Role of Angiotensin Type-1 and Angiotensin Type-2 Receptors in the Expression of Vascular Integrins in Angiotensin II–Infused Rats

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Abstract—Angiotensin II plays an important role in vascular remodeling through effects that involve, in part, interactions of vascular smooth muscle cells with extracellular matrix via integrins, which belong to a family of transmembrane receptors. We hypothesized that angiotensin (Ang) II regulates expression of vascular integrins and their ligands in experimental hypertension. Rats were infused subcutaneously with Ang II and received angiotensin type-1 (AT₁) receptor blocker losartan, the AT₁/angiotensin type-2 (AT₂) [Sar₁-Ile₈]-Ang II, or the vasodilator hydralazine for 7 days. Osteopontin and integrin subunit expression were evaluated immunohistochemically. Ang II enhanced vascular α₁, β₁, β₃ integrins and osteopontin expression, which were significantly reduced by losartan, [Sar₁-Ile₈]-Ang II, and hydralazine. Although Ang II increased vascular α₁ subunit expression, this was additionally increased by losartan. Losartan was the only treatment that induced α₁ subunit expression. These results demonstrate that AT₁ and AT₂ receptors have countervailing effects on vascular integrin subunit expression that may influence their effects on vascular remodeling and extracellular matrix composition. (Hypertension. 2006;47:1-6.)

Key Words: renin-angiotensin system ■ aorta ■ extracellular matrix ■ hypertension ■ osteopontin

Migration of vascular smooth muscle cells (VSMCs) within or from the media to the intima plays a major role in arterial remodeling in atherosclerosis and hypertension. Migration of VSMCs and remodeling require controlled degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs) and the activation or release of growth factors. VSMCs are embedded in ECM that includes collagens, fibronectin, laminin, elastin, and proteoglycans. The composition of the ECM can change in response to vascular injury. For example, the ECM protein tenascin, absent in normal rat aortic media, is expressed in the neointima in hypertension or after injury.²,³

Several receptor–ligand systems, such as integrins, are involved in cell–ECM interactions, which regulate cell phenotype and function. Integrins belong to a superfamily of transmembrane glycoprotein adhesion receptors consisting of 2 noncovalently linked subunits, α and β. To date, at least 18 α subunits and 8 β subunits have been described, with a resulting combination of >22 integrins with varying ligand specificity.⁴ The surrounding ECM, as well as integrin–matrix interactions, regulate a variety of cell behaviors, including migration, proliferation, proteinase production, and differentiation.⁸–¹⁰

During the processes of VSMC migration from the media to the intima, cells dissociate from and degrade the ECM proteins. This process involves 3 steps: a phenotypic change from the contractile to the synthetic state, proteolysis of ECM proteins, and cell migration through matrix digestion, a process that resembles tumor cell invasion.¹¹

Remodeling of the small arteries occurs in both human and experimental models of hypertension and involves changes in ECM and its interactions with VSMCs. Restructuring of VSMCs may be, in part, triggered by adhesion molecules, such as integrins, which transduce signals from the extracellular environment to cytoskeletal fibrillar components.¹²,¹³ Changes in ECM components and the corresponding adhesion receptors and interactions between VSMC and matrix proteins may result in rearrangement of these components of the vascular wall. Thus, fibronectin, laminin, and integrins participate in resistance artery remodeling.¹⁴ MMP activation is also necessary for VSMC migration. Recent evidence supports the concept that MMPs play an important role in VSMC migration into the intima in the balloon-injured carotid artery.¹⁵–¹⁷

We demonstrated previously that small artery remodeling in angiotensin (Ang) II–induced hypertension was mediated by both angiotensin type-1 (AT₁) and angiotensin type-2 (AT₂) receptors through alteration of activity of MMPs and of tissue inhibitors of metalloproteinases (TIMPs), which affected ECM content and vascular mechanics of resistance

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vessels.\(^{18}\) We now hypothesized that, as a result of changes in ECM components and their corresponding adhesion receptors, cell–matrix interactions would lead to a rearrangement of VSMCs and restructuring of the vascular wall. Accordingly, we questioned whether changes in the expression of the various vascular integrins in Ang II–infused rats result from regulation by AT\(_1\) and/or by AT\(_2\) receptors, using a selective AT\(_1\) or a combined AT\(_1\)/AT\(_2\) receptor antagonist.

**Methods**

**Antibodies and Reagents**

All of the reagents were from Sigma Chemicals unless otherwise noted. Sar-Ile was purchased from Bachem, whereas anti-\(\alpha_5\) subunit and anti-\(\beta_3\) subunit antibodies were from Chemicon. Osteopontin antibody was purchased from Santa Cruz Biotechnologies. Anti-\(\alpha_5\) and anti-\(\beta_3\) antibodies were generous gifts from Dr. R. Hynes (Howard Hughes Medical Institute, Chevy Chase, MD) and Dr. P. Liu (Toronto General Hospital, Toronto, Ontario, Canada), respectively. Antiserum to the \(\alpha_5\) subunit was generated as described elsewhere,\(^{19}\) and all of the secondary antibodies were from Vector Laboratories.

**Animal Experiments**

The study protocol was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and performed following recommendations of the Canadian Council of Animal Care. Male Sprague Dawley rats (Charles Rivers), housed under controlled conditions, were infused with Ile\(^5\)-Ang II (120 ng/kg per minute; Calbiochem) via osmotic minipumps (Alzet Corp.).\(^{18}\) The selective AT\(_1\) receptor antagonist losartan (10 mg/kg per day) and the vasodilator hydralazine (25 mg/kg per day) were given in the drinking water, whereas the combined AT\(_1\)/AT\(_2\) receptor antagonist Sar-Ile was infused via osmotic minipumps (10 \(\mu\)g/kg per minute), all for 7 days. Systolic blood pressure (SBP) was measured by the tail-cuff method as described previously,\(^{18}\) and rats were then killed humanely.

**Integrin and Osteopontin Immunohistochemistry**

Aortas were fixed in Russel fixative and embedded in paraffin. Endogenous peroxidase in 5-\(\mu\)m-thick sections was quenched by incubation with 0.3% \(\mathrm{H}_2\mathrm{O}_2\) in 0.03% Tween 20-Tris buffer (TBT) for 30 minutes. Nonspecific binding was blocked by incubation in 10% normal serum in TBT. All of the antigens were detected by overnight incubation with the appropriate antibody in TBT containing 10% normal serum in a humidified chamber. Primary antibodies were revealed by secondary antibodies coupled to a biotin–avidin–peroxidase complex (Vectorstain ABC kit, Vector Laboratories). Peroxidase activity was detected with 1 mg/mL dianimobenzidine tetrahydrochloride and 0.2% \(\mathrm{H}_2\mathrm{O}_2\). Sections were counterstained with hematoxylin (Vector Laboratories), visualized with Zeiss Axiophot 100M microscope (Carl Zeiss Microimaging Inc), and analyzed with Northern Eclipse image analysis software (Empix Imaging Inc). Staining was corrected by the surface area. Nonspecific staining was verified by the replacement of primary antibody by appropriate normal serum.

**Statistical Analysis**

Data are presented as mean±SEM. Immunohistochemistry quantification was analyzed by 1-way ANOVA followed by a Student–Newman–Keuls test. \(P<0.05\) was considered statistically significant.

**Results**

**Vascular Integrin Subunit Expression**

Vascular integrin \(\alpha_5\) subunit expression was unaffected by Ang II or concomitant treatment with either Sar-Ile or hydralazine but was significantly increased after AT\(_1\) receptor blockade with losartan (\(P<0.001\) versus all groups; Figure 1 and Figure I, available online at http://www.hypertensionaha.org). Vascular expression of integrin \(\alpha_5\) subunit was significantly increased by Ang II (\(P<0.05\) versus control; Figure 2 and Figure II, available online) and further increased by losartan (\(P<0.001\) versus control, \(P<0.01\) versus Ang II; Figure 2), whereas it decreased significantly with Sar-Ile (\(P<0.001\) versus Ang II and losartan, \(P<0.01\) versus control; Figure 2) and hydralazine (\(P<0.001\) versus Ang II and losartan, \(P<0.05\) versus control; Figure 2). Ang II induced integrin \(\alpha_5\) subunit expression (\(P<0.001\) versus control, Figure 3 and Figure III, available online), which was reduced by losartan (\(P<0.001\) versus Ang II; Figure 3). \(\alpha_5\) subunit expression was not decreased by either Sar-Ile (\(P<0.001\) versus Ang II, \(P<0.01\) versus control) or hydralazine (\(P<0.001\) versus Ang II; Figure 3). Ang II–induced expression of \(\beta_1\) and \(\beta_3\) subunits (Figures 4 and 5 and Figures IV and V, available online) was significantly lowered by losartan, Sar-Ile, and hydralazine (Figures 4 and 5).

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Vascular immunohistochemical staining of \(\alpha_5\) integrin subunits. Results are means±SEM. Los indicates losartan; Hyd, hydralazine. \(\ast P<0.001\) vs all groups.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Vascular immunohistochemical staining of \(\alpha_5\) integrin subunits. Results are means±SEM. Los indicates losartan. \(\dagger P<0.001\) vs control; \(\ddagger P<0.001\) vs Ang II; \(\ddagger\ddagger P<0.001\) vs Ang II+ Los; \(\S P<0.01\) vs Control; \(\| P<0.01\) vs Ang II; \(\|\| P<0.05\) vs Control.
Osteopontin Expression

Osteopontin expression was significantly increased by Ang II (P < 0.001 versus control; Figure 6 and Figure IV, available online), and this was partially blocked by losartan (P < 0.001 versus Ang II, P < 0.05 versus control) and abrogated by Sar-Ile and hydralazine (P < 0.001 versus Ang II, P < 0.05 versus losartan).

Discussion

We reported previously that dual blockade of AT1/AT2 receptors with Sar-Ile compared with AT1 receptor blockade with losartan differentially altered the structure, mechanics, and composition of small mesenteric resistance arteries in response to Ang II infusion, in association with changes in MMP-2 activity and TIMP-2 binding. These findings led us to investigate here the possible role of vascular integrins and their ligands in the changes observed in Ang II–infused rats treated with AT1 or combined AT1/AT2 receptor antagonists. In this previous study, we found that SBP was significantly increased by 7-day Ang II infusion (177 ± 7 versus 113 ± 2 mm Hg, controls; P < 0.001) and that this increase was significantly blunted by losartan (139 ± 4 mm Hg), hydralazine (134 ± 7 mm Hg), and Sar-Ile (154 ± 7 mm Hg). Despite no significant differences in the effectiveness of BP reduction between dual AT1/AT2 antagonism by Sar-Ile and that of specific AT1 antagonism by losartan, Sar-Ile–treated animals showed a trend to higher SBP values. The present study demonstrates a differential role for AT1 and AT2 receptors in the regulation of vascular integrin expression. Expression of integrin α and β subunits was altered after a blockade of AT1 receptors alone or both AT1 and AT2 receptors. In addition to differential integrin subunit expression, osteopontin, which has high affinity for several integrins, also increased after Ang II infusion and was differentially regulated by AT1 and AT2 receptors.

Increased VSMC proliferation and migration contribute to the pathogenesis of hypertension. A large number of agents and mechanisms (growth factors, ECM, cell–cell interactions, etc.) regulate VSMC growth and migration, but...
precise cellular signaling mechanisms involved have not been completely elucidated. Kohno et al.\textsuperscript{22} showed in vitro that Ang II via the AT\textsubscript{1} receptor stimulates migration and proliferation of human coronary artery smooth muscle cells. Ang II, via AT\textsubscript{1} receptors, increased cardiac fibroblast adhesion through the activation of multiple signaling pathways, which include upregulation of osteopontin and \(\alpha_5\), \(\beta_1\), and \(\beta_3\) integrins, leading to the development of left ventricular hypertrophy in spontaneously hypertensive rats.\textsuperscript{23}

In the present study, expression of \(\alpha_5\) integrin subunits, one of the major integrin receptors for collagen, was increased after AT\textsubscript{1} antagonism. This was prevented by a blockade of AT\textsubscript{1}, and AT\textsubscript{2} receptors with Sar-Ile, which suggests that AT\textsubscript{2} receptors are implicated in the expression of this integrin subunit. Because of opposing effects of AT\textsubscript{1} and AT\textsubscript{2} receptors on blood pressure (BP), AT\textsubscript{1} receptor stimulation could inhibit \(\alpha_5\) subunit expression. Thus, the simultaneous blockade of AT\textsubscript{1}, and AT\textsubscript{2} receptors would result in reduced effects relative to the selective blockade of AT\textsubscript{1} receptors. \(\alpha_5\) integrin mediates collagen-triggered signaling and has been proposed to signal through the Ras/Shc/mitogen-activated protein kinase pathway, which inhibits MMP synthesis.\textsuperscript{24} In \(\alpha_5\) integrin subunit null mice, angiogenesis was reduced.\textsuperscript{25} It has accordingly been suggested that this results from excess MMP activation with the inhibition of angiogenesis via the generation of angiotatin from circulating plasminogen, which demonstrates the potential of Ang II-regulated \(\alpha_5\) subunit–containing integrins to influence remodeling of the vasculature.

The \(\alpha_5\) integrin subunit, which is part of \(\alpha_\beta_3\) integrin, the fibronectin receptor, and involved in fibronectin polymerization,\textsuperscript{26} was significantly increased by Ang II and additionally enhanced by AT\textsubscript{1} antagonism but reduced by concomitant AT\textsubscript{1}, and AT\textsubscript{2} blockade. This result suggests a role for AT\textsubscript{2} receptors in the regulation of \(\alpha_5\) subunit subunits. The interaction between the fibronectin receptor \(\alpha_\beta_3\) integrin\textsuperscript{27} and the arginine-glycine-aspartic acid (RGD) site of fibronectin\textsuperscript{28} is required for matrix assembly in most cellular systems.\textsuperscript{29,30} This is important for VSMC proliferation, because inhibition of fibronectin matrix assembly inhibits VSMC proliferation,\textsuperscript{31} which may explain, in part, our previous result showing abrogation of growth in resistance arteries after Sar-Ile administration. This occurred despite significant increase in fibronectin, which we demonstrated is modulated by AT\textsubscript{2} receptors.\textsuperscript{18} In support of our findings, Chassagne et al.\textsuperscript{32} demonstrated that AT\textsubscript{1} receptor activation inhibited VSMC migration through fibronectin secretion and subsequent VSMC attachment. It is therefore possible that \(\alpha_5\) subunit expression, which can mediate cell attachment to fibronectin, is associated with a nonmigratory VSMC phenotype.

Ang II significantly increased vascular \(\alpha_5\) subunit expression, similar to what was shown previously with cardiac fibroblasts.\textsuperscript{33} This increase may be BP dependent, because it was prevented by losartan, Sar-Ile, and hydralazine. \(\alpha_5\) integrin expression is associated with induction of the contractile phenotype, because its upregulation was reported to occur in association with a differentiation of fibroblasts into myofibroblasts\textsuperscript{39} and a reduction in migratory phenotype.\textsuperscript{34}

The regulation of the 2 major \(\beta\) subunits participates in the overall expression and function of integrins. The expression of the \(\beta_1\) subunit was significantly increased after Ang II infusion. This increase was prevented by all of the treatments, suggesting that the regulation of \(\beta_1\) subunits is BP dependent, similar to that of \(\alpha_5\) subunits, which are associated with them.\textsuperscript{35} Expression of the \(\beta_1\) subunit, also increased by Ang II, was abrogated by losartan, in opposition to the \(\beta_1\) subunit, the expression of which was only partially blunted by AT\textsubscript{1} antagonism. \(\beta_1\) subunit expression was unaltered by dual AT\textsubscript{1}/AT\textsubscript{2} antagonism and hydralazine. Thus, \(\beta_1\) subunit expression may be under AT\textsubscript{1} receptor regulation, as reported previously by Kawano et al.\textsuperscript{23} \(\beta_3\) and \(\alpha_5\) subunits, which form the \(\alpha_\beta_3\) integrin, play a critical role in cell proliferation and migration and, accordingly, in vascular remodeling.\textsuperscript{36}

Another RGD-containing protein that may be associated with VSMC proliferation is osteopontin, a soluble secreted phosphoglycoprotein, which plays a role in VSMC adhesion with \(\alpha_\beta_3\) integrin.\textsuperscript{37,38} Although numerous functions have been attributed to osteopontin in vitro, the in vivo function remains less defined. Osteopontin may be regulated by Ang II and can modulate Ang II–induced fibrosis.\textsuperscript{39,40} Osteopontin may serve as a negative modulator of integrin–ECM interactions.\textsuperscript{41} Extending our previous work, we show that osteopontin expression is partially affected by AT\textsubscript{1} antagonism and additionally decreased by combined AT\textsubscript{1} and AT\textsubscript{2} receptor blockade and by hydralazine, which suggests that it is regulated by BP similar to the \(\alpha_5\) and \(\beta_1\) integrin subunits. It is noteworthy that most vascular integrin subunits, as well as osteopontin expression, were partially reduced by hydralazine despite similar SBP lowering.\textsuperscript{43} In addition, because \(\alpha_5\), \(\beta_1\), and \(\beta_3\) subunit expression levels were similar in these groups, this allows the conclusion to be drawn that these subunits do not play a role in vascular remodeling in this model.

VSMC behavior in the media of blood vessels and their role in vascular remodeling may be modulated by several ECM proteins, such as fibronectin and ligands for \(\alpha_\beta_3\), \(\alpha_\beta_1\), and \(\alpha_\beta_5\). These integrins have been shown to play a role in cellular events including differentiation, development, wound healing, adhesion, and cell migration. Hedin et al.\textsuperscript{44} demonstrated that fibronectin and MMP synthesis are involved in the conversion of the VSMC phenotype from contractile to synthetic. Therefore, it is plausible that, in the absence of Ang II receptor activation, VSMC phenotype is migratory rather than contractile, as shown when AT\textsubscript{1} receptors are blocked.\textsuperscript{45} When both AT\textsubscript{1} and AT\textsubscript{2} receptors were blocked, there was less TIMP-2 binding to MMP-2 and more MMP-2 activity, and, consequently, more ECM degradation and growth was abrogated. These results are supported by the present data, because there was a decrease of most integrin subunits, such as \(\alpha_5\beta_3\) integrin, responsible for a reduction in VSMC anchoring to the surrounding ECM, and conse-
quently, possible emergence of a synthetic, migratory, and nonproliferative phenotype.

There are limitations to this study. The use of a selective AT$_2$ receptor antagonist alone or in combination with losartan would have been informative, but because of limited availability and prohibitive high cost, this could not be performed and was replaced by Sar-Ile. By using Sar-Ile, a combined AT$_1$/AT$_2$ antagonist, we obtained evidence of a role for AT$_2$ receptors by comparison with the effect of a selective AT$_1$ receptor antagonist. We have discussed a change to a migratory phenotype represented by a differential regulation of integrins associated with this phenotype, but in this in vivo study we have not actually demonstrated the presence of this phenotype. The association of certain integrins with a VSMC migratory phenotype has been shown previously by others.34,46

In conclusion, the blockade of AT$_1$ or both AT$_1$ and AT$_2$ receptors in Ang II–infused rats differentially modulated integrin and osteopontin expression, which may result in a different VSMC phenotype. Whereas AT$_1$ receptors enhance vascular $\alpha_v$, $\beta_1$, and $\beta_3$ integrin subunit expression and osteopontin deposition and reduce $\alpha_1$ integrin expression, AT$_2$ receptors increase the expression of $\alpha_1$ and $\alpha_3$ integrin subunits.

**Perspectives**

These results demonstrate the differential importance of AT$_1$ and AT$_2$ receptors in the regulation of vascular integrins in Ang II–induced hypertension. Regulation of integrins and their implication in vascular remodeling or in cell phenotypic changes by AT$_1$ and AT$_2$ receptors may explain in part different effects of Ang-converting enzyme inhibitors and Ang receptor blockers on cardiovascular risk reduction in various cardiovascular conditions.

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Supplemental Figure Legends

**Figure I:** Vascular immunohistochemical staining of $\alpha_1$ integrin subunits. Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E) hydralazine. (F) Negative control. Brown represents $\alpha_1$ subunit staining whereas nuclei appear blue.

**Figure II:** Vascular immunohistochemical staining of $\alpha_5$ integrin subunits.
Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E) hydralazine. (F) Negative control. Brown represents $\alpha_5$ subunit staining whereas nuclei appear blue.

**Figure III:** Vascular immunohistochemical staining of $\alpha_8$ integrin subunits.
Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E) hydralazine. (F) Negative control. Brown represents $\alpha_8$ subunit staining whereas nuclei appear blue.

**Figure IV:** Vascular immunohistochemical staining of $\beta_1$ integrin subunits.
Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E) hydralazine. (F) Negative control. Brown represents $\beta_1$ subunit staining whereas nuclei appear blue.

**Figure V:** Vascular immunohistochemical staining of $\beta_3$ integrin subunits.
Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E)
hydralazine. (F) Negative control. Brown represents $\beta_1$ subunit staining whereas nuclei appear blue.

**Figure VI:** Vascular immunohistochemical staining of osteopontin. Representative images in (A) control, (B) Ang II (C) losartan (D) Sar-Ile or (E) hydralazine. (F) Negative control. Brown represents $\alpha_1$ subunit staining whereas nuclei appear blue.
Figure I
Figure II
Figure VI