Angiotensin Induces Inflammatory Activation of Human Vascular Smooth Muscle Cells

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Abstract—Multiple data suggest that the renin-angiotensin system contributes to the pathogenesis of atherosclerosis. The atherogenic effect of the renin-angiotensin system can only in part be explained by the influence of its effector angiotensin II on blood pressure, smooth muscle cell (SMC) growth, or antifibrinolytic activity. Because chronic inflammation of the vessel wall is a hallmark of atherosclerosis, we hypothesized that angiotensin II may elicit inflammatory signals in vascular SMCs. Human vascular SMCs were stimulated with angiotensin. Inflammatory activation was assessed by determination of interleukin-6 (IL-6) release into the culture medium, detection of IL-6 mRNA by RT-PCR, and demonstration of activation of nuclear factor-κB in electrophoretic mobility shift assays. Angiotensin II concentration-dependently (1 nmol/L to 1 μmol/L) stimulated IL-6 production by SMCs via activation of the angiotensin II type 1 receptor (demonstrated by the inhibitory action of the receptor antagonist losartan). Angiotensin I increased IL-6 production by SMCs, too. This effect was inhibited by captopril and ramiprilat, suggesting conversion of angiotensin I to angiotensin II by angiotensin-converting enzyme in SMCs. Steady-state mRNA for IL-6 was augmented after stimulation with angiotensin II, suggesting regulation of angiotensin-induced IL-6 release at the pretranslational level. Moreover, the proinflammatory transcription factor nuclear factor-κB, which is necessary for transcription of most cytokine genes, was also activated by angiotensin II. Pyrrolidine dithiocarbamate suppressed angiotensin II–induced IL-6 release, a finding compatible with involvement of reactive oxygen species as second messengers in cytokine production mediated by angiotensin. The data demonstrate the ability of angiotensin to elicit an inflammatory response in human vascular SMCs by stimulation of cytokine production and activation of nuclear factor-κB. Inflammatory activation of the vessel wall by a dysregulated renin-angiotensin system may contribute to the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 1999;19:1623-1629.)

Key Words: angiotensin ■ atherosclerosis ■ inflammation ■ interleukins ■ smooth muscle ■ nuclear factor-κB

Several lines of evidence suggest a role of the renin-angiotensin system in the pathogenesis of atherosclerosis. Immunohistochemical studies demonstrated increased accumulation of angiotensin-converting enzyme (ACE) in human atheroma.1 Molecular genetic studies showed an association of variants in the genes for ACE and angiotensinogen with an increased risk for myocardial infarction or coronary heart disease,2,3 although conflicting data exist.4 Prospective clinical investigations showed an association between plasma renin activity and the risk of myocardial infarction and ischemic heart disease.5–7 Large clinical trials investigating the effect of ACE inhibitors on survival after myocardial infarction showed convincingly a reduction of reinfarction rate mostly independent from blood pressure lowering.8–10 In animals, ACE inhibitors had a protective effect against atherosclerosis.11

Most studies on the mechanisms underlying the atherogenic effect of the renin-angiotensin system have focused on angiotensin-induced hyperplasia and hypertrophy of vascular smooth muscle cells (SMCs),12,13 presumably mediated by growth factors.14,15 Atherosclerosis, however, is also characterized by chronic inflammation of the vessel wall.16,17 Cytokines are regarded as important modulators of inflammatory events occurring during all stages of atherogenesis.18,19 Numerous studies have shown that SMCs, in addition to leukocytes, can be an important source of cytokines in the vessel wall.20–24 Some factors linked to atherosclerosis are known to augment cytokine production in SMCs, eg, oxidatively modified LDL or thrombin.25,26 This study tested the hypothesis that another pathophysiologically relevant mediator, angiotensin, stimulates inflammatory activation and cytokine production in human vascular SMCs. Production of

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 interleukin-6 (IL-6), which is a potent stimulus of the acute-phase reaction, an important activator of lymphocytes, and an inducer of collagen and glycosaminoglycan production in fibroblasts, was used as marker of the proinflammatory potential of SMCs.

**Methods**

**Cell Preparation and Culture**

Vascular SMCs were cultured by explant outgrowth from unused portions of human saphenous veins harvested for coronary bypass surgery. Cells were grown in DMEM (Eurobio) supplemented with 10% (vol/vol) FCS (Eurobio), 100 U/mL penicillin, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 2 mL/L l-glutamine. The cells exhibited the typical “hill and valley” growth morphology of SMCs and many reacted with the monoclonal antibody HHF-35 that selectively recognizes muscle forms of actin but does not react with endothelial cells or fibroblasts. From passages 2 to 5 were used for the experiments after being growth-arrested for 2 days in serum-free insulin–transferrin medium consisting of DMEM and Ham’s F12 (1:1, vol/vol; Eurobio) supplemented with 1 µmol/L insulin and 5 µg/mL transferrin. Fresh insulin–transferrin medium was used for the experiments with or without addition of stimuli.

**Materials**

Angiotensin II, angiotensin I, captopril, and pyrrolidine dithiocarbamate (PDTC) were from Sigma. Losartan was a gift from Merck, ramiprilat a gift from Astra. Recombinant human tumor necrosis factor-α (TNFα) was purchased from Endogen. Testing for bacterial endotoxin with the Limulus amebocyte lysate assay (BioWhittaker) revealed levels ≤0.25 EU/mL for all agents.

**Determination of IL-6 Release**

SMCs were grown in 96-well plates to confluency and kept in insulin–transferrin medium for 2 days before the experiment. After addition of the stimuli, cells were cultured for 24 hours, then the conditioned medium was collected and frozen. Assays for IL-6 were performed with an enzyme-linked immunosorbent assay kit (Endogen) according to the manufacturer’s instructions. The assay selectively recognizes IL-6, with a limit of detection of <1 pg/mL.

**RNA Isolation and RT-PCR**

Confluent SMCs in 10-cm Petri dishes were used for total RNA extraction, using RNAzol (Wak-Chemie) according to the manufacturer’s instructions. Complementary DNA was synthesized from extraction, using RNAzol (Wak-Chemie) according to the manufacturer’s instructions. The assay selectively recognizes IL-6, with a limit of detection of <1 pg/mL.

**Electrophoretic Mobility Shift Assay**

Protein extracts from SMCs were prepared as follows: After washing in ice-cold PBS 3 times, the cells were scraped off the tissue culture dish, resuspended, and sedimented by centrifugation. The cell pellet was lysed in a buffer composed of 20 mmol/L HEPES-KOH (pH 7.9), 0.35 mol/L NaCl, 20% glycerol, 1% NP-40, 1 mmol/L MgCl₂, 0.5 mol/L EDTA, 0.5 mmol/L EGTA, 10 µg/mL leupeptin, 0.5 mol/L DTT, and 0.2 mmol/L PMSF by incubation on ice for 30 minutes. After centrifugation, the supernatant containing the protein fraction was frozen at −80°C. For electrophoretic mobility shift assays, a double-stranded oligonucleotide (Promega) representing the consensus sequence for nuclear factor-κB (NF-κB) binding was labeled with [γ-32P]ATP (NEN), using T-4 polynucleotide kinase (Promega). Cell proteins (10 µg) and labeled oligonucleotide (50 000 to 70 000 cpm) were incubated for binding of active NF-κB for 20 minutes at room temperature in a buffer containing 20 µg poly(deoxyinosinic:deoxy-cytidylic acid), 8% Ficoll 400, 44 mmol/L HEPES-KOH (pH 7.9), 140 mmol/L KCl, 4% glycerol, 0.05% NP-40, 0.1 mmol/L EDTA, 4.4 mmol/L DTT, and 0.06 mmol/L PMSF. Immediately after binding, the protein/DNA complexes were separated from unbound oligonucleotide by electrophoresis on a native 5% polyacrylamide gel in TRIS boric acid EDTA buffer. Autoradiography was performed with the dried gels by using Hyperfilm (Amersham). For testing of specificity of NF-κB/DNA binding, in some experiments, antibodies (Santa Cruz Biotechnology) against the p65 or p50 subunits of NF-κB were added to the proteins, resulting in further retardation of electrophoretic mobility, or a 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction, leading to a decrease in NF-κB–bound radioactivity.

**Statistical Analysis**

Numeric results are expressed as arithmetic mean±SEM values. Statistical difference was analyzed by ANOVAs followed by Fisher’s exact test. A P value of <0.05 was considered significant.

**Results**

**Angiotensin II Induces Cytokine Release From Vascular SMCs**

We used measurement of IL-6 in cell supernatants to gauge the ability of angiotensin II to induce cytokine production, because this member of the cytokine family is rapidly secreted on its induction. Angiotensin II caused concentration- and time-dependent IL-6 release from SMCs from 1 mmol/L to 1 µmol/L (Figure 1). At 1 µmol/L angiotensin II, IL-6 release ranged between 61% and 79% of the release obtained with a maximally effective positive control stimulus, TNFα (20 ng/mL). Four experiments with cells from different donors gave similar results.

**Angiotensin II–Induced IL-6 Release From SMCs Depends on the Angiotensin II Type 1 Receptor**

Most effects of angiotensin on vascular SMCs are mediated by the angiotensin II type 1 receptor. Losartan (1 and 10 µmol/L), a selective angiotensin II type 1 receptor antagonist, reduced angiotensin II–stimulated IL-6 release from SMCs (Figure 2). This finding demonstrates both specificity of the angiotensin II effect and involvement of the type 1 receptor.

**Angiotensin I Stimulates IL-6 Release by SMCs via an Angiotensin-Converting Enzyme–Dependent Mechanism**

Because ACE is present in human atheroma, we investigated the effect of angiotensin I on cytokine production. In a similar manner to angiotensin II, angiotensin I (100 mmol/L to 10 µmol/L) stimulated IL-6 release from SMCs, with a concentration–response curve shifted to the right compared with angiotensin II. Angiotensin I–induced IL-6 production was suppressed by 2 different ACE inhibitors, captopril (10 µmol/L) and ramiprilat (1 µmol/L) (Figure 3). This finding demonstrates that the angiotensin I effect depends on conversion of angiotensin I to angiotensin II and is mediated by ACE present in active form in cultured SMCs. The
observation is of particular interest, because another enzyme, chymase, has been implicated in angiotensin conversion in blood vessels.31

Angiotensin Stimulates Accumulation of IL-6 mRNA
Most cytokines are regulated mainly at the transcriptional level. Therefore, we examined the effect of angiotensin II on IL-6 mRNA in SMCs by RT-PCR. Both TNFα (20 ng/mL) and angiotensin II (1 μmol/L) increased steady-state IL-6 mRNA levels compared with control conditions, whereas the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene was not upregulated by these agents (Figure 4). Actinomycin D (5 μg/mL) completely blocked angiotensin II– and TNFα-stimulated increase of IL-6 mRNA, suggesting that IL-6 expression is dependent on transcriptional regulation (data not shown).

Angiotensin II Activates the Transcription Factor NF-κB
Activation of NF-κB was probed by electrophoretic mobility shift assay (Figure 5). Both TNFα and, to a lesser extent, angiotensin II activated NF-κB in SMCs. Active NF-κB was already present after 30 minutes of stimulation. Maximal NF-κB activation was found after 1 hour of stimulation and was still present after 2 hours (Figure 5A). The specificity of the shifted autoradiographic bands was ascertainment in 2 ways. (1) Addition of antibodies against the p65 subunit or the p50 subunit (not shown) of NF-κB resulted in a further retardation of the mobility of the NF-κB/oligonucleotide complex (“supershift”). (2) An excess of unlabeled oligonucleotide reduced the signal intensity of the band associated with active NF-κB (Figure 5B).

PDTC Inhibits Angiotensin II–Induced IL-6 Release
Activation of NF-κB (and subsequent production of cytokines) can also be mediated by reactive oxygen species.32,33 We therefore examined the effect of the radical scavenger PDTC on angiotensin II–induced IL-6 release by vascular SMCs (Figure 6). At 10 μmol/L, PDTC reduced IL-6 accumulation both under control conditions and with stimulation by TNFα or angiotensin II; 25 μmol/L PDTC virtually abolished IL-6 release caused by either angiotensin II or TNFα. The results suggest the involvement of oxygen radicals in both basal and stimulated IL-6 secretion.

Discussion
The present data demonstrate the potential of angiotensin to induce inflammatory activation and cytokine production in human vascular SMCs. Angiotensin II dose-dependently increased IL-6 release by SMCs via the angiotensin type 1 receptor. Moreover, angiotensin I also increased IL-6 production by SMCs via an ACE-dependent mechanism. Stimulation of SMCs by angiotensin II resulted in increased accumulation of IL-6 mRNA, suggesting interaction of angiotensin with IL-6 release at the pretranslational level. Finally, angiotensin II potently activated NF-κB, a transcription factor that promotes the production of IL-6 and other cytokines in SMCs.
tion factor system commonly involved in inflammatory and immune responses.

Mechanisms usually ascribed to angiotensin’s atherogenic action include stimulation of SMC mitogenesis or hypertrophy, augmented extracellular matrix synthesis, and interaction with the fibrinolytic system. These observations were made in rat SMCs, leaving some uncertainty as to whether the same mechanisms apply to human tissue. A recent study demonstrated increased mRNA expression for the chemokine MCP-1 in rat vascular SMCs on angiotensin II stimulation. In a similar manner, Moriyama et al described IL-6 release from mouse mesangial cells stimulated by angiotensin II. To our knowledge, the present study is the first to describe inflammatory activation of human vascular SMCs by angiotensin. Other potentially important inflammatory actions of angiotensin II include stimulation of TNFα release by blood monocytes and increased adherence of monocytes to endothelial cells. Inflammatory responses mediated by cytokines are presumably important in all stages of atherosclerosis. Monocyte adherence to the endothelium and infiltration of the vessel wall, probably the first step leading to the development of the fatty streak, depends on endothelial expression of adhesion molecules, an event that is regulated by cytokines. In advanced stages of atherosclerosis, cytokines may promote destabilization and rupture of plaques by induction of matrix-degrading enzymes, ultimately leading to thrombosis and complete obstruction of the vessel. Stimulation of cytokine production by angiotensin could contribute to these events. Increased production of IL-6 may be of particular clinical relevance, because the acute-phase reaction, eg, synthesis of C-reactive protein by the liver, is regulated mainly by IL-6. Data from the Physician’s Health Study showed that the plasma level of C-reactive protein in apparently healthy men predicts the risk of future myocardial infarction and stroke. Moreover, increased blood concentrations of IL-6 in patients with unstable angina correlated with C-reactive protein levels. IL-6 production by the vessel wall may be an important mediator of local and generalized inflammatory reactions in the evolution of acute coronary syndromes. Moreover, stimulation of lymphocytes by IL-6 may be equally important, because activated T lymphocytes are present in human atheroma and probably contribute to ongoing inflammation within the plaque, ultimately leading to its rupture.

**Figure 3.** Angiotensin I induces interleukin-6 (IL-6) release from human vascular smooth muscle cells after conversion to angiotensin II. Cells were grown to confluency in 96-well plates and growth-arrested in insulin–transferrin medium for 2 days. For the experiment, the medium was replaced by fresh insulin–transferrin medium with or without angiotensin I or the positive control stimulus tumor necrosis factor-α (TNFα; 20 ng/mL) in the absence (open columns) or presence (hatched columns) of the angiotensin-converting enzyme inhibitors captopril (A) or ramiprilat (B). The medium was collected after 24 hours and assayed for IL-6 concentration by enzyme-linked immunosorbent assay. Results are expressed as mean±SEM values, n=3; *P<0.05.

**Figure 4.** Angiotensin II induces mRNA for interleukin-6 (IL-6) in human vascular smooth muscle cells. Confluent cells were growth-arrested with insulin–transferrin medium for 2 days and stimulated with tumor necrosis factor-α (TNFα; 20 ng/mL) or angiotensin II (ANG II; 1 μmol/L) for the indicated time periods. Total RNA (1 μg) was reverse-transcribed. The reverse transcription product was subjected to 35 cycles of PCR, using primer pairs specific for human IL-6 and the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, yielding PCR products of 622 and 212 bp, respectively. Representative ethidium bromide–stained agarose gels of RT-PCR products for IL-6 mRNA (top) and GAPDH (bottom) are shown. Results are representative for 3 independent experiments with cells from different donors.
Another interesting result of this study is that angiotensin I also stimulated IL-6 production in SMCs by an ACE-dependent mechanism, indicating the presence of active ACE in cultured human SMCs. Suppression of inflammatory responses in the vessel wall can explain in part the beneficial action of ACE inhibitors on myocardial reinfarction rates observed in the Studies of Left Ventricular Dysfunction and Survival and Ventricular Enlargement (Study) trials. However, in the intact artery lined with endothelium, the situation may be more complicated. Hernández-Presa et al demonstrated in a rabbit model of early atherosclerosis that the ACE inhibitor quinapril reduced monocyte accumulation, MCP-1 expression, and NF-κB activation in the vessel wall. These findings could result from either decreased stimulation of vessel wall cells by angiotensin II or increased accumulation of bradykinin because of suppression of its breakdown by ACE inhibitors. Bradykinin is known to stimulate endothelial production of nitric oxide, a molecule that can suppress inflammatory activation of vascular SMCs. Our data provide evidence for an antiinflammatory action of ACE inhibitors affecting SMCs directly. Further in vivo experiments comparing the effects of ACE inhibitors with those of angiotensin II type 1 receptor blockers and bradykinin receptor antagonists could clarify the contribution of the different pathways.

IL-6 production, as well as the synthesis of other cytokines, is regulated at the transcriptional level. The promoter regions of cytokine genes commonly contain binding sequences for the transcription factor NF-κB. Transcription of the IL-6 gene also depends on NF-κB. Activated NF-κB is present in human atheroma and human vascular SMCs express inducible NF-κB activity. We report here activation of NF-κB in human vascular SMCs by angiotensin II. Activation of NF-κB is a point of convergence by which different atherogenic agents cause inflammatory activation of the vessel wall. At present, it remains unclear by which intracellular signaling pathway angiotensin induces NF-κB and IL-6 production. NF-κB can possibly be activated through phosphorylation by protein kinase C, which is stimulated by angiotensin II. Preliminary data presented in abstract form suggest involvement of the JAK/STAT pathway for induction of IL-6 production in SMCs. Another way in which angiotensin may signal NF-κB activation is through stimulation of...
NADH and NADPH oxidases, enzymes that generate \( \text{O}_2^− \). Reactive oxygen species are regarded as second messengers for the activation of NF-κB and for the expression of cytokines. Our work demonstrated inhibition of angiotensin II–induced IL-6 release by the radical scavenger PDTDC. This finding is compatible with the hypothesis that reactive oxygen intermediates participate in angiotensin II–stimulated cytokine production by vascular SMCs. Further work is required to dissect the intracellular signal pathways that transmit angiotensin-mediated inflammatory activation.

In summary, the present data show that dysregulation of the local renin-angiotensin system may initiate and promote atherosclerosis by inflammatory activation of the vessel wall. Thus, angiotensin must be regarded as more than a regulator of vascular tone, but as a mediator affecting the local biology of the arterial wall by triggering inflammatory pathways. Suppression of angiotensin’s proinflammatory action on vascular tissue could in part explain the beneficial effect of ACE inhibitors on recurrence of myocardial infarction.

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