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Abstract—Previous studies have shown that angiotensin II stimulates the synthesis of plasminogen activator inhibitor-1 in cultured vascular cells, which suggests that activation of the renin-angiotensin system may impair fibrinolysis. We have investigated the effects of angiotensin II and of valsartan, a recently developed angiotensin II antagonist that is highly specific and selective for the angiotensin II subtype 1 receptor, on plasminogen activator inhibitor-1 secretion by smooth muscle cells isolated from rat and human vessels. Angiotensin II induced a time- and concentration-dependent increase of plasminogen activator inhibitor activity in supernatants of rat aortic cells, which reached a plateau after 6 hours of incubation with 100 nmol/L angiotensin II (2.4±0.6-fold over control value; $P<0.001$). The angiotensin II–induced plasminogen activator inhibitor activity was inhibited, in a concentration-dependent manner, by valsartan with an IC_{50} value of 21 nmol/L. Valsartan fully prevented the angiotensin II–induced increase in plasminogen activator inhibitor-1 protein and mRNA. Furthermore, angiotensin II doubled the secretion of plasminogen activator inhibitor-1 by smooth muscle cells obtained from human umbilical and internal mammary arteries, and valsartan fully prevented it. Angiotensin II did not affect the secretion of tissue plasminogen activator antigen by any of the cell systems tested. Thus, valsartan effectively inhibits angiotensin II–induced plasminogen activator inhibitor-1 secretion without affecting that of tissue plasminogen activator in arterial rat and human smooth muscle cells. **(*Hypertension*. 2001;37:961-966.)**

Key Words: plasminogen ■ fibrinolysis ■ angiotensin II ■ receptors, angiotensin ■ valsartan ■ muscle, smooth

Activation of the renin-angiotensin system (RAS) is associated with an increased risk of ischemic cardiovascular events independently of its effects on blood pressure.[1] Conversely, the inactivation of RAS, with angiotensin-converting enzyme (ACE) inhibitors, reduces the risk of recurrent myocardial infarction in patients with left ventricular dysfunction,[2] reduces intimal thickening after vascular injury,[3] and in experimental models decreases progression of atherosclerosis.[4]

Angiotensin II (Ang II) is responsible for the RAS-mediated cardiovascular complications,[5] and therefore the beneficial effects of ACE inhibitors derive mainly from their reduction of the biosynthesis of this autacoid. A non–ACE-dependent Ang II formation resulting from the action of a chymase enzyme occurs in the heart and in normal and to a greater extent in atherosclerotic vessel walls.[6,7] This may limit the effectiveness of ACE inhibitors.

Ang II receptor antagonists, and in particular antagonists of the Ang II subtype 1 (AT_1) receptor, form a recently developed class of drugs that suppress both the ACE- and non–ACE-dependent vascular effects of Ang II and that are therefore better at preventing cardiovascular effects than simple ACE inhibitors.[8]

Plasminogen activator inhibitor-1 (PAI-1) is a key regulator of fibrinolysis, and accumulating data suggest that elevated PAI-1 levels in plasma are a risk factor for arterial thrombotic disease.[9] The interaction of Ang II with the fibrinolytic system has recently gained attention.[10] Infusion of Ang II, in both normotensive and hypertensive subjects, increases the circulating levels of PAI-1.[11] Moreover, Ang II increases the levels of PAI-1 antigen and mRNA in cultured bovine and rat aortic endothelial cells as well as the level of PAI-1 mRNA in human umbilical vein endothelial cells.[12,13] In addition, Ang II has been shown to increase PAI-1 expression in smooth muscle cells (SMCs) isolated from rat aorta, through an interaction with the AT_1 receptor subtype.[13,14] No information is available, however, on the effect of Ang II on PAI-1 biosynthesis by human SMCs.

SMCs represent the most abundant cell type in the vessel wall, and their contribution to the local and systemic fibrino-

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From the Institute of Pharmacological Sciences (L.S., A.M.C., L.A., A.C., E.T., L.M.) and the Department of Cardiac Surgery (A.P.), University of Milan (Italy), and the Pharmaceutical Division, Novartis, Basel, Switzerland (M. de G.).

Correspondence to Elena Tremoli, Laboratory of Pharmacology of Thrombosis and Atherosclerosis, Institute of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy. E-mail Elena.Tremoli@unimi.it

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lytic balance is of particular relevance. Increased PAI-1 expression was noted in the thickened intima at the base of human atherosclerotic plaques.[15] This may result in an impairment of the plasmin-mediated proteolytic cascade, which controls extracellular matrix degradation and vessel wall function. Interestingly, inhibition of ACE has been shown to reduce PAI-1 expression in the neointima of rat balloon-injured aortas.[16] In this condition, enhancement of luminal fibrinolysis and extracellular matrix turnover may occur. Indeed, plasmin generation is part of the proteolytic machinery regulating the activation of matrix metalloproteinases within the vessel wall.[17] Interestingly, Ang II–forming activity has been found in atherosclerotic as well as in aneurysmal lesions.[6]

In view of the relevance of the effects of Ang II on the fibrinolytic potential of vascular SMCs, we investigated the effects of valsartan,[18] a highly specific and selective antagonist of the AT_1 receptor, on the formation of PAI-1 and tissue plasminogen activator (tPA) by rat aortic SMCs isolated from normal and hypertensive rats. We have extended the study to human SMCs isolated from various arterial locations.

Methods

Cell Culture and Experimental Procedures

Rat aortic SMCs were obtained according to Ross,[19] from intima-media layers of aorta of Sprague-Dawley rats, Wistar-Kyoto rats (WKY), or spontaneously hypertensive rats (SHR). Procedures involving animals and their care were conducted in conformity with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Cells were grown as previously described and used at passages 3 to 9.[20] Human SMCs were isolated from pieces of internal mammary arteries obtained from patients undergoing cardiopulmonary bypass surgery and from arteries of human umbilical cords obtained at normal deliveries. This was approved by the Institutional Referee Board of the Cardiology Center Walter Monzino (Milan, Italy). Human SMCs were cultured in M199 supplemented with 20% FCS and used at passages 2 to 8. Cells were confirmed to be SMCs by morphology and positive staining to α-actin.[21]

Experimental Procedures

Rat aortic SMCs (2.5×10^5) were seeded in 35-mm dishes and cultured to subconfluence in complete medium (modified Eagle's medium). Human SMCs (3×10^4) were seeded in 24-well plates and cultured to confluence in complete medium 199. Cells were incubated for different times in serum-free medium with the appropriate substances after 24 hours in serum-free medium for rat cells and 48 hours in 0.4% FCS medium for human cells. At the end of the incubation, the conditioned medium (CM) was collected and stored in aliquots at −20°C until analyzed. Cells were dissolved with 0.2 mol/L NaOH to determine cellular protein. Valsartan, kindly provided by Novartis Pharma (Basel, Switzerland), was dissolved in KOH (0.1 mol/L), adjusted to pH 8 with HCl, and diluted in sterile PBS at 1 mmol/L concentration. Equivalent volume of the solvents was added in control cells. Captopril, PD123319, saralasin, and Ang II (Sigma Chemical Co) were dissolved in sterile PBS at 1 mmol/L concentration. Unless otherwise stated, drugs were incubated with cells for 10 minutes before the addition of Ang II.

Determination of Plasminogen Activator and PAI-1

PAI was measured by an indirect chromogenic assay (pPAR/L, Biopool, DK). Briefly, different amounts (5 to 20 μL) of CM were incubated in 96-well plates for exactly 10 minutes at room temperature with a fixed concentration of tPA standard (1 IU/mL). After 1 to 2 hours of incubation at 37°C, plasmin generation was assessed at 405 nm in a spectrophotometric plate reader. PAI activity is expressed as arbitrary units (AU/mL) using reference curves of standard tPA. Plasminogen activator (PA) activity of the conditioned medium was evaluated in the same assay described for PAI activity except that tPA was omitted from the reaction mixture. Data are expressed as PA activity using reference curves of standard tPA.

Determination of PAI-1 and tPA Antigen

PAI-1 and tPA were measured in medium collected from human SMCs by specific ELISAs (Imulyse, Biopool). Data are expressed as nanograms PAI-1 or tPA antigen per microgram cell protein.

Immunoblotting for PAI-1

CM (1 mL) of cells incubated for 6 hours with vehicle or Ang II in the presence or absence of valsartan was precipitated, separated by SDS-PAGE, and transferred to nitrocellulose as described.[13] PAI-1 protein was recognized with a specific monoclonal antibody (MA-33H1), a kind gift of Professor P.J. Declerck, Katholieke Universiteit Leuven,[22] and revealed with anti-mouse IgG (Amersham Pharmacia Biotech), conjugated with peroxidase. Positive bands were visualized after exposure to Hyperfilm ECL (Amersham).

Measurements of Rat PAI-1 mRNA by Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from rat cells was prepared with the use of TRIzol (Gibco Life Technologies) in accordance with the manufacturer's instructions. The levels of rat PAI-1 and GAPDH mRNA in the differently treated samples were detected using a reverse transcriptase–polymerase chain reaction (RT-PCR) performed as previously described.[23]

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed with ANOVA followed by Tukey's test. Differences were taken as statistically significant at $P<0.05$.

Results

Ang II–Induced PAI-1 in Human and Rat Arterial SMCs

Initial studies were performed to assess the effects of Ang II on PAI activity of rat aortic SMCs. PAI activity in CM of unstimulated rat SMCs was measurable at 2 hours (1.4±0.39 AU/mL; n=6) and did not change up to 16 hours (1.7±0.3 AU/mL; n=3). Ang II incubated with rat SMCs for 6 hours concentration-dependently increased PAI activity, reaching the maximum effect at 100 nmol/L (2.4±0.3 times the control value; n=9; $P<0.01$) (Figure 1B). The effect of Ang II was time dependent, starting at 2 hours, reaching a plateau after 6 hours, and remaining constant up to 16 hours (Figure 1A).

The PA activity of rat SMCs, by contrast, was very low, being barely detectable in both unstimulated and Ang II–stimulated rat SMCs.

To determine whether the increase in PAI activity exerted by Ang II was due to increased amounts of PAI-1 released from cells, we detected PAI-1 antigen by Western blotting of CM collected from rat SMCs unstimulated or incubated 6 hours with Ang II (100 nmol/L) (Figure 1C). The Ang II–induced PAI-1 antigen increases in CM from rat SMCs paralleled the increase of PAI-1 mRNA steady state levels as assessed by RT-PCR (Figure 1D). The capacity of Ang II to modulate the synthesis of PAI-1 in human SMCs obtained from 2 arterial locations (internal mammary and umbilical arteries) was then evaluated. To this end, human SMCs were

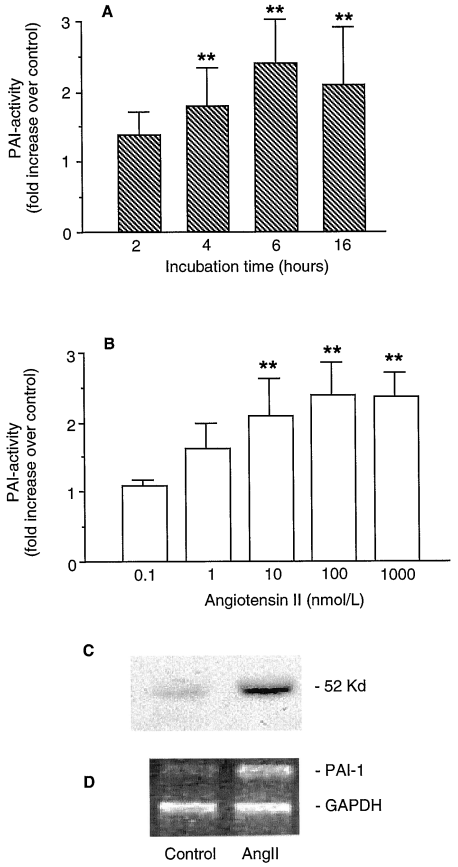

Figure 1. Effects of Ang II on PAI activity, protein accumulation, and mRNA synthesis by rat aortic SMCs. The cells were incubated with 100 nmol/L of Ang II for the indicated time (A) or for 6 hours with different concentrations of Ang II (B). C and D, Rat aortic SMCs were incubated for 6 hours in medium with or without Ang II 100 nmol/L. CM was then precipitated, separated by SDS-PAGE, and immunoblotted with a monoclonal antibody against rat PAI-1 (C). Total RNA was extracted, and PAI-1 and GAPDH mRNA levels were estimated by RT-PCR (D). A and B, Each bar represents the mean±SEM of at least 6 independent experiments performed in duplicate (**$P<0.01$ vs vehicle-treated rats). C and D, One typical experiment of 4 is represented.

incubated for 6 hours in serum-free medium with or without Ang II (100 nmol/L). Ang II doubled PAI-1 antigen secretion by the umbilical artery SMCs ($P<0.01$) and, to a lesser extent, by SMCs isolated from internal mammary artery ($P<0.05$) (Figure 2).

tPA antigen levels in unstimulated SMCs obtained from human arteries were only weakly detectable and did not change after exposure to 100 nmol/L of Ang II (data not shown).

Modulation of PAI Ang II Induced in Rat and Human SMCs by Valsartan

The involvement of Ang II receptor subtypes in the stimulatory effect of Ang II on PAI-1 secretion was then investigated. In rat aortic SMCs, [Sar^1-Ile^8]Ang II (saralasin), a nonspecific Ang II receptor subtype antagonist, and PD123319, a selective antagonist of the AT_2 receptors, did not affect basal levels of PAI activity up to 1 μmol/L concentration. In cells exposed for 6 hours to 100 nmol/L

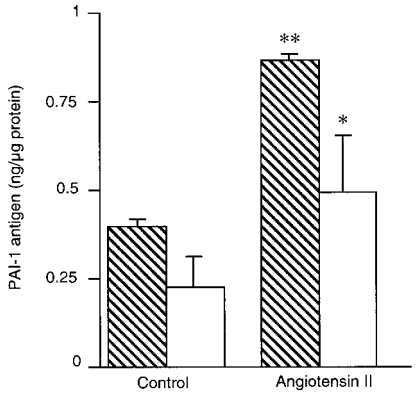

Figure 2. Ang II–induced PAI-1 release into the supernatants of human SMCs isolated from umbilical (▨) and internal mammary (□) arteries. Cells were incubated for 6 hours with or without Ang II (100 nmol/L). PAI-1 antigen levels were determined in CM with specific ELISAs. Each bar represents the mean±SEM of 3 independent experiments performed in duplicate (**$P<0.01$, *$P<0.05$ vs the corresponding control).

Ang II instead, saralasin completely prevented the increase in PAI activity induced by Ang II, whereas PD123319 (100 to 1000 nmol/L) had no effect (data not shown). The role of the AT_1 receptor subtype was therefore examined by incubating rat SMCs with valsartan. Valsartan (0.01 to 1000 nmol/L) prevented the increase of PAI activity induced by Ang II in a concentration-dependent manner, with an IC_{50} of 21 nmol/L (Figure 3A). In the presence of 100 nmol/L valsartan, a parallel rightward shift of the concentration-response curve of Ang II–induced PAI activity was recorded (EC_{50} from 1.7 to 75 nmol/L with Ang II alone and in the presence of valsartan, respectively), with no change in the maximal effect of Ang II (Figure 3B). The *R*-enantiomer of valsartan failed to affect PAI activity induced by Ang II (data not shown), indicating that the effect of the drug is stereospecific.

Prior treatment (14 to 16 hours) of rat SMCs with captopril (1 μmol/L), which prevents the conversion of angiotensin I to Ang II, did not influence the effects of either Ang II or valsartan on PAI activity. This observation rules out the possibility of an effect of endogenous Ang II in our experimental system. Valsartan at 100 nmol/L prevented the effect of Ang II (100 nmol/L) on the levels of rat PAI-1 antigen as well as on PAI-1 mRNA (Figure 4A and 4B). Moreover, valsartan completely prevented the effect of Ang II on PAI-1 secretion by human SMCs from either internal mammary or umbilical arteries (Figure 5).

The effect of valsartan on the Ang II–induced PAI activity was also investigated in SMCs isolated from the aorta of SHR. Ang II (100 nmol/L) increased PAI activity of SHR SMCs by 3.3±0.2-fold, and this effect was significantly greater than that exerted by Ang II on SMCs isolated from aorta of WKY (2.06±0.1-fold; n=3; $P<0.01$ versus SHR SMCs), which is the related control strain. Valsartan (100 nmol/L) completely prevented the increase in PAI activity induced by Ang II in SMCs isolated from aorta of both rat strains (data not shown).

Discussion

In this study we show that Ang II impairs the fibrinolytic potential of human and rat arterial SMCs by increasing the

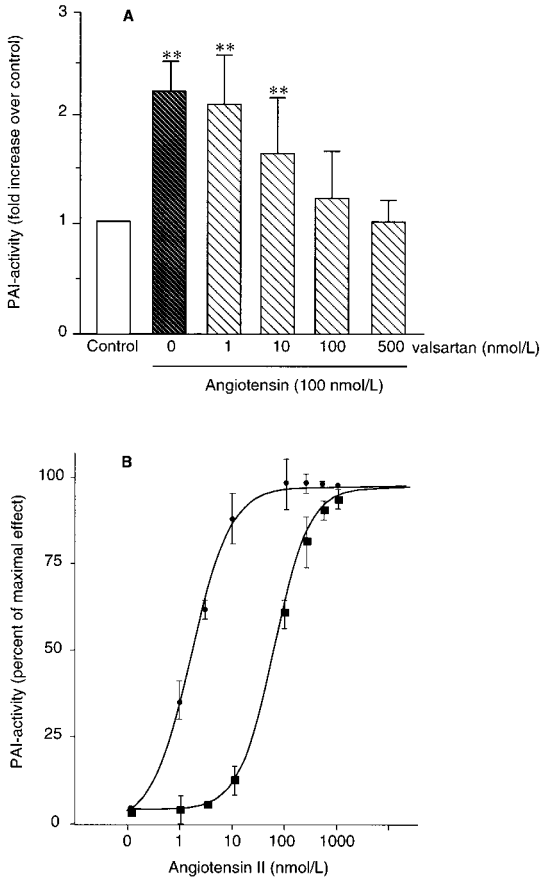

Figure 3. Effects of valsartan on the PAI activity induced by Ang II in rat aortic SMCs. A, Cells were incubated for 10 minutes with different concentrations of valsartan (1 to 500 nmol/L) before the addition of 100 nmol/L Ang II for an additional 6 hours. CM of cells was then collected, and PAI activity was measured. Values are the mean±SEM of 9 individual experiments (**$P<0.01$ vs control). B, Cells were incubated for 10 minutes with a fixed concentration of valsartan (100 nmol/L) and then challenged for an additional 6 hours with different concentrations of Ang II (1 to 1000 nmol/L). The curves obtained with Ang II alone (●) or in the presence of valsartan (■) represent the mean±SEM of 4 individual experiments performed in duplicate. Results are expressed as percentages of the maximum response obtained with Ang II alone.

synthesis of PAI-1 and that this effect is reversed by valsartan, a highly specific AT_1 receptor antagonist.

In situ hybridization and immunohistochemical studies have demonstrated the presence of PAI-1 on the vascular wall, with a significant increase in its concentration during the progression from normal vessel to fatty streaks and to atherosclerotic plaques.[15] Recent studies have shown an increase of local PAI-1 mRNA expression, predominantly in SMCs, within human atherosclerotic lesions.[24]

PAI-1 production by SMCs is increased by platelet-associated growth factors and Ang II.[13,25] In particular, Ang II exerts hypertrophic and proliferative growth actions for SMCs.[26] These observations suggest a relationship between PAI-1 expression and cell proliferation and support the concept that high expression of PAI-1 may correlate with the progression of atherosclerosis.

PA, whether measured as tPA antigen in the CM of human SMCs or as PA activity in rat SMCs, was barely detectable,

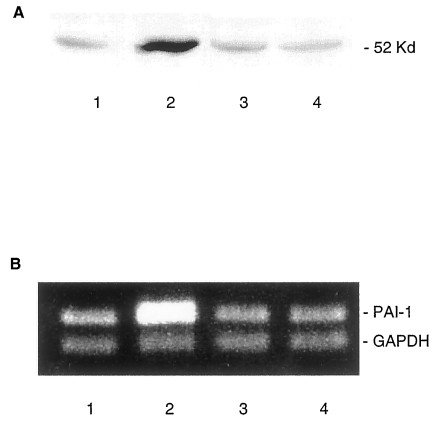

Figure 4. Effect of valsartan on PAI-1 protein accumulation and mRNA synthesis in rat SMCs. Rat SMCs were incubated for 6 hours with vehicle (1) and in the presence of Ang II 100 nmol/L (2), Ang II 100 nmol/L plus valsartan 100 nmol/L (3), or valsartan alone (4), then CM was precipitated, separated by SDS-PAGE, and immunoblotted with a monoclonal antibody against rat PAI-1 (A). Total RNA was extracted from the treated cells, and PAI-1 and GAPDH mRNA levels were estimated by RT-PCR (B). A and B are representative of 3 experiments with similar results.

and these levels did not change after stimulation of cells with Ang II. Thus, Ang II reduces the fibrinolytic potential of SMC as the result of a specific increase in PAI-1 biosynthesis.

The positive effect of Ang II on PAI-1 biosynthesis in cultured rat aortic SMC is mediated by an interaction of this autacoid with the AT_1 receptor.[13,14] Our data indicate that Ang II increases PAI-1 biosynthesis in human arterial SMCs by a similar mechanism. Valsartan, a highly specific AT_1 receptor antagonist, completely prevented the enhancing effects of Ang II on PAI-1 secreted by human arterial SMCs.

ACE inhibitors have been widely used to block RAS and to treat hypertension and congestive heart failure; however, despite their recognized clinical efficacy, they have some

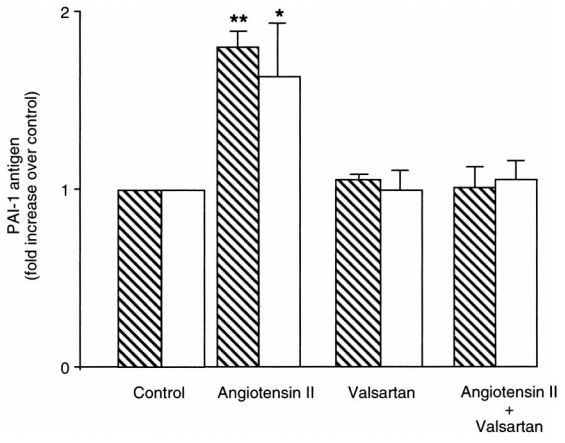

Figure 5. Effect of valsartan on Ang II–induced PAI-1 release into the medium of human SMCs from umbilical (▨) and internal mammary (□) arteries. The cells were incubated for 6 hours with or without Ang II (100 nmol/L), in the presence or absence of valsartan (100 nmol/L). PAI-1 antigen levels were determined in CM with specific ELISAs. Each bar represents the mean±SEM of 3 independent experiments performed in duplicate (**$P<0.01$, *$P<0.05$ vs corresponding control).

weaknesses. ACE inhibition results in bradykinin accumulation, which is a source of side effects such as cough and angioedema. In addition, during ACE inhibition, plasma Ang II levels decrease, but some Ang II is still measurable within the circulation because of non-ACE biosynthesis of this autacoid.[27] Since most known effects of Ang II are mediated by the AT_1 receptor, AT_1 receptor antagonists might be useful as a local antagonist of the effects of Ang II that are also derived from non-ACE pathways.[28] Previous reports have suggested that valsartan, a highly selective antagonist of the AT_1 receptor, is useful in lowering cardiovascular risk.[18,29,30] This drug, beyond its blood pressure–lowering activity, improves heart failure in rats with myocardial infarction, reduces endothelial dysfunction and intimal thickening in atherosclerotic rabbits, and induces a significant regression of left ventricular hypertrophy in patients with essential hypertension.[18,31–33] The effects of AT_1 antagonists on fibrinolytic balance, and in particular on PAI-1 and tPA plasma levels, in normal subjects as well as in patients with cardiovascular disease, are still controversial.[34] Moreover, no clinical data on valsartan, which possesses greater specificity and selectivity for the AT_1 receptor than losartan,[35] are yet available. This study shows that valsartan, at clinically relevant concentrations,[18] is effective in preventing Ang II–mediated PAI-1 biosynthesis in rat aortic SMCs isolated from normotensive or hypertensive animals. This is consistent with the observation that valsartan was effective in preventing PAI-1 overexpression in kidneys of a rat model of high human renin hypertension.[36] Moreover, valsartan prevented the reduction in fibrinolysis of hypercholesterolemic rabbits (M. de Gasparo, personal communication).

Ang II receptor antagonists not only may control plasma fibrinolytic potential but may also influence vessel wall proteolytic properties, with a most prominent role within the atherosclerotic plaque and or at the level of the migrating and/or proliferating SMCs. This effect may be important in preventing reduced plasmin generation that is due to the Ang II–mediated increase in PAI-1 biosynthesis. As previously mentioned, Ang II can be generated through a specific chymase, and this is not affected by ACE inhibition.[6] In conclusion, we show that Ang II modulates the release of PAI-1 from SMCs isolated from rat and human arteries and that this effect is mediated by the interaction of Ang II with AT_1 receptors. Ang II may influence the synthesis of fibrinolytic factors in the medial layer of arterial vessels, thus inhibiting the natural lysis of arterial thrombi and promoting atherogenesis; these effects of Ang II are antagonized by valsartan. We speculate that in vivo, AT_1 receptor antagonists represent a potential way to locally block the PAI-1 biosynthesis induced by the Ang II that is generated not only by ACE but also via non-ACE pathways.

Acknowledgments

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