Angiotensin II Increases Plasminogen Activator Inhibitor Type 1 and Tissue-Type Plasminogen Activator Messenger RNA in Cultured Rat Aortic Smooth Muscle Cells

René T.J. van Leeuwen, MSc; Amir Kol, MD; Felicita Andreotti, MD, PhD; Cornelis Kluit, PhD; Attilio Maseri, MD; Giovanni Sperti, MD

Background The role of angiotensin as a vasoconstrictor is well established. Lately, several other actions of this hormone on vascular smooth muscle (VSM) cells have been recognized including the induction of hypertension and/or DNA synthesis. Platelet-derived growth factor (PDGF), a mitogen recently shown to increase plasminogen activator inhibitor type 1 (PAI-1) synthesis in VSM cells, shares with angiotensin II (Ang II) several steps of its intracellular signaling pathway.

Methods and Results The expression of PAI-1 and tissue-type plasminogen activator (TPA) mRNA in cultured rat VSM cells was studied. Northern blot analysis demonstrated a severalfold increase in the PAI-1 mRNA 3 to 8 hours after stimulation with 300 nmol/L Ang II. A similar response for TPA mRNA was observed. This induction did not require the synthesis of an intermediate protein or peptide because it was not affected by cycloheximide. In the cell-conditioned supernatant, the net result was an increase in PAI-1 activity from 4.18±1.8 to 13.2±6.8 IU/mL 6 hours after the addition of 300 nmol/L Ang II (mean±SD, P≤.008, n=6). The Ang II-induced increase in PAI activity was dose related, with a maximal effect at a concentration of 23 nmol/L (n=3) and an ED50 of 3.3±1.5 nmol/L (n=3). [Sar1-Ile4]angiotensin II, a specific competitive antagonist of Ang II, blocked 90±5% (n=3) of the PAI activity induced by 10 nmol/L Ang II. In basal conditions, fibrin overlay zymography demonstrated the presence of free TPA. After stimulation with Ang II, lysis caused by the in situ dissociation of TPA was also present in the region of the TPA/PAI-1 complex. Angiotensin I (Ang I) elicited an increase in PAI activity similar to that obtained with equivalent doses of Ang II. Captopril (5 μg/mL), an inhibitor of the angiotensin-converting enzyme (ACE), completely prevented the Ang I effect, demonstrating that VSM cells display an ACE-like activity.

Conclusions Recent research has demonstrated the existence of a localized vascular renin-angiotensin system. The finding that Ang II can potentially modulate the plasminogen activation in the arterial wall has important biological and therapeutic implications for the evolution of arterial wall thrombi and the migration of cells through the vessel wall in the genesis of atherosclerotic lesions. We speculate that the reduction in thrombotic events observed in patients with a previous myocardial infarction and in high-renin, hypertensive patients treated with ACE inhibitors could be due at least in part to the decreased production of PAI-1 by VSM cells caused by these agents. (Circulation. 1994;90:362-368.)

Key Words • PAI-1 • TPA • angiotensin • smooth muscle

The renin-angiotensin system (RAS) plays a major role in vascular homeostasis. The physiologic actions of angiotensin II (Ang II), the final common product of this pathway, of directly regulating vascular resistance and fluid and electrolyte balance through the modulation of aldosterone secretion have long been recognized. In recent years, two major findings have added new interest to the study of the RAS: (1) the description of several other aspects of Ang II and in particular its possible role as growth factor and/or "hypertrophy" hormone for vascular wall myocytes and (2) the demonstration of the existence of a local vascular RAS.

Several clinical observations have linked the RAS to an increased risk of thrombotic diseases. Angiotensin-converting enzyme (ACE) inhibitors can reduce the risk of a second coronary event in patients with heart failure caused by a previous myocardial infarction, and patients with "high-renin" hypertension appear to sustain a higher risk of myocardial infarction compared with the "low-renin" ones. Accordingly, a genetically determined high activity of the ACE, as observed in the presence of the DD genotype, is associated with a higher risk of myocardial infarction. In vitro, a link between Ang II and the fibrinolytic system has been demonstrated, as Ang II increases plasminogen activator inhibitor type 1 (PAI-1) mRNA and protein in cultured rat astrocytes. Recently we and others demonstrated that vascular smooth muscle (VSM) cells both in culture and in vivo can synthesize tissue-type plasminogen activator (TPA) and its specific inhibitor PAI-1. Interestingly, PAI-1 mRNA expression has recently been reported to be higher in the thickened
media of atherosclerotic arteries compared with normal vessels. In cultured VSM cells, the synthesis of PAI-1 can be modulated by substances such as thrombin and platelet-derived growth factor (PDGF), which share with Ang II several steps of its intracellular signaling pathway, namely, the generation of inositol triphosphate and diacylglycerol and the increase in intracellular Ca<sup>2+</sup> Finally, infusion of Ang II in healthy volunteers substantially increases the level of PAI-1 antigen in the bloodstream. Taken together, these observations led us to formulate the hypothesis that Ang II could modulate the production of fibrinolytic factors in VSM cells.

In this report, we demonstrate for the first time that Ang II modulates TPA and PAI-1 mRNA and activity in a cultured VSM cell system. If confirmed in vivo, these findings could have important implications for the lysis of arterial wall thrombi and for the migration of VSM cells from the media to the intima.

**Methods**

**Materials**

Captopril, angiotensin I (Ang I), Ang II, and the angiotensin receptor antagonist [Sar<sup>1</sup>-Ile<sup>8</sup>]angiotensin II were purchased from Sigma. Recombinant PDGF BB was obtained from Peninsula Laboratories Europe. The chromogenic plasmin substrate p-nitroanilide-conjugated tripeptide (Val-Leu-Lys-pNA) and plasminogen were purchased from Kabi. Plasminogen was purchased from Eurostas. TPA and PAI-1 standards were obtained from Kabi.

**Cell Culture**

Rat aortic VSM cells were obtained from male Sprague-Dawley rats by enzymatic dispersion according to Gunther et al<sup>23</sup> and cultured as described by Speriti et al<sup>24</sup> The cells used in the experiments (passages 5 to 20) were dissociated with trypsin from T75 stock flasks and seeded at a density of 7000 to 10 000 cells/cm<sup>2</sup> in 24 multiwell dishes. Cells were grown to confluence (4 to 6 days), rinsed twice, and left in serum-free (SF) medium for another 48 hours. The cells were then rinsed twice, and fresh SF medium with or without the specified drugs was added to the cultures. The medium was sampled at specified time intervals and aliquots snap frozen in liquid nitrogen.

For gene induction experiments, cells were cultured in a similar way in 60-mm dishes. In some experiments, the protein synthesis inhibitor cycloheximide (10 μg/mL) was added 30 minutes before the addition of Ang II. Preliminary experiments showed that this concentration of cycloheximide completely abolished Ang II–induced labeled amino acid incorporation.

**PAI Activity Assay**

PAI activity in the cell-conditioned medium was determined as previously described.<sup>25</sup> A standard curve was obtained with known amounts of two-chain standard TPA. PAI activity was assessed by titration with known amounts of two-chain TPA in the presence of CNBr-digested fibrin fragments and plasminogen. Plasminogen generation was assessed by monitoring at different time points the cleavage of the chromogenic substrate Val-Leu-Lys-pNA in a spectrophotometer at a wavelength of 405 nm.

**Fibrin Overlay Zymography**

This was performed according to the method of Granelli-Piperno and Reich.<sup>26</sup> SDS-PAGE was performed using 10% polyacrylamide and 0.1% SDS in 0.383 mol/L Tris, pH 8.8 with a stacking gel containing 5% polyacrylamide, 0.124 mol/L Tris, pH 6.8. After electrophoresis, SDS was removed by washing the gel with water containing 2.5% Triton X-100 followed by a wash in a buffer containing 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.7. The gel then was layered onto a fibrin indicator gel containing 30 μg/mL of human plasminogen and incubated at 37°C in a humidified atmosphere.

**<sup>3</sup>H-Thymidine and <sup>3</sup>H–Amino Acid Incorporation**

Preconfluent cultures in 24-multiwell dishes were kept SF for 48 hours. At time 0, after two washes with SF medium, 0.5 mL of SF medium with or without the specified drugs was added to each well. After 20 hours, the cultures were pulsed for 4 hours by adding 0.5 μCi per well of <sup>3</sup>H-thymidine. The experiment was terminated by washing twice with 0.9% NaCl, once with 0.5 mL per well of 10% trichloroacetic acid, and twice again with saline. The cells were then dissolved in 0.4N NaOH, neutralized with HCl, and counted in a liquid scintillation counter (Packard 1600 TR) with quench correction in a Du Pont Aquasolve scintillation cocktail.

**RNA Extraction and Analysis**

RNA was extracted according to the guanidinium-thiocyanate method of Chomczynski and Sacchi.<sup>27</sup> Ten micrograms per lane of total RNA were run on an 0.8% agarose and 8% formaldehyde gel according to Maniatis (Sambrook et al<sup>28</sup>) and transferred (VacuGene System, Pharmacia) onto a nylon membrane (Hybond N, Amersham International). The blot was hybridized with a random primer <sup>32</sup>P-DCTP–labeled probe (Amersham multiprime labeling kit) in 0.5 mol/L sodium phosphate, 7% SDS at 60°C. Autoradiography was performed on Kodak X-OMat S film with intensifying screens. The films were scanned with a Hewlett-Packard IIC image scanner and quantified using a dedicated software program written by Dr Richard Prevost, Faculty of Pharmacy, University of Utrecht, The Netherlands. Scan values for TPA and PAI-1 mRNA signals were normalized for the scan reading of the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ratios were expressed in arbitrary units.

**DNA Fragments Used for Hybridization**

The PAI-1 probe was a 1.1-kb Pst I fragment of a human PAI-1 cDNA with a high homology to the rat PAI-1 gene.<sup>29</sup> The TPA probe was a 2.0-kb Bgl II fragment from the human TPA cDNA.<sup>30</sup> A 1.2-kb Pst I fragment from the rat GAPDH cDNA was used as an internal standard.

**Statistical Analysis**

Data are expressed as mean±SD from the mean. Differences between experimental conditions were assessed by ANOVA and the Bonferroni t test. A value of P<0.05 was considered significant.

**Results**

**PAI Activity**

Ang II (300 nmol/L) stimulation induced a time-dependent increase in PAI activity in the supernatant of VSM SF cultures. A PAI activity increase compared with controls was detectable after 3 hours of Ang II treatment; PAI activity reached a plateau at 6 hours and remained constant for at least 24 hours (Fig 1). The Ang II–induced increase in PAI activity was dose dependent, reaching a maximum at a concentration of 23±11 nmol/L (n=3) and a 50% maximal effect at 3.3±1.5 nmol/L (n=3) (Fig 2).

[Sar<sup>1</sup>-Ile<sup>8</sup>]angiotensin II, a specific competitive antagonist of Ang II, inhibited the maximal Ang II effect by 90±9% (n=3, P<.001, Fig 3).
Recently, evidence has been accumulating that most of the components of the RAS can be synthesized by vascular cells. In particular, preliminary reports have described an ACE-like activity in VSM cultures. For this reason we compared the effects of Ang II and Ang I on PAI production in VSM cell cultures. Ang I increased PAI activity in the supernatant of VSM cell-conditioned medium in a dose-dependent fashion (Fig 4). The maximal effect of Ang I was comparable to the effect of a maximal dose of Ang II. To determine whether the Ang I effect was due to a direct effect or to the Ang II generated by the cleavage of Ang I, experiments were performed in the presence of [Sar\(^1\)-Ile\(^8\)]angiotensin II and of the ACE inhibitor captopril. [Sar\(^1\)-Ile\(^8\)]angiotensin II (300 nmol/L) inhibited the Ang I effect by 47±10% (P<.02, n=3). Captopril (5 \(\mu\)g/mL) completely abolished the Ang I effect (Fig 3). [Sar\(^1\)-Ile\(^8\)]angiotensin II and captopril in the absence of Ang II caused a small nonsignificant change in PAI activity from SF controls (−3.5±25% and 9.0±34%, respectively).

**3\(^{\text{H}}\)**-Thymidine and **3\(^{\text{H}}\)**-Amino Acid Incorporation

Ang II has been reported to cause hypertrophy and/or hyperplasia of VSM cells in vitro. In our system, under the described conditions, Ang II did not increase DNA synthesis as measured by \(^{3}\text{H}\)-thymidine incorporation. Ang II stimulation increased \(^{3}\text{H}\)-amino acid incorporation by

![Fig 2. Line plot: Dose-dependent increase in plasminogen activator inhibitor (PAI) activity in rat aortic smooth muscle cell-conditioned medium.](image)

![Fig 3. Bar graph showing the effect of angiotensin I (Ang I, 10 nmol/L) and II (Ang II, 10 nmol/L) on plasminogen activator inhibitor (PAI) activity in rat aortic smooth muscle cell-conditioned medium. The Ang II receptor antagonist [Sar\(^1\)-Ile\(^8\)]angiotensin II (Sar, 1 \(\mu\)mol/L) significantly reduced both the Ang I- and Ang II-induced increase in PAI activity (P<.02, n=3 and P<.001, n=3, respectively). The inhibitor of the angiotensin-converting enzyme captopril (Cap, 5 \(\mu\)g/mL) completely abolished the Ang I effect. Data are expressed as percent change from control and represent the means of three independent experiments±SD.](image)

![Fig 4. Line plot: Dose-dependent increase in plasminogen activator inhibitor (PAI) activity in rat aortic smooth muscle cell-conditioned medium.](image)

**Fibrin Overlay Zymography**

This method allows the detection of free plasminogen activators. However, even plasminogen activators in complex with PAI-1 can be detected by this method when they are present in relatively large amounts. This is most likely the result of in situ dissociation of the plasminogen activator from the inhibitor in the gel. In control conditions, only TPA was detected, whereas after Ang II treatment, an area of lysis was seen in the region of the TPA/PAI-1 complex (Fig 5).

**PAI-1 and TPA Gene Expression**

Ang II stimulation of VSM cell cultures increased PAI-1 and TPA mRNA compared with controls as
assessed by Northern analysis. The mRNA increase was time dependent, with a similar time course for both PAI-1 and TPA. The maximum was reached at 3 hours, and the signal remained higher than controls for at least 18 hours (Fig 6). The Ang II–induced PAI-1 mRNA increase was dose dependent, with a maximum effect at 100 nmol/L (Fig 7A). This dose-response curve closely followed the PAI activity dose-response curve. The Ang II–induced TPA gene expression also was dose dependent, displaying a dose-response relation similar to that of PAI-1 mRNA. Since Northern blotting is a semiquantitative technique, comparisons between different experimental situations are heavily dependent on the amount of total RNA loaded in each lane. This is particularly important in a dose-response experiment in which the induction of the signal at the intermediate and low doses of the agonist is relatively weak. For this reason, we normalized the scan readings of the TPA and PAI-1 mRNA signals for the scan readings of the internal standard GAPDH. Data are shown as the ratios between the TPA or the PAI-1 signals divided by the GAPDH signal and expressed in arbitrary units (Fig 7B).

To ascertain whether the observed effects of Ang II on PAI-1 and TPA mRNA were direct or required the synthesis of an intermediate mediator, experiments were performed in the presence of the protein synthesis inhibitor cycloheximide. The Ang II–induced increase in PAI-1 and TPA mRNA was unaffected by this agent, demonstrating that the Ang II effect is direct.

Discussion

These data show that the RAS can powerfully regulate the fibrinolytic balance in VSM cell cultures. PAI-1 and TPA mRNA were increased by Ang II stimulation in a dose-dependent fashion, and [Sar^1-Ile^8]angiotensin II, a competitive Ang II antagonist, was capable of blocking 90% of the Ang II–induced increase in PAI activity in the cell-conditioned supernatant. These data suggest that the Ang II effect is specific, i.e., acting through a specific receptor(s). Future experiments using the new selective nonpeptidic receptor antagonists should lead to the identification of the Ang II receptor subtype(s) involved. The concentrations used in our in
In the absence of TPA stimulation, Ang II (100 nM) increased PAI-1 mRNA levels by approximately 8 fold in VSM cells. Northern blot analysis of these changes revealed a more sensitive semiquantitative method to assess plasminogen activator inhibitor activity. In basal conditions, small amounts of TPA were detected with this method in the cell-conditioned medium. After Ang II stimulation, when PAI activity was markedly increased, TPA also appeared in complex with PAI-1. Taken together, these data demonstrate that the soluble phase the net Ang II effect was an increase in PAI activity. However, the Ang II effect on TPA production could play an important role at a local level. Cell migration requires extracellular matrix digestion, and plasminogen activation is an important cellular mechanism in the digestion of extracellular matrix proteins. We have demonstrated that cultured rat VSM

![Fig 7. A, Northern blot analysis of dose-related increase in plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (TPA) mRNA induced by angiotensin II (Ang II). Vascular smooth muscle cell cultures were incubated for 6 hours in the presence of the specified concentrations of Ang II. Ten micrograms of total RNA was loaded in each lane. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) signal is shown as an internal standard. Lane 1, Control (C); lanes 2 through 8, Ang II at the specified concentrations. B, Graph showing the dose-related increase in PAI-1 (●) and TPA (●) mRNA induced by Ang II. The Northern blot films shown in A were scanned and the values obtained were normalized for the GAPDH reading. Data are in arbitrary units.](http://circ.ahajournals.org/)

![Fig 8. Northern blot analysis demonstrating the lack of effect of the protein synthesis inhibitor cycloheximide (CHX, 10 μg/mL) on angiotensin II (Ang II)-induced (0.1 μmol/L) increase in plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (TPA) mRNA. Vascular smooth muscle cell cultures were incubated for 3 hours in the absence (−) or in the presence (+) of the specified agents. Ten micrograms of total RNA was loaded in each lane. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) signal is shown as an internal standard.](http://circ.ahajournals.org/)
cells can digest extracellular matrices, with a mechanism that is at least in part plasminogen dependent.24 Plasminogen activation was possible even in the presence of excess PAI activity but required direct contact of the cells with the extracellular matrix substrate.24 Cell surface receptor molecules have been demonstrated for plasminogen and for the two natural plasminogen activators, urokinase-like plasminogen activator (u-PA) and TPA.39–42 This cell-bound fibrinolytic system would provide both localization and, in the case of the TPA receptor, protection of the cell-associated proteolysis from inhibition by PAI-1.41–43 In vivo, TPA is expressed by VSM cells during migration after balloon denudation in rat carotid arteries,44 and ACE inhibition can prevent intimal hyperplasia in rat carotid arteries after balloon denudation.45 In vitro, Bell and Madri46 have shown an increase in cell motility and in cell-associated plasminogen activator activity after stimulation with Ang II in cultured aortic bovine smooth muscle cells. These observations raise the possibility that the RAS could increase cell-surface-associated proteolysis while at the same time inhibiting plasminogen-dependent proteolysis in the surrounding milieu.

Clinical data have suggested an association between the RAS and the occurrence of myocardial infarction. Hypertensive patients with high plasma renin activity and presumably high Ang II levels have a higher risk of myocardial infarction compared with hypertensive patients with low renin activity.7 The DD genotype of the ACE is associated with increased plasma levels of Ang II10 and represents a potent risk factor for myocardial infarction.10 Similarly, raised plasma PAI activity has been shown to be more prevalent in young survivors of myocardial infarction48 and to represent a risk factor for reinfarction in this patient population.49 Recently, it has been reported that Ang II infusion increases the circulating levels of PAI-1 antigen in normal volunteers without appreciable changes in the circulating TPA antigen levels.22 Preliminary data from the same group report an increase in the production of PAI-1 by cultured endothelial cells after stimulation with Ang II, and the authors hypothesize that the PAI-1 increase seen in vivo could be of endothelial origin.23 These data, however, are not necessarily in contrast with our in vitro data. Ang II could well have a similar effect on both endothelial and VSM cells. VSM cells represent the most abundant cell type in the vessel wall, and their contribution to the systemic fibrinolytic balance could be important.

Recently, our understanding of the RAS has greatly expanded. The improvements in cellular and molecular biology approaches have shown the existence of several local renin-angiotensin systems. The vessel wall has been shown to be equipped with all the enzymes necessary to synthesize Ang II.1 In particular, both renin51 and ACE activities52 have been described in cultured VSM cells, and these cells also have been shown by in situ hybridization to express the mRNA for angiotensinogen.52 We have confirmed that cultured rat VSM cells express ACE activity because Ang I was similar to Ang II in eliciting a PAI-1 response that could be blocked by ACE inhibitors. Interestingly, both the ACE activity and the angiotensinogen mRNA expression have been shown to be increased by endothelial balloon denudation or by hormonal stimuli, which could be expected to be released after vessel wall injury.32,52

Conclusions

A "vascular" RAS may profoundly influence medial VSM cells, and the novel finding that these cells produce PAI-1 and TPA in response to Ang I and II has several potential implications. First, the modulation of plasminogen activation in the medial layer of arterial vessels is likely to affect the process of cell migration through it. This could influence the infiltration of inflammatory and tumor cells and also the migration of the VSM cells from the media to the intima during atherogenesis. Second, the lysis of a vessel wall thrombus depends on the amount of plasminogen activators and inhibitors incorporated in the thrombus. The fibrinolytic factors produced at the interface between the clot and the vessel wall could therefore significantly affect the natural or exogenous lysis of arterial thrombi. It can be speculated that if confirmed in vivo, the effect of ACE inhibitors on PAI-1 production by smooth muscle cells could account at least in part for the reduction in cardiovascular events observed in certain patient subsets treated with these agents.

References


Angiotensin II increases plasminogen activator inhibitor type 1 and tissue-type plasminogen activator messenger RNA in cultured rat aortic smooth muscle cells.
R T van Leeuwen, A Kol, F Andreotti, C Kluft, A Maseri and G Sperti

_Circulation_. 1994;90:362-368
doi: 10.1161/01.CIR.90.1.362

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/90/1/362

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/