were no signs of peritonitis. Animal experiments were performed in accordance with the German animal protection law.

mRNA Isolation and Northern Blot Analysis

Cells were treated as indicated. IL-6 treatment did not affect cellular viability of the cultured cells, as determined by cell count, morphology, and trypan blue exclusion. Total cellular RNA was isolated with RNA-clean according to the manufacturer's protocol. Then, 10 μg aliquots were electrophoresed and Northern analysis was performed with a [³²P]-dCTP-labeled, rat AT₁ receptor cDNA probe, as described previously.¹⁷ Blots were exposed to film, and autoradiographic signals were quantified by laser densitometry. The AT₁ receptor mRNA expression levels were normalized to the corresponding RNA loading.

Real-Time Polymerase Chain Reaction

For assessment of vascular gene expression, mouse aortas were excised, quickly frozen in liquid nitrogen, and homogenized with a motorized homogenizer. RNA from aortic homogenates was isolated with RNA-clean, and 1-μg aliquots were electrophoresed as described above. Then, 1 μg of the isolated total RNA was reverse transcribed using random primers and MMLV reverse transcriptase

for 60 minutes at 42°C and 10 minutes at 75°C. The single-stranded cDNA was amplified by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) with the TaqMan system (ABI-Prism 7700 Sequence Detection System, Applied Biosystems) using SYBR Green dye. For AT₁ receptor, the primers were 5'-GGGTGGACAATGGCCAGGTAG-3' and 5'-CTCGCCCTGGCTGACTTATGC-3' (murine AT1A receptor). For GAPDH, the primers were 5'-CCTGGACCACCCAGCCAGCAA-3' and 5'-TGTTATGGGGTCTGGGATA-3'. For quantification, AT₁ receptor mRNA expression was normalized to the expressed housekeeping gene GAPDH.

Nuclear Run-On Assays

VSMCs were treated as indicated, collected, and washed. After lysis for 10 minutes on ice, nuclei were isolated by centrifugation through 0.6 mol/L sucrose, as described previously.¹⁷ The nuclei (≈3 to 5×10⁸/reaction) were used to carry out the in vitro transcription in a reaction mixture containing 40% glycerol, 50 mmol/L Tris/HCl, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 0.5 mmol/L levels of CTP, GTP, ATP, and UTP at 30°C for 30 minutes. Reactions were terminated by addition of RNA-clean. Immediately before transcription, a sample of each condition was removed. Total RNA before and after transcription was isolated, and AT₁ receptor and GAPDH mRNA were quantified by real-time RT-PCR, as described above. The extent of AT₁ receptor mRNA transcription was determined by subtracting the amount of AT₁ receptor mRNA standardized to GAPDH mRNA before transcription from the amounts after transcription.

Western Blotting

After treatment, VSMCs were homogenized in ice-cold lysis buffer containing additional leupeptin and aprotinin. Membrane proteins were isolated, and 30 μg-aliquots were separated on SDS/PAGE, as described previously.²⁰ Blot membranes were stained with Ponceau red to verify appropriate 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 by 2',7'-dichlorofluorescein (DCF; 10 μmol/L) fluorescence using confocal laser-scanning microscopy techniques (Zeiss Axiocvert 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 and identical parameters, such as contrast and brightness, for all samples. Five groups of 25 cells for each sample were randomly 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 luminol 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, NaHEPES 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 ($20\ \mu\text{m}$) and placed on glass slides. Krebs-HEPES buffer containing $2\ \mu\text{mol/L}$ 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 $514\ \text{nm}$, 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, CaCl_2 2.5, KCl 4.73, MgCl_2 1.2, KH_2PO_4 1.2, NaHCO_3 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% O_2 and 5% CO_2), 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 $1\ \mu\text{mol/L}$, phenylephrine 1 nmol/L to $10\ \mu\text{mol/L}$, carbachol 10 nmol/L to $100\ \mu\text{mol/L}$, and nitroglycerin 1 nmol/L to $10\ \mu\text{mol/L}$. 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 \pm 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 AT_1 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 AT_1 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 AT_1 receptor mRNA expression to a maximum of $166 \pm 25\%$ 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 ($139 \pm 15\%$ of control; $P < 0.05$ versus control; $n = 3$), as demonstrated in Figure 1C.

AT_1 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 AT_1 receptor expression on protein level ($165 \pm 20\%$ 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 ($115 \pm 24\%$ of control).

IL-6 Increases AT_1 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

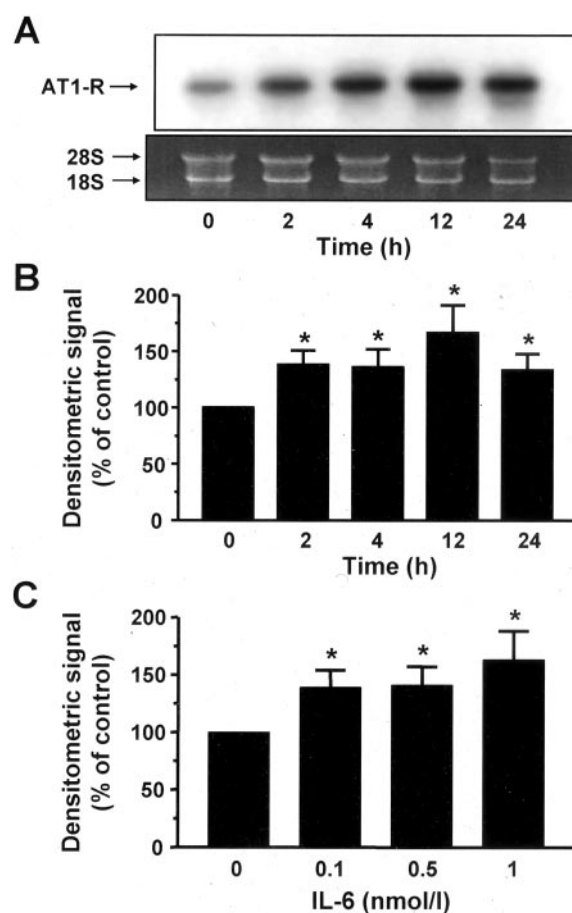


Figure 1. Effect of IL-6 on AT_1 receptor mRNA expression in VSMCs. A, Rat aortic VSMCs were incubated with 1 nmol/L IL-6 for the indicated time points. AT_1 receptor mRNA expression was assessed by Northern analysis. Representative autoradiogram and corresponding ethidium bromide-stained ribosomal RNA. B, Densitometric analysis of 4 separate experiments. Each column represents the relative hybridization signal (mean \pm SEM) normalized to the treatment with vehicle (100%). $*P < 0.05$ vs control. C, VSMCs were incubated for 12 hours with the indicated concentrations of IL-6 before AT_1 receptor mRNA was assessed by Northern analysis. Densitometric analysis. Mean \pm SEM, $n = 3$, $*P < 0.05$ vs control.

run-on assays. Figure 3A demonstrates that IL-6 induced an increase of AT_1 receptor mRNA transcription rate to $129 \pm 3\%$ 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 $50\ \mu\text{g/mL}$ of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole (DRB), which blocks de novo synthesis of mRNA.²² Figure 3B shows the quantification of AT_1 receptor mRNA decay and reveals that IL-6 had no influence on AT_1 receptor mRNA stability ($n = 4$).

IL-6-Induced AT_1 Receptor Overexpression Enhances Reactive Oxygen Species Production in VSMCs

VSMCs were preincubated for 24 hours with 1 nmol/L IL-6 or vehicle, before $1\ \mu\text{mol/L}$ angiotensin II was added for an

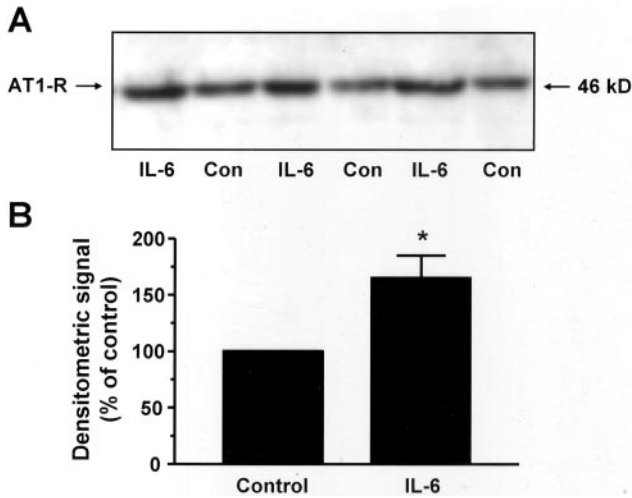


Figure 2. Effect of IL-6 on AT₁ receptor protein expression in VSMCs. VSMCs were stimulated for 24 hours with 1 nmol/L IL-6 or vehicle and AT₁ receptor protein expression was assessed by Western blotting. A, Representative immunoblot. B, Densitometric analysis. Mean ± SEM, n=6, **P*<0.05 vs control.

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

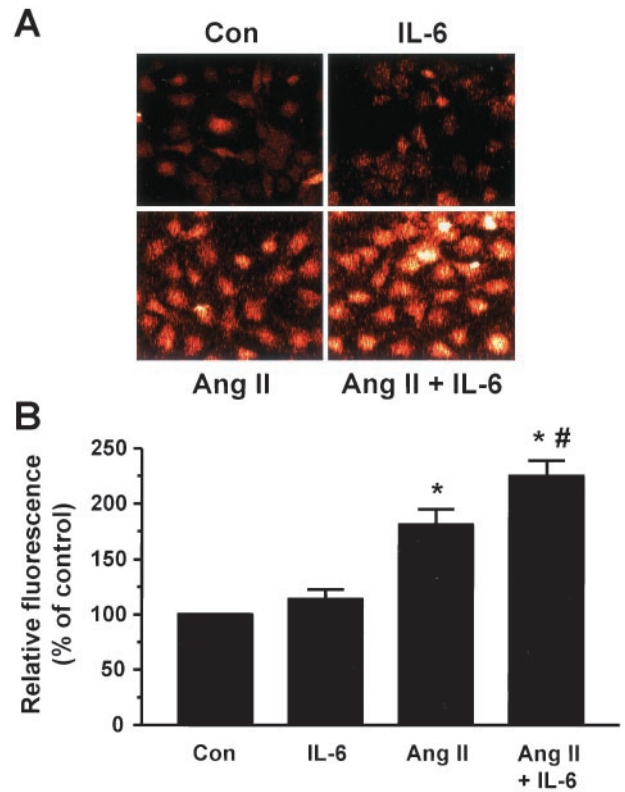


Figure 4. Effect of IL-6 on reactive oxygen species production in VSMCs. VSMCs were preincubated for 24 hours with 1 nmol/L IL-6 or vehicle (Con), followed by a 3-hour incubation with 1 μmol/L angiotensin II (Ang II). Reactive oxygen species production was visualized by DCF fluorescence confocal laser 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, n=4). IL-6 had no significant effect on basal production of reactive oxygen species (114 ± 9% of control).

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

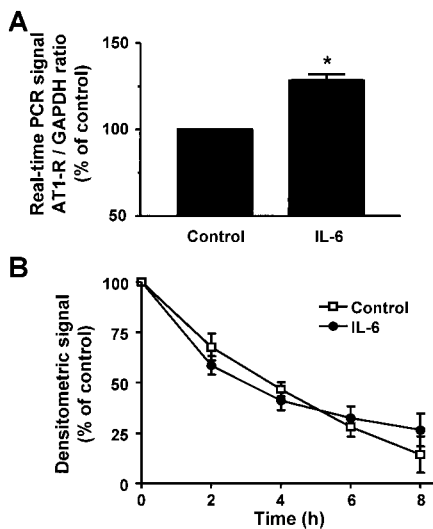


Figure 3. Effect of IL-6 on AT₁ receptor mRNA transcription rate and mRNA stability. A, VSMCs were incubated for 12 hours with 1 nmol/L IL-6 or vehicle, before nuclear mRNA de novo synthesis was assessed by nuclear run-on assays using real-time RT-PCR. AT₁ receptor mRNA synthesis was normalized to the 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.

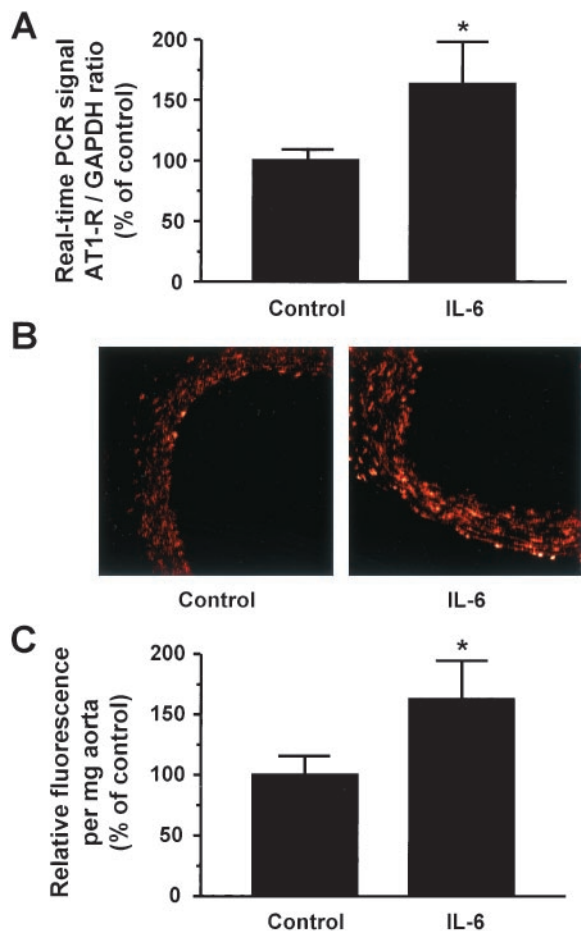


Figure 5. Effect of IL-6 on aortic AT₁ receptor expression and superoxide production in vivo. Male wild-type C57BL/6J mice were treated intraperitoneally with increasing doses of murine IL-6 or vehicle for 18 days (40 ng/day, days 1 to 4; 80 ng/day, days 5 to 12; 160 ng/day; days 13 to 18), and aortas were excised after treatment. **A**, Aortic AT₁ receptor mRNA expression was measured by real-time quantitative RT-PCR. AT₁ receptor mRNA expression was normalized to GAPDH expression. Mean±SEM, n=5 per group, **P*<0.05 vs control group. **B**, Vascular superoxide production was assessed by dihydroethidium (DHE) fluorescence of aortic tissue sections (n=4 per group). In situ production of superoxide was visualized by confocal laser-scanning microscopy. Representative microscopic scans. **C**, In addition, superoxide production in intact aortic segments was quantified by L-012 chemiluminescence assays. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue. Mean±SEM, n=6 per group, **P*<0.05 vs control group.

(n=4 with 10 rings per group). Treatment with IL-6 induced a marked impairment of endothelium-dependent vasodilatation, as assessed by stimulation with carbachol (*P*<0.05 versus control group; 10 and 100 μmol/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 μmol/L angiotensin II), whereas vasoconstriction induced by phenylephrine or KCl was not affected by IL-6 treatment (data not shown).

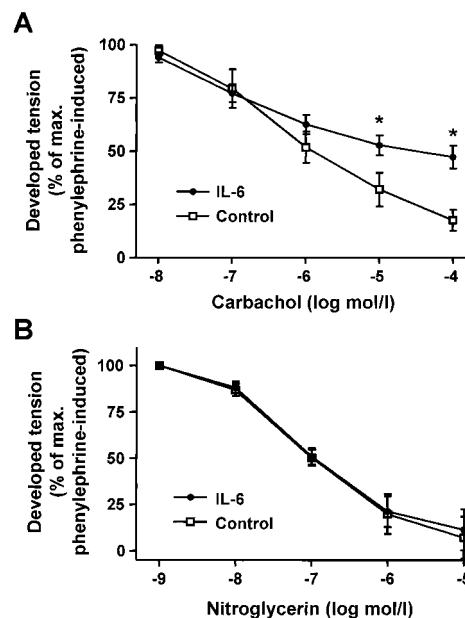


Figure 6. Effect of IL-6 on aortic vasodilatation. Wild-type mice were treated intraperitoneally with increasing doses of IL-6 or vehicle for 18 days. Aortic segments were isolated and their functional performance was assessed in organ chamber experiments. Endothelium-dependent vasodilatation induced by carbachol (**A**) and endothelium-independent vasorelaxation induced by nitroglycerin (**B**), both expressed in percent of maximal phenylephrine-induced vasoconstriction. Aortic preparations of vehicle-treated animals (control) were compared to IL-6-treated mice (IL-6). Mean±SEM, n=4 with 10 rings per group, **P*<0.05 vs control group.

IL-6–Induced Oxidative Stress and Endothelial Dysfunction Are Omitted in AT₁ Receptor Knockout Mice

Mice lacking the AT_{1A} receptor (AT_{1A}^{-/-} mice) with the same genetic background as the wild-type animals were treated with IL-6 or vehicle for 18 days identical to the wild-type group, and oxidative stress (n=4 to 7 per group) and functional performance of aortic ring preparations (n=4 with 10 rings per group) were assessed as described above. Figures 7A and 7B demonstrate that in contrast to wild-type animals, treatment with IL-6 did not significantly enhance vascular superoxide production in AT_{1A}^{-/-} mice (119±16% of vehicle-treated AT_{1A}^{-/-} mice, L-012 chemiluminescence). Accordingly, endothelium-dependent vasorelaxation was not significantly different after IL-6 treatment compared with vehicle-treated AT_{1A}^{-/-} mice (Figure 7C). As expected, angiotensin II-induced vasoconstriction was almost absent in AT_{1A}^{-/-} mice and was not affected by IL-6 (data not shown). Finally, phenylephrine-induced vasoconstriction and nitroglycerin-mediated vasorelaxation were not altered by IL-6 treatment in the AT_{1A}^{-/-} mice (data not shown).

Discussion

The data of the presented study demonstrate that the proinflammatory cytokine IL-6 induces an upregulation of AT₁ 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-

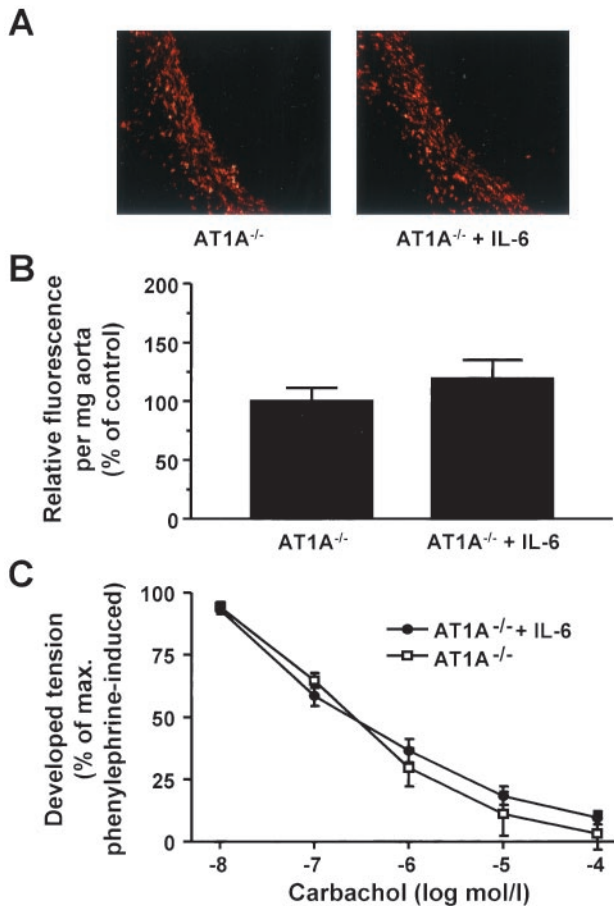


Figure 7. Oxidative stress and endothelial function in AT₁ receptor knockout mice. Male AT₁ receptor knockout mice (AT1A^{-/-}) with the same genetic background as the wild-type animals were treated with IL-6 or vehicle for 18 days identical to the wild-type group. **A**, Aortic superoxide production 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 vasodilatation assessed in aortic ring preparations, expressed in percent of maximal phenylephrine-induced vasoconstriction. Mean±SEM, n=4 with 10 rings per group.

xygen radical production in vitro and in vivo, and to the development of endothelial dysfunction. It is well established that oxidative stress plays a major role in the pathogenesis of endothelial dysfunction and that AT₁ receptor activation leads to an increased production of reactive oxygen species in vascular cells.^{11,23–25} The reduction of oxidative stress by antioxidants or genetic disruption of reactive oxygen species-producing enzymes was shown to be associated with an improvement of endothelial function.^{11,24–26}

IL-6 increases AT₁ receptor expression by enhancement of the mRNA transcription rate, whereas alteration of mRNA stability is not the underlying gene regulatory mechanism. It is known that AT₁ receptor expression is influenced by many agonists, such as angiotensin II, LDL cholesterol, hormones such as estrogen, progesterone and insulin, and growth factors.^{27–32} Numerous experimental studies have confirmed this regulative pathway which leads to either reduced effects

of AT₁ receptor activation (eg, angiotensin II, estrogen, growth factors) or to an enhancement of AT₁ receptor-mediated effects (eg, progesterone, LDL cholesterol, insulin).^{27–32} Frequently, this is caused by alteration of AT₁ receptor mRNA stability, but changes in AT₁ receptor mRNA transcription rate have also been described, as for example for angiotensin II and progesterone, involving numerous signal transduction pathways, such as cytosolic calcium, MAP kinases, and nitric oxide.^{11,12}

Proinflammatory cytokines such as IL-6 have been implicated in the pathogenesis of atherosclerosis.^{1,2} 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.^{1,2} In a murine model of atherosclerosis, IL-6 was shown to increase atherosclerotic lesion size.³³ 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.^{1,10} 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.¹⁴ Activation of the latter enhances IL-6 production which suggests important interactions between IL-6 and the AT₁ receptor.^{13–16} 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.^{11,12,34} 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.^{35–40}

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.^{27–32} 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

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 could putatively identify patients who especially benefit from a medical treatment that diminishes AT₁ receptor activation.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft. The excellent technical assistance of Sybille Richter is greatly appreciated.

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