Angiotensin II Induces Vascular Cell Adhesion Molecule-1 Expression In Rat Vasculature
A Potential Link Between the Renin-Angiotensin System and Atherosclerosis

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Background—Cardiovascular ischemic events may occur more frequently in hypertensive patients with activated renin-angiotensin systems. We tested the hypothesis that angiotensin II (Ang II) may contribute to atherosclerosis by increasing expression of vascular inflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1).

Methods and Results—Rats infused with norepinephrine or Ang II for 6 days developed similar hypertensive responses, but only Ang II-treated rats exhibited significant increases in aortic VCAM-1 protein and mRNA expression. Oral losartan treatment (50 mg kg\(^{-1}\) d\(^{-1}\)) inhibited Ang II-induced hypertension and aortic VCAM-1 mRNA expression. Ang II treatment significantly increased VCAM-1 mRNA expression in cultured rat aortic smooth muscle cells (RASMCs). Ang II also induced nuclear NF-κB-like binding activity and transactivated an NF-κB–driven VCAM-1 promoter. Losartan and proteasome inhibitors blocked Ang II-induced NF-κB activation and VCAM-1 mRNA accumulation. IκB-α overexpression in RASMCs inhibited Ang II-induced VCAM-1 promoter transactivation.

Conclusions—Ang II may contribute to atherogenesis by activation of VCAM-1 through proteasome dependent, NF-κB-like transcriptional mechanisms. (Circulation. 1999;100:1223-1229.)

Key Words: angiotensin ■ hypertension ■ VCAM-1 ■ NF-κB

The molecular mechanisms underlying hypertension as a risk factor for atherosclerosis are poorly understood.\(^1\);\(^2\) Atherosclerosis may be characterized as an inflammatory disease involving the upregulation of mononuclear leukocyte recruiting mechanisms in response to oxidative stress in the vessel wall.\(^1\) Neointimal monocyte infiltration is a key initial step in atheroma formation.\(^3\) Monocyte recruitment is mediated, in part, by vascular cell adhesion molecule-1 (VCAM-1), a cell surface protein expressed by endothelial and smooth muscle cells. VCAM-1 is induced by a variety of inflammatory signals, and it mediates monocyte adherence via interaction with the integrin counter-receptor very late antigen-4.\(^4\);\(^5\) VCAM-1 is observed in endothelium and smooth muscle of early atherosclerotic lesions.\(^6\);\(^7\) VCAM-1 gene expression is activated through an oxidation-reduction sensitive mechanism that involves activation of the transcription factor NF-κB.\(^8\);\(^9\);\(^10\)

Epidemiological studies suggest that hypertensive patients with activated renin-angiotensin systems have a higher risk for myocardial infarction than other forms of hypertension.\(^11\);\(^12\) Treatment of patients with left ventricular dysfunction with angiotensin converting enzyme (ACE) inhibitors reduces recurrent myocardial infarctions and mortality.\(^13\);\(^14\)

These data suggest a potential role of the renin-angiotensin system in contributing to the atherosclerotic process. Angiotensin II (Ang II), an important component of the renin-angiotensin system, can induce oxidative stress in the vasculature via generation of oxygen-free radicals.\(^15\) Superoxide anion is generated by membrane-bound NADH/NADPH oxidase in aortas from rats made hypertensive with Ang II but not norepinephrine.\(^16\) Therefore, oxidative signaling is a feature shared by Ang II’s effects on cellular metabolism and regulation of VCAM-1 gene expression. In this study, we found that Ang II directly stimulates VCAM-1 mRNA and protein expression in aortic tissue of rats. This induction appears to be mediated via the AT1 receptor. In cultured rat aortic smooth muscle cells (RASMCs), Ang II induces VCAM-1 gene expression and NF-κB activation. Our data suggest that Ang II may contribute to the atherosclerotic process by inducing the expression of inflammatory genes.
such as VCAM-1, through NF-κB-like transcriptional mechanisms.

**Methods**

**Materials**

All preparations of Ang II (Sigma and Calbiochem) tested negative for endotoxin by Limulus amoebocyte lysate assay (ICN Pharmaceuticals Inc.). Other materials included losartan (a gift from Dr Ronald Smith, Du Pont Pharmaceuticals and Merck, Inc.), lactacystin (Dr E.J. Corey, Harvard University), PDI23319 (Research Biochemicals International), N-Ac-leu-leu-norleucinal (ILNleL; Calbiochem), antibodies to p65 and c-fos (Santa Cruz Biotechnology Inc., Santa Cruz, Calif), and IeB-α expression vector (a gift from Dr Dean Ballard, Vanderbilt University).

**Animal Studies**

Chronic arterial catheters were placed into male Sprague-Dawley rats (250 to 300 g) as previously described.18 Osmotic minipumps were implanted subcutaneously to deliver drugs at a constant infusion rate as previously described:20 Ang II (0.7 mg · kg⁻¹ · d⁻¹), norepinephrine (2.8 mg · kg⁻¹ · d⁻¹) or vehicle (0.9% NaCl). Arterial pressures were measured while rats were conscious.18 After lethal pentobarbital injection but before death, 2500 U intracardiac heparin was given. Aortic arches and thoracoabdominal aortas were collected for immunohistochemistry and Northern analysis, respectively.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.19 Antibodies were used at the following dilutions: goat anti-rat VCAM-1 (Berkeley Antibody Company, Richmond, Calif) and nonimmune goat IgG (Sigma, St. Louis, Mo): 1:200 goat anti-human Von Willebrand’s Factor (VWF, which cross-reacts with Rat VWF; Incstar, Stillwater, Minn): 1:1000. Biotinylated rabbit anti-goat secondary antibody (Dako, Carpinteria, Calif): 1:2000. All slide micrographs were digitized and images developed with Adobe Photoshop in uniform manner.

**Cell Culture**

RASMCs were grown in DMEM supplemented with 10% FBS, 2 mmol/L-glutamine, penicillin (100 U/mL) and streptomycin (100 mg/mL). Experiments were conducted on cells at passages 5 to 15. Confluent RASMCs were quiesced in DMEM with 0.1% FBS for 48 hours. Cell cultures were incubated at 37°C in 5% CO₂-95% humidified air.

**Northern Analysis**

Northern analysis was performed as previously described.20 Hybridizations with a 1.2-kb EcoR I-Xho I fragment of murine VCAM-1 cDNA were performed at 60°C for 2 hours in QuickHyb solution (StrataGene). Filters were washed with a final stringency of 0.2×SSC at 55°C for 30 minutes. Autoradiography was performed with a PhosphorImager 445Si (Molecular Dynamics).

**Electrophoretic Mobility Shift Analysis (EMSA)**

Nuclear extracts from RASMCs were prepared as described previously.21 The oligonucleotide containing the κB NF-κB consensus sequence (GGGATTTC) was labeled and EMSA performed as described previously.20,21 Specificity of NF-κB-like binding was confirmed by incubation with 100-fold molar excess of unlabelled competitor probe and mutated probe. These data were further confirmed by using an oligonucleotide containing the HIV LTR canonical κB sequence: (GTTACAAAGGGACTTCTCGGTGGG-GCTTCCAGGGG).

**Analysis of VCAM-1 Promoter Activation**

RASMCs were grown to 60% to 80% confluence in 60 mm² tissue culture plates and transfected with 5 μg of p85VCAM-CAT by calcium phosphate coprecipitation as described previously.20 p85VCAM-CAT is a chimeric reporter gene containing coordinates −85 to +12 of the human VCAM-1 promoter linked to a chloramphenicol acetyl transferase reporter (CAT) gene. With cotransfection studies, RASMCs were cotransfected with p85VCAM-CAT (5 μg/dish) plus either empty vector (5 μg/dish) or IeB-α expression vector22 (5 μg/dish). After 24 hours, the cells underwent quiescence in medium containing 0.1% FBS for 24 hours. RASMCs were then treated with Ang II for 16 hours. CAT activities were determined as previously described.20 Acetylated and unacylated forms of chloramphenicol were separated by thin-layer chromatography and imaged by the PhosphorImager 445Si.

**Statistical Analysis**

VCAM-1 mRNA accumulation was quantified via densitometry of bands on Northern analyses. Measurements were expressed as fraction of control levels and compared among groups using an unpaired Student’s t test with Bonferroni protection. Mean aortic pressure measurements were also compared using an unpaired Student’s t test.

**Results**

**Effects of Ang II and Norepinephrine on Blood Pressure and Aortic VCAM-1 mRNA Accumulation**

Rats treated with Ang II (0.7 mg · kg⁻¹ · d⁻¹) or norepinephrine (2.8 mg · kg⁻¹ · d⁻¹) developed increased mean arterial pressures from 100 to 182 and 185 mm Hg, respectively, 6 days after infusion (Figure 1C). Northern analysis of whole aortic RNA showed a marked increase in VCAM-1 (2.8±0.6× control) mRNA levels in Ang II treated rats (n=6) when compared with controls (n=6, P<0.05; Figure 1A and 1B). Norepinephrine-treated rats exhibited no signif-
icant increase (1.3±0.1 × control) in aortic VCAM-1 mRNA levels (n=4, P=NS; Figure 1A and 1B).

**Ang II Infusion Induces VCAM-1 Expression in Rat Aortas**

Rats treated with Ang II for 6 days demonstrated a marked increase in VCAM-1 protein expression localized predominantly to the adventitia (Figure 2A, arrow) and endothelium (arrowhead). A low level of increased expression was detected in the media. The specificity of VCAM-1 localization was demonstrated by the lack of staining in a serial section with nonimmune goat IgG (Figure 2B). No VCAM-1 protein immunoreactivity was detected in a section of aorta from a sham-treated rat (Figure 2C). The lack of VCAM-1 staining in the sham-treated control was not due to lack of intact endothelium as evidenced by robust endothelial staining of a serial section with an antibody to VWF (Figure 2D; arrowhead).

**Ang II-Induced VCAM-1 Gene Expression is Mediated By the AT1 Receptor**

Rats were treated with the AT1 receptor antagonist losartan 2 days before Ang II infusion and throughout the 6 days of Ang II treatment. Oral losartan treatment (50 mg · kg⁻¹ · d⁻¹) inhibited Ang II-induced hypertension (MAP = 108 mm Hg, n=4, Figure 3, top) and aortic VCAM-1 mRNA expression (P<0.05, Figure 3, bottom). Lower dose losartan treatment (25 mg · kg⁻¹ · d⁻¹) also blocked Ang II-induced hypertensive response (MAP = 107 mm Hg, n=6, Figure 3, top) but had no effect on Ang II-induced VCAM-1 gene expression (P=NS, n=4). Rats infused with the AT2 receptor antagonist PD123319 (30 µg · kg⁻¹ · min⁻¹) for 6 days increased aortic VCAM-1 mRNA levels. PD123319 treatment had no effects on Ang II induced aortic VCAM-1 mRNA accumulation (data not shown).

**Ang II Induces VCAM-1 mRNA Accumulation Via the AT1 Receptor in Cultured RASMCs**

Although our in vivo study shows that Ang II-induced VCAM-1 expression occurs predominantly in the endothelium and the adventitia, a low level of increased expression occurred in smooth muscle cells of the media. Cultured, passaged endothelial cells do not express AT1 receptors in a stable manner and did not express VCAM-1 in response to Ang II (data not shown). We therefore used RASMCs to study the signaling mechanisms of Ang II-induced VCAM-1 gene expression.

RASMCs were treated with or without Ang II for 6 hours. There was some basal VCAM-1 mRNA expression in unstimulated RASMCs (Figure 4A). Treatment with 1 nmol/L
Ang II caused a 1.9-fold increase in VCAM-1 mRNA expression. Maximal induction occurred at 10 nmol/L. Ang II-induced VCAM-1 mRNA accumulation appeared at 2 hours, peaked at 4 to 8 hours, and disappeared at 24 hours (Figure 4B).

RASMCs were pretreated for 15 minutes with losartan (10 μmol/L), which blocked the increase in VCAM-1 mRNA caused by Ang II (100 nmol/L, Figure 4C). Losartan had no effect on TNF-induced VCAM-1 expression (data not shown). The AT2 receptor antagonist PD123319 had no effect on basal or Ang II-induced VCAM-1 mRNA accumulation (data not shown).

**LLnL and Lactacystin Inhibit Ang II-Induced VCAM-1 mRNA Accumulation in RASMCs**

Nuclear translocation of NF-κB in endothelial cells is dependent on its dissociation from and degradation of IκB-α via the ubiquitin-proteasome pathway. RASMCs were treated with the proteasome inhibitors LLnL or lactacystin for 1 hour before and throughout a 6-hour exposure to Ang II. Ang II-induced VCAM-1 mRNA accumulation was abolished by pretreatment with both LLnL and lactacystin (Figure 5).

**Ang II Induces NF-κB Binding Activities in RASMCs Through an AT1 Receptor Mediated Mechanism**

Nuclear extracts were prepared from RASMCs after 1-hour exposure to Ang II (10 and 100 nmol/L); EMSA for NF-κB-like binding activity to the VCAM-1 NF-κB consensus sequence elements, κL-κR, was performed. Ang II treatment significantly increased NF-κB binding activity compared with the low basal activity in nonstimulated cells (Figure 6A). The specificity of NF-κB-like binding activity was confirmed by competition with either 100-fold molar excess of unlabelled probe or mutated probe. Furthermore, the NF-κB band is specifically bound and disappears in the presence of antibody to p65 but not antibody to c-fos (Figure 6A). Similar results were obtained using an HIV LTR canonical κB sequence (data not shown). Losartan (10 μmol/L) blocked Ang II-induced (100 nmol/L) nuclear NF-κB binding activity (Figure 6B).

**IκB-α Overexpression Inhibits Ang II-induced VCAM-1 Promoter Transactivation**

RASMCs were cotransfected with 5 μg/dish p85VCAM-1-CAT, whose activation is dependent on a κL-κR NF-κB consensus sequence, plus either an empty vector (5 μg/dish) or IκB-α expression vector (5 μg/dish). Ang II treatment significantly increased p85VCAM-CAT promoter activity compared with the low basal activity in nonstimulated cells (Figure 6A).
Proteasome Inhibitors Block Ang II-induced NF-κB Activation

RASMCs were treated with LLnL or lactacystin for 1 hour before and throughout 1-hour exposure to Ang II. Ang II-induced nuclear NF-κB binding activity was abolished by pretreatment with LLnL or lactacystin (Figure 8).

Discussion

In this study we established that Ang II-mediated experimental hypertension in rats is associated with increased expression of aortic VCAM-1, an inducible adhesion protein associated with inflammatory events in atherosclerosis. Whether hypertension per se is sufficient to activate vascular inflammatory responses remains an important unresolved question. The present study showed that for similar degrees of hypertension, Ang II induced much greater vascular VCAM-1 gene expression than norepinephrine. These results suggest that although hypertension alone could play some role in upregulating VCAM-1 expression, it is not sufficient to fully activate vascular VCAM-1 expression. Several studies corroborate our data that Ang II influences vascular pathophysiology in addition to its pressor effects. Rats made hypertensive by Ang II, but not norepinephrine, infusion exhibit an increase in aortic superoxide generation via activation of membrane-bound NADH/NADPH oxidase. Similarly, Ang II stimulates NADH/NADPH oxidase in vascular smooth muscle cells. These studies, in concert with our data, suggest that Ang II and not hypertension alone may initiate oxidative signaling mechanisms; these mechanisms in turn activate redox-sensitive transcription factors such as NF-κB and upregulate the expression of NF-κB driven genes such as VCAM-1.

Our data suggests that the AT1 receptor mediates Ang II-induced VCAM-1 gene expression. Losartan treatment at 25 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\) inhibited Ang II-induced hypertension, but not Ang II-mediated VCAM-1 expression. Higher doses (50 mg \(\text{kg}^{-1} \cdot \text{d}^{-1}\)) are required to inhibit Ang II-induced VCAM-1 mRNA expression in rat aortas. Therefore, Ang II induces VCAM-1 gene expression through a mechanism that is distinct from its role in the development of hypertension. A potential explanation for this finding is that the AT1 receptor may have a higher threshold for inducing signaling mechanisms for vasoconstriction compared with mechanisms for NF-κB activation. Alternatively, Ang II may use other types of receptors to activate VCAM-1 gene expression. We explored the possibility of Ang II cleaved products such as angiotensin IV in mediating the upregulation of VCAM-1 expression, perhaps via other receptors, but found that RASMCs treated with angiotensin IV did not increase VCAM-1 mRNA levels; furthermore, the Ang IV receptor antagonist divalinal-Ang IV did not inhibit Ang II-induced VCAM-1 mRNA accumulation in RASMCs (data not shown). PD123319 treatment did not effect basal or Ang II-induced VCAM-1 mRNA accumulation in RASMCs, suggesting that the AT2 receptor may not be involved in Ang II-induced VCAM-1 mRNA accumulation. Interestingly, PD123319 increased aortic VCAM-1 mRNA levels in the whole animal study. The mechanism for this finding is unclear, but it is possible that AT2 receptor blockade in vivo may upregulate AT1 receptor-mediated signaling. However, there is no published data to support this notion, and further exploring this mechanism is beyond the scope of this study.

To explore the mechanisms of Ang II-induced VCAM-1 gene expression, we initially used cultured endothelial cells and found that they did not express the VCAM-1 gene in response to Ang II because they lose their ability to express AT-1 receptors in early passages (data not shown). This may explain the differences in published data regarding Ang II actions in endothelial cells. Grafe and coworkers reported that Ang II induces E-selectin gene expression in, and leukocyte adhesion to, human coronary endothelial cells. However, Ang II may have a higher threshold for inducing signaling mechanisms for vasoconstriction compared with mechanisms for NF-κB activation. Alternatively, Ang II may use other types of receptors to activate VCAM-1 gene expression. We explored the possibility of Ang II cleaved products such as angiotensin IV in mediating the upregulation of VCAM-1 expression, perhaps via other receptors, but found that RASMCs treated with angiotensin IV did not increase VCAM-1 mRNA levels; furthermore, the Ang IV receptor antagonist divalinal-Ang IV did not inhibit Ang II-induced VCAM-1 mRNA accumulation in RASMCs (data not shown). PD123319 treatment did not effect basal or Ang II-induced VCAM-1 mRNA accumulation in RASMCs, suggesting that the AT2 receptor may not be involved in Ang II-induced VCAM-1 mRNA accumulation. Interestingly, PD123319 increased aortic VCAM-1 mRNA levels in the whole animal study. The mechanism for this finding is unclear, but it is possible that AT2 receptor blockade in vivo may upregulate AT1 receptor-mediated signaling. However, there is no published data to support this notion, and further exploring this mechanism is beyond the scope of this study.

Figure 8. Proteasome inhibitors block Ang II-induced NF-κB activation. EMSA demonstrating NF-κB binding activity in RASMCs pretreated with LLnL (100 μmol/L) or lactacystin (100 μmol/L) for 1 hour and Ang II (100 nmol/L) for 1 hour.
II did not induce VCAM-1 expression in this study. Kim et al. reported that Ang II induces monocyte adherence to endothelial cells without inducing VCAM-1, ICAM-1, or E-selectin gene expression. Because Ang II was unable to stimulate VCAM-1 gene expression in cultured endothelial cells, we used RASMCs, which express the AT-1 receptor in a stable manner through many passages, to study the mechanisms of VCAM-1 gene activation by Ang II.

We demonstrated that Ang II induces VCAM-1 gene expression at physiological concentrations (1 nmol/L) in RASMCs. Peak responses occurred at concentrations of 10 and 100 nmol/L, which are similar to those in other reports of Ang II actions. In endothelial and vascular smooth muscle cells, cytokine mediated VCAM-1 gene expression occurs through NF-κB mediated transcriptional mechanisms. The VCAM-1 promoter contains κB-like elements that are essential for its activation by cytokines. We demonstrated that Ang II activates nuclear NF-κB-like DNA binding activity that is functional in its ability to transactivate κB containing promoters. Li and coworkers reported that Ang II can activate NF-κB and stimulate angiotensinogen gene expression through NF-κB-mediated transcriptional mechanisms in rat hepatocytes. Consistent with this mechanism, we found that Ang II-induced VCAM-1 mRNA accumulation is blocked by inhibitors of the proteasome, an intracellular multicatalytic proteinase complex that degrades ubiquitinated IkB-α and enables nuclear translocation of NF-κB. Furthermore, overexpression of IkB-α inhibited Ang II-induced VCAM-1 promoter transactivation in RASMCs. These data suggest a potentially significant role of a proteasome sensitive, NF-κB-like transcriptional mechanism in Ang II mediated VCAM-1 gene expression in vascular smooth muscle cells.

Numerous studies suggest the importance of the renin-angiotensin system in atherosclerosis. Hypertensive patients with high renin profiles and the deletion polymorphism DD ACE genotype, which is associated with higher levels of plasma ACE, may have a higher risk for myocardial infarction than those with low renin profiles. Treatment of patients with ACE inhibitors after suffering a myocardial infarction decreases the number of recurrent myocardial infarctions and overall mortality. These reductions in ischemic events are not fully explained by the drugs’ hemodynamic effects. ACE inhibitors reduce atherosclerotic lesions in several animal models including Watanabe hyperlipidemic rabbits, cholesterol-fed monkeys, and mice. Several studies suggest a direct role of Ang II in atherosclerosis. Our data demonstrates VCAM-1 protein expression localized predominantly to the adventitia and endothelium in Ang II infused rats. Capers and coworkers have demonstrated infiltration of monocytes/macrophages predominantly in aortic adventitia in rats made hypertensive by Ang II infusion. Monocyte infiltration occurs in the arterial walls of spontaneously hypertensive rats but is abolished with treatment with ACE inhibitors. Because VCAM-1 plays a significant role in monocyte adhesion to endothelial cells, the data from the present study and others suggest that the proatherogenic properties of the renin-angiotensin system may be mediated, in part, through Ang II-mediated induction of vascular inflammatory gene expression.

In summary, our findings demonstrate that Ang II, a potent vasoconstrictor, growth factor, and activator of vascular oxidant signals, stimulates NF-κB activation and VCAM-1 gene expression in the vasculature. Ang II induced VCAM-1 expression serves as a useful model for a novel, growth factor-mediated mechanism in upregulating NF-κB-driven vascular inflammatory genes. As such, this model begins to link hypertension, a principal risk factor for developing cardiovascular ischemic events, with the inflammatory mechanisms involved in the pathogenesis of atherosclerosis.

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