Role of the Angiotensin AT₁ Receptor in Rat Aortic and Cardiac PAI-1 Gene Expression

Hong-Chi Chen, Julie L. Bouchie, Alexandra S. Perez, Allen C. Clermont, Seigo Izumo, James Hampe, Edward P. Feener

Abstract—Although the renin-angiotensin system has been implicated in increasing plasminogen activator inhibitor-1 (PAI-1) expression, the role of the angiotensin type 1 (AT₁) receptor is controversial. This report examines the effects of angiotensin peptides, angiotensin-converting enzyme inhibition, and AT₁ antagonism on rat aortic and cardiac PAI-1 gene expression. In vitro, angiotensin (Ang) I, Ang II, and angiotensin Arg²-Phe⁸ (Ang III) were potent agonists of PAI-1 mRNA expression in rat aortic smooth muscle cells (RASMCs), and stimulation of PAI-1 by these peptides was blocked by the AT₁ antagonist candesartan. Angiotensin Val³-Phe⁶ (Ang IV) and angiotensin Asp¹-Pro⁷ (Ang [1-7]) did not affect PAI-1 expression in RASMCs. In neonatal rat cardiomyocytes, Ang II increased PAI-1 mRNA expression by 4-fold (P<0.01), and this response was completely blocked by AT₁ receptor antagonism. Continuous intrajugular infusion of Ang II into Sprague-Dawley rats for 3 hours increased aortic and cardiac PAI-1 mRNA expression by 17- and 9 fold, respectively, and these Ang II responses were completely blocked by coinfusion with candesartan. Aortic and cardiac PAI-1 expressions were compared in spontaneously hypertensive rats and Wistar-Kyoto rats. PAI-1 expression in the aorta and heart from spontaneously hypertensive rats was 5.8-fold and 2-fold higher, respectively, than in control Wistar-Kyoto rats (P<0.05). Candesartan treatment for 1 week reduced aortic and cardiac PAI-1 expression in spontaneously hypertensive rats by 94% and 72%, respectively (P<0.05), but did not affect vascular PAI-1 levels in Wistar-Kyoto rats. These results demonstrate a role for the AT₁ receptor in mediating the effects of Ang II on aortic and cardiac PAI-1 gene expression. (Arterioscler Thromb Vasc Biol. 2000;20:2297-2302.)

Key Words: angiotensin II ■ plasminogen activator inhibitor ■ hypertension ■ aorta ■ vascular smooth muscle cells

Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of tissue and urokinase plasminogen activators and thereby reduces the conversion of plasminogen to plasmin, an extracellular protease that mediates fibrinolysis and activates matrix metalloproteinases.¹⁻³ An elevated level of PAI-1, which occurs in diabetes, insulin resistance, obesity, and hypertension, has been implicated as a contributing risk factor for cardiovascular disease.⁴⁻⁷ Recent studies suggest that the renin-angiotensin system (RAS) may exert an important role in the regulation of circulating and vascular PAI-1 expression and may thereby affect the fibrinolytic balance. Reports from our laboratory and others have demonstrated that angiotensin II (Ang II) is a potent stimulator of PAI-1 mRNA and protein expression in both cultured endothelial and vascular smooth muscle cells.⁸⁻¹¹ The physiological importance of the RAS in modulating PAI-1 levels is supported by in vivo studies, which have demonstrated that treatment of rats with the angiotensin-converting enzyme (ACE) inhibitor captopril suppresses the induction of PAI-1 expression in the aortic neointima induced by balloon catheter injury,¹² and a number of clinical studies, which have shown that the ACE inhibitors and the angiotensin type 1 (AT₁) receptor antagonist reduce plasma PAI-1 antigen and activity.⁷,¹³⁻¹⁷ Although these reports provide substantial evidence for an important role of the RAS in PAI-1 expression, the specific effects of angiotensin-related peptides and the role of the AT₁ receptor in regulating PAI-1 expression remain controversial.

The RAS involves the proteolytic conversion of angiotensinogen to Ang I by renin, followed by its conversion to the octapeptide Ang II (Asp¹-Phe⁸) by either ACE or chymase.¹⁸,¹⁹ Ang II activates 2 receptor subtypes, including AT₁ and AT₂.²⁰,²¹ In addition, Ang I and Ang II can undergo further processing to generate other biologically active peptides, including angiotensin Arg²-Phe⁸ (Ang III), angiotensin Val³-Phe⁶ (Ang IV), and angiotensin Asp¹-Pro⁷ (Ang [1-7]), which may partially activate AT₁ and AT₂ or bind additional vascular receptors.²¹⁻²³ Previous studies have shown that the AT₁ antagonist losartan partially blocks Ang II–induced PAI-1 expression in rat aortic smooth muscle cells (RASMCs) and rat microvessel endothelial cells.⁹ In contrast, other reports have suggested that the AT₁ receptor does not
mediate Ang II–stimulated PAI-1 expression.24,25 Establishing the role of the AT1 receptor in the regulation of PAI-1 expression may have important clinical significance related to potential differences between ACE inhibitors and AT1 antagonists on the fibrinolytic system.

In this report, the effects of angiotensin peptides and the role of the AT1 receptor on PAI-1 mRNA expression in RASMCs and in rat cardiomyocytes were evaluated by using candesartan, previously described as an insurmountable AT1 antagonist.26 The in vivo role of the AT1 receptors in aortic and cardiac PAI-1 expression was evaluated in rats subjected to short-term intrajugular Ang II infusion and in spontaneously hypertensive rats (SHRs) chronically treated with candesartan. These in vitro and in vivo studies demonstrate that the AT1 receptor influences vascular PAI-1 gene expression.

Methods

Cell Culture

RASMCs were isolated from Sprague-Dawley rats, cultured in Dulbecco’s modified Eagle’s medium (DMEM), 100 mg/dL D-glucose (Gibco-BRL), and 10% fetal bovine serum (Gibco-BRL), as described previously,9 and used between passages 8 and 15. Confluent monolayers of cells were deprived of serum in DMEM containing 0.1% (wt/vol) bovine serum albumin for 18 hours before stimulation. Neonatal rat cardiac myocyte primary cultures were prepared as described previously.27 A total of 8×10⁶ cells were plated onto 100-mm gelatin-coated culture dishes in supplemented DMEM/F12 medium27 containing 5% horse serum and 100 μmol/L bromodeoxyuridine. After the first 24 hours, cultures were serum-deprived for 48 hours in DMEM/F12 containing 0.1% bovine serum albumin. Cells were stimulated with angiotensin peptides (Sigma) in the absence or presence of pretreatment with candesartan (CV 11974), kindly provided by Dr Peter Morsing (Astra Hassle AB, Mölndal, Sweden).

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by using Tri reagent (Molecular Research Center). RNA was separated by agarose gel electrophoresis, and PAI-1 mRNA was probed with a cDNA probe against rat PAI-1, as described previously.9 Expression of acidic ribosomal phosphoprotein (36B4) RNA levels was determined by Northern analysis with a 32P-labeled oligonucleotide probe. Levels of mRNA were visualized and quantified by PhosphorImager analysis (Molecular Dynamics Inc).

Intrajugular Infusion

A catheter was securely inserted into the left jugular vein of male Sprague-Dawley rats, and the animals were allowed to recover for at least 18 hours. Awake rats were then connected to a multisyringe pump to provide an intravascular infusion of saline alone (control) or saline containing Ang II and/or candesartan. The infusion rates were 100 ng·kg⁻¹·min⁻¹ Ang II and 25 μg·kg⁻¹·min⁻¹ candesartan (a 50-fold molar excess over Ang II) at 10 μL/min. After 3 hours of infusion, the rats were killed by CO₂ inhalation. The aorta from the aortic arch to the renal artery and the heart ventricles were excised and immediately frozen in LN₂.

Blood Pressure Measurements

Systolic, diastolic, and mean blood pressures of 10-week-old SHRs (Taconic, Germantown, NY) and weight-matched Wistar-Kyoto (WKY) control rats were measured by tail-cuff plethysmography (Ueda Electronics) as described previously.12 Blood pressure measurements were made before and after 1-week treatments with either captopril (100 mg·kg⁻¹·d⁻¹; Sigma) or candesartan-cilexetil TCV-116 (10 mg·kg⁻¹·d⁻¹; provided by Dr Peter Morsing, Astra Hassle AB) delivered in the drinking water.

Statistics

All statistical analyses were performed by 1-way ANOVA with SigmaStat software (Jandel Scientific). Values of P<0.05 were considered significantly different.

Results

Role of the AT1 Receptor on PAI-1 Expression in RASMCs and in Rat Cardiomyocytes

The effects of angiotensin peptides Ang I (Asp¹-Leu⁰), Ang II (Arg¹-Phe⁰), Ang III (Val¹-Phe⁰), Ang (1-7) (Asp¹-Pro⁰), and Ang IV (Val¹-Phe⁰) on PAI-1 expression in RASMCs were examined and compared. Cells were treated with 100 nmol/L of these peptides for 3 hours followed by RNA isolation and Northern blot analysis of PAI-1 expression, as described previously.9,28 This study demonstrated that Ang I, Ang II, and Ang III were potent stimulators of PAI-1 mRNA expression (Figure 1). In contrast, Ang IV and Ang (1-7) did not significantly affect PAI-1 mRNA levels. To examine the role of the AT1 receptor on PAI-1 gene expression, cells were pretreated for 15 minutes with 1 μmol/L of the AT1 antagonist candesartan followed by stimulation with angiotensin peptides. This study demonstrated that Ang I; antagonism completely blocked Ang I–, Ang II–, and Ang III–stimulated PAI-1 expression in RASMCs (Figure 1). To compare the effects of Ang I, Ang II, and Ang III on PAI-1 expression in RASMCs, cells were stimulated with 1, 5, 10, 50, or 100 nmol/L of Ang I, Ang II, or Ang III for 3 hours. Northern blot analysis revealed that the ED₅₀ for Ang II–induced PAI-1 mRNA was 1 nmol/L, compared with 10 nmol/L for Ang I and Ang III (online Figure; see www.atvbaha.org).

The potential effects of Ang II and the AT1 receptor on PAI-1 mRNA expression were also examined in primary culture of neonatal rat cardiomyocytes. Cells were stimulated with Ang II (100 nmol/L, 2 hours) in the presence or absence of 1 μmol/L candesartan. Northern blot analysis revealed that Ang II increased PAI-1 mRNA levels by 4-fold (P<0.001), and this response was blocked by AT1 antagonism (Figure 2).

Effect of Short-Term Intrajugular Ang II Infusion on Aortic and Cardiac PAI-1 Expression

A possible concern from these in vitro studies is that the effects of the Ang II/AT1 receptor pathway on PAI-1 expression in RASMCs and cardiomyocytes may not reflect the role of this pathway in vascular PAI-1 gene expression in vivo. The effect of short-term Ang II administration on aortic and cardiac PAI-1 expression was examined in awake Sprague-Dawley rats subjected to continuous Ang II infusion (100 ng·kg⁻¹·10 μL⁻¹·min⁻¹) for 3 hours via an intrajugular catheter. Control animals were infused with saline at 10 μL/min for 3 hours. After these infusions, the aorta and ventricles were harvested, and PAI-1 mRNA levels were examined by Northern blot analyses. This study demonstrated that Ang II infusion increased aortic and heart PAI-1 mRNA expression by 17- and 9-fold, respectively, compared with saline infusion (Figure 3). To examine the role of the AT1 receptor in these Ang II responses, candesartan (25 μg·kg⁻¹·min⁻¹) was coinfused with Ang II as described above. This study showed that AT1 antagonism completely blocked the Ang II–induced PAI-1 expression in both aorta and ventricles (Figure 3). Candesartan infusion alone did not affect PAI-1 mRNA levels. These studies show that short-term adminis-
tration of Ang II increases aortic and cardiac PAI-1 mRNA expression and that these effects of Ang II are mediated via the AT$_1$ receptor.

Effects of ACE Inhibition and AT$_1$ Antagonism on Aortic and Cardiac PAI-1 Expression in SHRs and WKY Rats

To determine whether the Ang II/AT$_1$ receptor pathway may also affect vascular PAI-1 expression in hypertension, the effects of ACE inhibition and AT$_1$ antagonism on aortic and cardiac PAI-1 expression in SHRs were investigated. The SHR is a genetic model of hypertension that is sensitive to ACE inhibition and AT$_1$ antagonism, as well as gene therapies targeted at RAS inhibition. $^{29,30}$ Mean blood pressures of 10-week-old SHRs and weight-matched control WKY rats were 142.6±2.8 and 110.9±1.8 mm Hg, respectively (the Table). Treatment of these SHRs for 1 week with 100 mg · kg$^{-1}$ · d$^{-1}$ captopril and 10 mg · kg$^{-1}$ · d$^{-1}$ candesartan TCV-116 reduced mean blood pressure to 123.5±2.9 mm Hg ($P<0.031$) and 96.5±3.5 mm Hg ($P<0.001$), respectively, compared with untreated SHRs. Treatment of WKY rats with captopril did not significantly affect mean blood pressure; however, treatment with candesartan reduced mean blood pressure to 79.8±2.8 mm Hg ($P<0.001$; the Table). Comparison of vascular PAI-1 expression in untreated SHRs and WKY rats revealed that the aortic and cardiac PAI-1 mRNAs in SHRs were elevated by 5.8-fold ($P<0.05$) and 2-fold ($P<0.05$), re-

**Figure 1.** Effects of angiotensin peptides and AT$_1$ antagonist candesartan (Cand) on PAI-1 mRNA expression in RASMCs. Confluent cultures of RASMCs were treated with the angiotensin peptides (100 nmol/L, 3 hours) in the absence or presence of 1 µmol/L candesartan, as indicated. The dose responses of Ang I, Ang II, and Ang III on PAI-1 mRNA in RASMCs were compared after a 3-hour stimulation with the peptide concentrations from 1 to 100 nmol/L (see the online Figure at www...). After these incubations RNA was isolated, and the mRNA expressions of PAI-1 and ribosomal phosphoprotein (36B4) were determined by Northern blot analysis. Representative blots and bar graph quantification of PAI-1 mRNA normalized to 36B4 are shown. Results are expressed as mean±SEM from 3 or 4 experiments. For the online Figure, statistical differences vs untreated cells are indicated as *$P<0.05$ and **$P<0.01$.}
spectively, compared with WKY rats (Figure 4). Treatment of SHRs with captopril or candesartan similarly reduced aortic PAI-1 mRNA by 94% ($P < 0.05$, Figure 4). In the heart, candesartan treatment of SHRs reduced PAI-1 expression by 72% ($P < 0.05$) compared with untreated SHRs. Captopril treatment of SHRs, at a dose that reduced aortic PAI-1 expression and mildly reduced mean blood pressure, did not significantly affect cardiac PAI-1 levels. Neither captopril nor candesartan significantly altered PAI-1 expression in WKY rats in these vascular tissues.

**Discussion**

This report demonstrates that the AT$_1$ receptor pathway mediates the effects of Ang II on PAI-1 gene expression in the rat aorta and heart ventricle. Comparison of the effects of angiotensin peptides on PAI-1 expression in RASMCs revealed that Ang I, Ang II, and Ang III increased PAI-1 mRNA through an AT$_1$-dependent pathway, whereas Ang IV and Ang (1-7) did not affect PAI-1 levels. In addition, we have shown that Ang II–induced PAI-1 expression in cultured cardiomyocytes was mediated by the AT$_1$ receptor. These results are in contrast to those of other studies, which have suggested that the effects of Ang II are mediated by an Ang IV/AT$_4$ pathway.$^{24}$ The complete blockade of Ang II–induced PAI-1 expression by the AT$_1$-selective antagonist candesartan may be attributed to its higher expression by 72% ($P < 0.05$) compared with untreated SHRs. Captopril treatment of SHRs, at a dose that reduced aortic PAI-1 expression and mildly reduced mean blood pressure, did not significantly affect cardiac PAI-1 levels. Neither captopril nor candesartan significantly altered PAI-1 expression in WKY rats in these vascular tissues.

**Blood Pressure of SHRs and WKY Rats**

<table>
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<tr>
<th></th>
<th>Mean Blood Pressure, mm Hg</th>
<th>$P$, Pre vs Post</th>
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<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
<td>Posttreatment</td>
</tr>
<tr>
<td>WKY rats</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>101.9±2.0</td>
<td>110.9±1.8</td>
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<tr>
<td>Captopril</td>
<td>107.7±4.0</td>
<td>92.5±1.3</td>
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<tr>
<td>Candesartan</td>
<td>104.1±1.4</td>
<td>79.8±2.8</td>
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<tr>
<td>SHR</td>
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<td></td>
</tr>
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<td>Control</td>
<td>146.0±4.4</td>
<td>142.6±2.8</td>
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<td>142.5±2.1</td>
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<tr>
<td>Candesartan</td>
<td>133.8±2.6</td>
<td>96.5±3.5</td>
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affinity and slower dissociation kinetics than other AT₁ antagonists tested, such as losartan.²⁶,³¹ Because candesartan reduces maximal Ang II binding to the AT₁ receptor, whereas losartan causes a rightward shift in the Ang II dose response without reducing maximal Ang II binding,³¹ it is likely that the partial reduction of Ang II–induced PAI-1 expression by losartan⁹ was due to incomplete antagonism of the AT₁ receptor. Because RASMCs express ACE-like activity in culture,⁸ the ability of candesartan to block Ang I–induced PAI-1 expression suggests that Ang I is converted to Ang II, followed by subsequent activation of the AT₁ receptor. The observation that Ang III–stimulated PAI-1 expression is also blocked by candesartan is consistent with previous reports that have shown that the N-terminal aspartate of Ang II is not essential for AT₁ activation.³² These results suggest that a combination of Ang II and Ang III stimulation of AT₁ may contribute to increased vascular PAI-1 mRNA levels. Because aminopeptidase A, a zinc metallopeptidase that converts Ang II to Ang III,²⁷ it is possible that Ang III may contribute to the effects of the AT₁ receptor on vascular PAI-1 expression in vivo. Processing of Ang III to Ang IV by aminopeptidase N would be expected to diminish AT₁–induced PAI-1 gene expression. Further characterization of AT₁ receptors and development of specific Ang IV antagonists are needed to establish the role of the Ang IV/AT₁ pathway on vascular PAI-1 expression.

Coinfusion of candesartan blocked Ang II–induced PAI-1 gene expression in both the rat aorta and heart ventricle. This study has demonstrated that short-term administration of Ang II rapidly induces PAI-1 expression in these vascular tissues and that this Ang II response is mediated by the AT₁ receptor. Because previous studies have shown that PAI-1 mRNA expression is correlated with PAI-1 protein synthesis,⁹,¹² it is likely that the AT₁–induced changes in PAI-1 mRNA expression result in an increase of PAI-1 protein production and secretion from the vasculature. Although the role of vascular PAI-1 expression in affecting circulating PAI-1 levels is not known, the effects of Ang II and AT₁ antagonism on vascular PAI-1 expression described in this study are consistent with reports that have shown that Ang II infusion elevates plasma PAI-1 antigen¹⁴ and that ACE inhibition and AT₁ antagonism lower plasma PAI-1 in human subjects.¹³–¹⁵,³⁵ Although changes in vascular PAI-1 production may affect circulating PAI-1 levels, it is likely that other sites of PAI-1 synthesis, its release from platelets, and its clearance are also major determinants of plasma PAI-1 levels.

Recent studies have shown that plasma PAI-1 levels are positively correlated with blood pressure⁵ and are reduced in hypertensive subjects after treatment with an ACE inhibitor and AT₁ antagonist.⁷,¹⁷ To investigate the potential effect of hypertension on vascular PAI-1 mRNA levels in rats, we compared aortic and cardiac PAI-1 expression in the SHR with that of normotensive WKY control rats. This study demonstrated that PAI-1 expression in these vascular tissues was elevated by 2- to 5-fold in the SHR compared with WKY rats. Furthermore, because candesartan treatment normalized PAI-1 mRNA expression in both the aorta and heart in the SHR, it is likely that the AT₁ receptor contributed to the elevated vascular PAI-1 expression in this rat model of essential hypertension. Although Ang II contributes to elevated blood pressure in the SHR, the precise mechanism by which Ang II does so in this model is unknown. Plasma and tissue levels of Ang II appear similar in the SHR and normotensive control rats.³⁶ However, it is possible that local cellular increases in Ang II production contribute to an enhanced Ang II action in an autocrine/paracrine manner. Recent studies have shown that cultured vascular smooth
muscle cells from SHRs are capable of generating Ang II, which leads to autocrine stimulation of AT1 receptors, whereas Ang II production was undetectable in vascular smooth muscle cell cultures from WKY rats. Alternatively, enhanced pressor sensitivity to Ang II and Ang III may contribute to the increased vascular AT1 action in SHRs.

In summary, this report demonstrates that the effects of Ang II on PAI-1 gene expression in the rat aorta and heart ventricle are mediated by the AT1 receptor. In addition, studies on SHRs suggest that vascular PAI-1 expression is elevated in hypertension and that both AT1 antagonism and ACE inhibition can reduce elevated vascular PAI-1 gene expression in this model. These findings suggest that the RAS influences vascular PAI-1 expression in rats primarily through the Ang II/AT1 pathway.

Acknowledgments

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