Angiotensin II Regulates the Expression of Plasminogen Activator Inhibitor-1 in Cultured Endothelial Cells

A Potential Link between the Renin–Angiotensin System and Thrombosis

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Abstract

Plasminogen activator-inhibitor C-1 (PAI-1) plays a critical role in the regulation of fibrinolysis, serving as the primary inhibitor of tissue-type plasminogen activator. Elevated levels of PAI-1 are a risk factor for recurrent myocardial infarction, and locally increased PAI-1 expression has been described in atherosclerotic human arteries. Recent studies have shown that the administration of angiotensin converting enzyme inhibitors reduces the risk of recurrent myocardial infarction in selected patients. Since angiotensin II (Ang II) has been reported to induce PAI-1 production in cultured astrocytes, we have hypothesized that one mechanism that may contribute to the beneficial effect of angiotensin converting enzyme inhibitors is an effect on fibrinolytic balance. In the present study, we examined the interaction of Ang II with cultured bovine aortic endothelial cells (BAECs) and the effects of this peptide on the production of PAI-1. ^{125}I–Ang II was found to bind to BAECs in a saturable and specific manner, with an apparent K_d of 1.4 nM and B_{max} of 74 fmol per mg of protein. Exposure of BAECs to Ang II induced dose-dependent increases in PAI-1 antigen in the media and in PAI-1 mRNA levels. Induction of PAI-1 mRNA expression by Ang II was not inhibited by pretreating BAECs with either Dup 753 or [Sar^1, Ile^8]-Ang II, agents that are known to compete effectively for binding to the two major angiotensin receptor subtypes. These data indicate that Ang II regulates the expression of PAI-1 in cultured endothelial cells and that this response is mediated via a pharmacologically distinct form of the angiotensin receptor. (*J. Clin. Invest.* 1995. 95:995–1001.) Key words: Plasminogen activator inhibitor-1 • fibrinolysis • angiotensin II • endothelial cell • renin–angiotensin system

Introduction

Plasminogen activator inhibitor-1 (PAI-1)[1] is a member of the superfamily of serine protease inhibitors (serpins) and is the main physiological inhibitor of tissue-type plasminogen activator (t-PA) (1, 2). The endothelium is likely to be a major source of PAI-1 in the circulation, although the liver and platelets also contribute to the PAI-1 levels measured in plasma (3, 4). Elevated levels of PAI-1 have been implicated in the pathogenesis of thromboembolic disease and, in particular, may contribute to the risk of reinfarction in patients that have suffered a previous myocardial infarction (5, 6). Furthermore, increased PAI-1 expression has been observed in atherosclerotic human arteries (7). Conversely, PAI-1 deficiencies are associated with a bleeding tendency in humans (8, 9). Multiple factors have been identified that play a role in the regulation of PAI-1 synthesis and secretion. PAI-1 is an acute-phase reactant (10), and plasma levels of the protein are elevated postoperatively (11) and during septicemia (12). Agents that are known to regulate endothelial PAI-1 production include endotoxin (13), TNF (14), TGF-β (14), thrombin (15), and lipoprotein (a) (16).

We have recently shown that the infusion of physiological amounts of angiotensin II (Ang II) promotes a rapid and dose-dependent increases in plasma PAI-1 levels in humans (17). Ang II is a potent effector peptide of the renin–angiotensin system and exerts a wide variety of actions on the cardiovascular, renal, endocrine, and central nervous systems (18, 19). Furthermore, the biological responses induced by Ang II are varied and tissue dependent (18). Ang II exerts its effects via binding to specific receptors located on cell membranes (19), and at least two major classes and several additional subtypes have been identified (20). These receptor subtypes can be differentiated by their biochemical properties and by their affinity for synthetic ligands. The cDNA for the type 1 (AT_1) and type 2 (AT_2) Ang II receptors have been cloned (21–24). These receptors are both members of the superfamily of G-protein–coupled receptors containing seven transmembrane domains and are ~ 32% homologous at the protein level (23). Although both receptors bind Ang II saturably and specifically, the AT_1 appears to mediate most of the physiologic effects of Ang II (20).

Endothelial cells possess many of the constituents of the renin–angiotensin system including angiotensin converting enzyme (ACE), angiotensinogen, renin, and receptors for angiotensin (25–27). Ang II saturably and reversibly binds to the surface of cultured vascular endothelial cells via a single class of high affinity binding sites (27), although the characteristics of the endothelial binding do not allow it to be categorized as typical of an AT_1 or AT_2 receptor subtype. There is some evidence to suggest that this interaction has physiological consequences, since endothelial prostaglandin production is enhanced after exposure to Ang II (28). Furthermore, there is evidence of a regulatory linkage between the renin–angiotensin system and fibrinolysis. Interruption of the endothelial autocrine angiotensin system with ACE inhibition promotes endothelial migration and urokinase production in vitro (29). Recently, Ang II

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1. *Abbreviations used in this paper:* ACE, angiotensin converting enzyme; Ang II, angiotensin II; BAEC, bovine aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; PAI-1, plasminogen activator-inhibitor-1; t-PA, tissue plasminogen activator.

has been reported to stimulate selectively the production of PAI-1 in cultured murine astrocytes (30). The present study was designed to determine whether Ang II plays a role in the regulation of the expression of PAI-1 in cultured endothelial cells.

Methods

Materials. FBS and bovine calf serum were obtained from Hyclone Laboratories (Logan, UT). Tissue culture medium was from BioWhittaker (Walkersville, MD). Endothelial mitogen and Dil-acetyl LDL were from Integrated Biotechnology (Stoughton, MA). Ang II, [Sar^1, Ile^8]-Ang II, gelatin, penicillin, streptomycin, antibody against human von Willebrand factor, endotoxin-free water, and trypsin/EDTA were from Sigma Chemical Co. (St Louis, MO). Ang II was also obtained from Bachem (Torrence, CA). Dup 753 was kindly provided by Ronald D. Smith, PhD (Du Pont Pharmaceutical Co., Wilmington, DE). Collagenase was from Worthington Biochemical Corp. (Freehold, NJ). [^{32}P]-dCTP and ^{125}I–Ang II were from New England Nuclear (Boston, MA).

Cell culture. Bovine aortic endothelial cells (BAECs) were harvested, using 0.1% collagenase, from fresh bovine aortas (31); primary cultures were initiated simultaneously from six to eight aortas. After reaching confluence, cells were detached using trypsin/EDTA, pooled, and serially propagated in DME supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 20% bovine calf serum. The cells were incubated at 37°C in humidified 95% air/5% CO_2 and routinely passaged with a split ratio of 1:4. The cells exhibited typical endothelial cell morphology, growing as confluent monolayers of polygonal, closely apposed cells with a "cobblestone" appearance, and by immunofluorescence staining with antibodies against von Willebrand factor and by uptake of Dil-acetyl-LDL. Only cultures composed of > 90% endothelial cells were selected for use in these studies, and only early passage cells were used (passage 2 or earlier). Human umbilical vein endothelial cells (HUVECs) were propagated from pooled primary cultures of human umbilical veins (gift of M. Gimbrone, Brigham and Women's Hospital, Boston, MA) as previously described (32). HUVECs were grown on gelatin-coated plates in DME containing 25 mM Hepes buffer and 2.0 mM glutamine supplemented with 20% FBS, 50 μg/ml endothelial mitogen, 50 U/ml penicillin, and 50 μg/ml streptomycin. For experimental studies, BAECs were grown to confluence in 100-mm plastic culture dishes, washed with sterile PBS, and incubated overnight in serum-free medium that was otherwise complete. The next day, the confluent monolayers were washed two times with PBS and incubated with serum-free media supplemented with Ang II freshly reconstituted in DME at the concentrations and for the durations indicated in the following section.

Endotoxin assay. The Ang II preparations used in these experiments were > 98% pure and generated single peaks by HPLC analysis performed by the manufacturers. These peptides were assayed for the presence of contaminating endotoxin using the Limulus Amebocyte Lysate assay (Pyrogent^R, BioWhittaker), according to the manufacturer's instructions (33). This assay has a sensitivity of 0.06 endotoxin units per ml, which corresponds to a concentration of 6.0 pg/ml, with 10 endotoxin units equal to 1.0 ng. The assay is calibrated using a preparation of purified endotoxin from *Escherichia coli* strain 055:B5. There was no detectable endotoxin present in a 1.0-μM/liter concentration of Ang II reconstituted in endotoxin-free water.

Binding assays. Specific binding of ^{125}I–Ang II with BAECs was performed as described by Patel et al. (27). Confluent cultures of BAECs, grown in six-well tissue culture plates, were washed free of maintenance media by rinsing twice with PBS, pH 7.4, 1% BSA. Duplicate cultures of BAECs were incubated at room temperature with variable concentrations of ^{125}I–Ang II with a specific activity of 2,200 Ci/mmol. After a 30-min incubation period, the cells were washed three times with ice-cold PBS, pH 7.4. The attached cells were dissolved with 1.5 ml of 0.2 N NaOH and transferred to polypropylene tubes. Cellular protein content was determined according to the method of Bradford (34). Radioactivity was determined in a gamma counter (Packard Instrument Co., Meriden, CT) with a counting efficiency of 74%. Specific binding was defined as the difference between total and nonspecific binding. Nonspecific binding was determined by the addition of a 100-fold molar excess of unlabeled Ang II.

PAI-1 antigen measurements. Conditioned medium was collected after the indicated times, centrifuged to remove cellular debris, and stored for ≤ 48 h at −20°C until assays were performed. PAI-1 antigen concentrations were determined with a commercially available ELISA (TintElize^R PAI-1, Biopool, Umea, Sweden) specific for PAI-1 (free or complexed) (35) using a double antibody technique, in accordance with the manufacturer's instructions, as previously described (36).

RNA isolation and measurement. Total cellular RNA was prepared from confluent cultures of BAECs by the acid guanidinium thiocyanate method (37), followed by isopropanol precipitation (RNAzol, Cinna Biotecx, Houston, TX). RNA pellets were resuspended in DEPC-treated H_2O, and their concentration was determined by absorbance at 260 nm. The relative amount of specific mRNA present in the samples was quantified via either slot blot (38) or Northern (39) hybridization analysis. For slot blot studies, total RNA was denatured with 6 M glyoxal for 1 h at 50°C. The RNA (10 μg) was applied to a nylon membrane (Zeta Probe^R, Bio-Rad Laboratories, Richmond, CA) using a slot blot filtration apparatus (Bio-Dot SF, Bio-Rad Laboratories). After application of the samples, membranes were baked for 2 h at 80°C under vacuum. For Northern hybridization studies, RNA (10 μg) was size fractionated on 1.2% formamide agarose gels, transferred to nylon filters, and baked as previously described. The membranes were prewashed in 0.1× SSC, 0.5% SDS for 1 h at 65°C followed by prehybridization overnight at 42°C in the following mixture: 50% formamide, 5× SSC, 5× Denhardt's solution, 25 mM phosphate buffer, pH 7.4, 10% dextran sulfate, 1 mM EDTA, 1% SDS containing heat-denatured transfer RNA (500 μg/ml), and sonicated, heat-denatured salmon sperm DNA (200 μg/ml). Membranes were hybridized with cDNA probes labeled with ^{32}P-labeled deoxycytidine triphosphate ([^{32}P]dCTP) for 16–24 h at 42°C in a shaking water bath, washed using 0.1× SSC, 0.1% SDS at 65°C, air dried, and exposed to XAR film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −70°C. The relative intensity of the autoradiographic images was determined using transmission densitometry. Relative RNA loading was determined by examination of ethidium bromide–stained gels. Reflectance densitometry of the ethidium bromide–stained 28S bands was used for normalizing autoradiographic data.

cDNA probes. The PAI-1 probe consisted of a 2.3-kb EcoRI fragment from pSP65, containing 32 nucleotides of 5′ untranslated sequence and ~ 800 bp of 3′ untranslated sequence (40), and was kindly provided by Paul Declerck (University of Leuven, Belgium). The cDNA probe for t-PA consisted of a 1.9-kb BglII fragment that was also in pSP65. cDNA probes were labeled with [^{32}P]dCTP by the random primer method (41).

Statistical analyses. The effects of Ang II on PAI-1 mRNA levels were compared using Student's t test for unpaired values. To test for the significance of a trend between increasing doses of Ang II and PAI-1 production, a simple linear regression model was constructed after logarithmic transformation of the Ang II dose as suggested by graphical display. All reported P values are two-tailed. A P value < 0.05 was considered significant.

Results

Binding of ^{125}I–Ang II to BAECs. The time course of ^{125}I–Ang II binding to BAECs was measured at 24°C. The results shown in Fig. 1 were obtained using a concentration of 0.2 nmol/liter ^{125}I–Ang II and indicate that the ligand associates with the cells in a time-dependent manner. Binding reached an apparent equilibrium within 90 min, with a $t_{1/2}$ of 45 min.

Equilibrium binding studies were performed by incubating various concentrations of ^{125}I–Ang II with BAECs for 90 min at 24°C in the presence or absence of a 100-fold molar excess of unlabeled Ang II. Evidence of saturable, specific binding

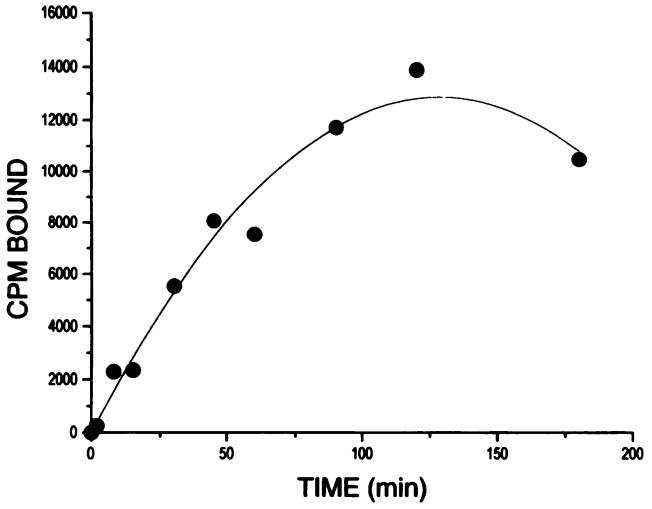

Figure 1. Time dependence of saturable binding of ^{125}I–Ang II to BAECs. Cells were incubated with ^{125}I–Ang II (0.2 nM) for the times indicated, and total binding was determined as described.

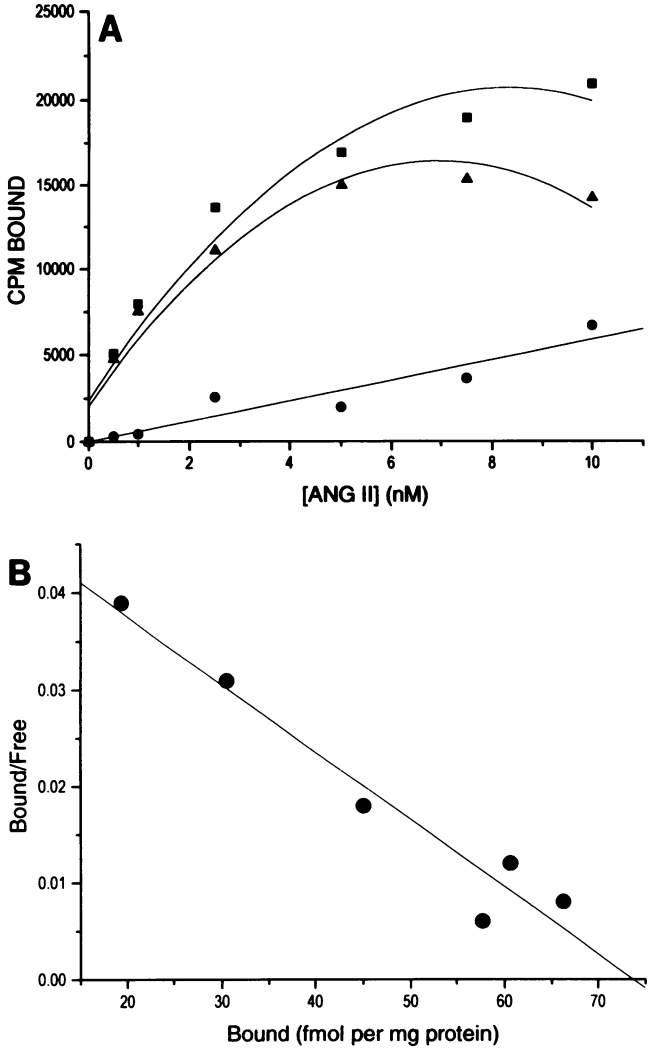

Figure 2. Equilibrium binding isotherms for the interaction of ^{125}I–Ang II with BAECs. (*A*) Confluent cultures of BAECs in six-well tissues culture dishes were incubated with the indicated concentrations of ^{125}I–Ang II, in the presence or absence of a 100-fold molar excess of unlabeled peptide for 90 min at room temperature. Each point represents the average of two determinations, with total (*closed squares*), nonspecific (*closed circles*), and specific (*closed triangles*) binding isotherms depicted. (*B*) Scatchard transformation of specific binding data. Scatchard plot yielded a straight line (r = 0.972), with a K_d and B_{max} of 1.4 nM and 74 fmol per mg of cell protein, respectively.

was obtained (Fig. 2 *A*). Nonspecific binding averaged 16±9% (mean±SD). Scatchard analysis of the specific binding data was performed as shown in Fig. 2 *B*. The data could be adequately fitted with a straight line ($r = -0.972$), which is consistent with a single class of binding sites. At saturation, ~ 44,000 molecules of Ang II bound per endothelial cell, with an apparent K_d = 1.4 nM.

Additional studies were performed to provide a pharmacologic characterization of the BAEC receptor for Ang II. Duplicate samples of BAECs were incubated with 0.2 nM ^{125}I–Ang II for 15 min at room temperature. Increasing amounts of unlabeled competitors were added and incubated for a total of 60 min. Total binding was measured and relative inhibitory activities were determined, as shown in Fig. 3. ^{125}I–Ang II was readily displaced by unlabeled Ang II ($IC_{50} < 10$ nM), whereas the IC_{50} for Dup 753 (a highly specific antagonist for the AT_1) (21, 22) approached 1.0 μM, and the IC_{50} for [Sar^1, Ile^8]-Ang II (a high affinity ligand for the AT_2 receptor) (23, 24) was ~ 10 μM.

Effects of Ang II on the accumulation of PAI-1 antigen in the media. In these experiments, Ang II was added to confluent cultures of BAECs under serum-free conditions, as previously described. BAECs were incubated in the presence of Ang II for 18 h, at which point the conditioned medium was harvested and assayed for PAI-1 antigen levels. As shown in Fig. 4, Ang II induced a dose-dependent increase in PAI-1, maximally inducing an approximate threefold increase in PAI-1 protein. The dose–response relationship between Ang II and PAI-1 production was highly statistically significant ($P < 0.001$).

Effects of Ang II on the expression of PAI-1 mRNA. In these experiments, confluent cultures of BAECs were incubated for 6 h in the presence of various concentrations of Ang II. At the end of the experimental period, the cells were washed and solubilized, and total RNA was extracted as described. A representative autoradiogram derived from a Northern blot is shown in Fig. 5 *A*. Low but detectable levels of PAI-1 mRNA were present in the control cells not exposed to Ang II. In comparison, cells treated with Ang II exhibited a marked dose-dependent increase in PAI-1 message. In these experiments, the PAI-1 probe consistently hybridized with a single species of RNA of ~ 3.3 kb. Levels of t-PA mRNA were barely detectable and were unchanged by treatment of cells with Ang II (data not shown). The dose dependence of PAI-1 mRNA expression was further quantified via performance of a slot blot analysis of triplicate samples of total mRNA isolated from BAECs exposed to similar concentrations of Ang II (Fig. 5 *B*). In these experiments, 10 μg of total RNA was applied to each slot, with each slot containing RNA from separate dishes of BAECs. The results of these experiments are shown in Fig. 5 *B*. Densitometric analysis of this autoradiographic data indicated that a twofold increase in PAI-1 mRNA was present in cells treated with 0.1 nM Ang II ($P < 0.05$), and under maximal conditions, PAI-1 mRNA levels increased by nearly fourfold versus levels in control cells ($P < 0.01$). Similar experiments have been performed using Ang I instead of Ang II in order to exclude the possibility

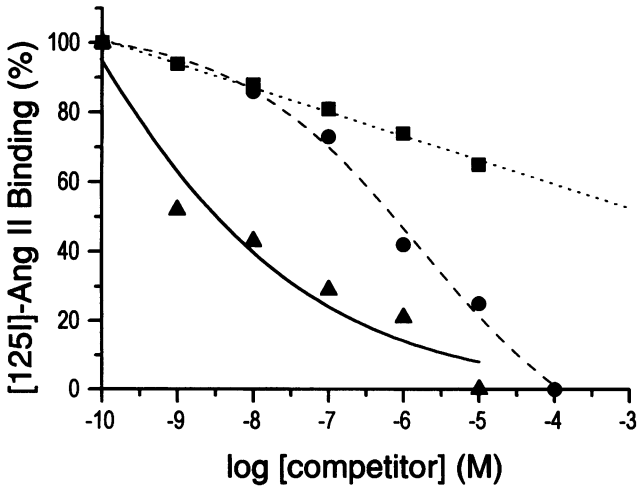

Figure 3. Competitive displacement studies. Dose-dependent inhibition of ^{125}I–Ang II binding to BAECs by unlabeled Ang II (*closed triangles*), Dup 753 (*closed circles*), and [Sar^1, Ile^8]-Ang II (*closed squares*). ^{125}I–Ang II (0.2 nM) was incubated with BAECs with increasing concentrations of unlabeled competitor for 60 min at room temperature. Measurements were performed in duplicate.

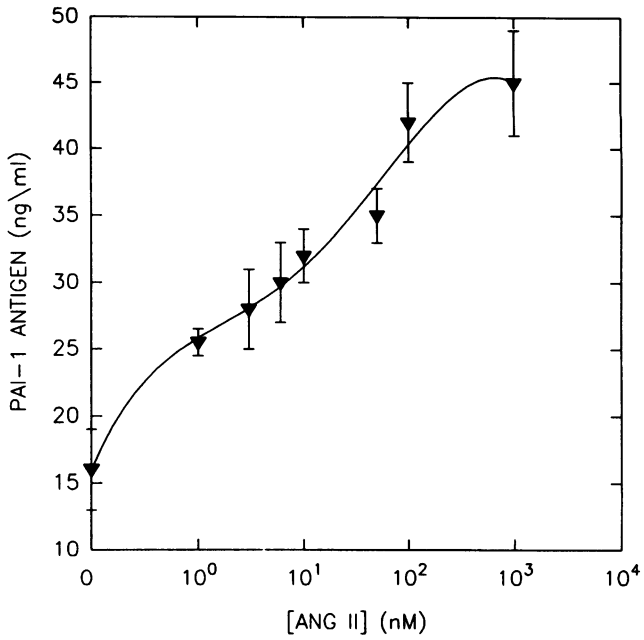

Figure 4. Effect of Ang II on PAI-1 production by cultured endothelial cells. Confluent cultures of BAECs were washed and incubated in serum-free DME for 18 h in the presence of Ang II at the indicated concentrations. PAI-1 antigen levels in the conditioned media were measured using a specific ELISA. Experiments were performed in triplicate and mean ±SD are shown.

that the effect of Ang II on PAI-1 expression is due to other contaminating factors present in the peptide preparation. Ang I (20 nM) induced PAI-1 mRNA levels to nearly the same extent as Ang II, but this effect was inhibited when the experiment was performed in the presence of captopril (1.0 μM) (data not shown).

To exclude the possibility that the effect of Ang II in inducing PAI-1 mRNA expression was species specific, similar experiments were performed using HUVECs (P2). These experiments were identical to those performed using BAECs except that the cells were not serum starved during the preinduction period, and the experiment was performed in the presence of 5% FBS. The results of a representative experiment are shown in Fig. 6 and indicate that exposure to Ang II increases PAI-1 mRNA levels in HUVECs in a dose-dependent manner. Both PAI-1 mRNA species were identifiable in the experiments performed using HUVECs.

Effects of Ang II on the accumulation of PAI-1 mRNA as a function of time. In these experiments, cells were placed in serum-free media and exposed to 20 nM Ang II at time 0. Cells were lysed and total RNA was harvested after the indicated incubation periods. Northern blotting of total RNA revealed induction of a single species of PAI-1 mRNA (~ 3.3 kb). A representative autoradiogram is shown in Fig. 7 *A* and exhibits an increase in PAI-1 mRNA as a function of time after exposure of BAECs to Ang II. The autoradiograms were scanned by densitometry, and the values were normalized for RNA loading at each time point (Fig. 7 *B*). To control for variability in gel loading, relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide, photographed, and quantified via reflectance densitometry. The results of this analysis indicate that the PAI-1 mRNA increased rapidly after exposure to Ang II, peaking ~ 8 h after addition of Ang II.

Effect of competitive receptor antagonists on PAI-1 induction by Ang II in BAECs. Confluent cultures of BAECs were incubated for 6 h with Ang II (20 nM) in the absence or presence of a 50-fold molar excess of high affinity ligands for the AT_1 and AT_2 receptors. Antagonists were added 30 min before the addition of Ang II. Total RNA was extracted and analyzed by Northern blotting as described. As seen in Fig. 8, neither [Sar^1,Ile^8]-Ang II (lane *3*) nor Dup 753 (lane *4*) was effective in preventing the induction of PAI-1 by Ang II.

Discussion

These studies demonstrate that Ang II binds to BAECs and induces an increase in PAI-1 secretion and expression of PAI-1 mRNA. This effect of Ang II is both dose and time dependent and is specific for the expression of PAI-1 mRNA that is ~ 3.3 kb in size. This is the first demonstration of this effect of Ang II on endothelial cells.

Although ^{125}I–Ang II appears to bind saturably and specifically to BAECs, the failure of Dup 753 and [Sar^1, Ile^8]-Ang II to displace it effectively indicates that the endothelial angiotensin receptor does not meet strict pharmacologic criteria as either an AT_1 or AT_2 receptor subtype. This confirms and extends previous observations that endothelial cells bind a variety of Ang peptides with high affinity, including Ang III (Ang II (2–8)) and Ang IV (Ang II (3–8)) (27, 42). The lack of potency of [Sar^1, Ile^8]-Ang II as a competitive antagonist in these studies is unexpected, but suggests that one or both of the terminal residues of the peptide play an important role in the interaction of Ang II with endothelial cells. Additional studies will be required in order to clarify this observation. The previously reported failure of Ang II to alter cytosolic calcium levels in cultured endothelium (43) also supports the contention that the endothelial angiotensin receptor is not an AT_1 subtype, since binding of Ang II to the AT_1 is generally associated with the mobilization of intracellular calcium (20). In recent studies, we have failed to identify the presence of AT_1 receptor mRNA in Northern blots of total RNA from BAECs using a cDNA

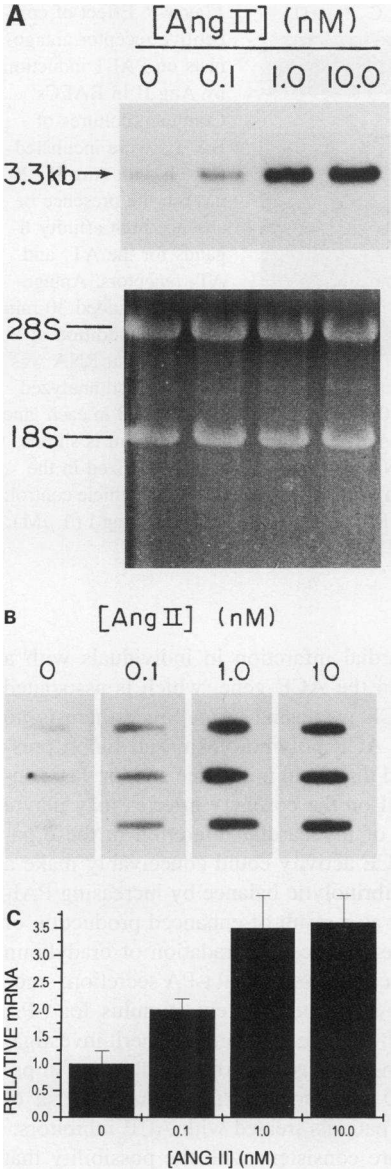

Figure 5. Ang II induction of PAI-1 mRNA in cultured endothelial cells. (*A*) Northern blot analysis showing relative amounts of PAI-1 transcript from BAECs treated with Ang II. Cells were incubated for 6 h in fresh serum-free DME in the presence of the indicated concentrations of Ang II. Total RNA was extracted and analyzed by Northern blotting as described. A representative autoradiogram is shown, with relative amounts of RNA loaded in each lane visualized in the lower panels by staining with ethidium bromide. (*B*) Slot blot analysis. In similar experiments, total cellular RNA was extracted and applied to membranes using a slot blot apparatus. A total of 10 μg of total RNA was applied to each slot under vacuum, with each slot receiving RNA from separate dishes of BAECs. (*C*) Graphic representation of data from the slot blot analysis previously described. Autoradiographic data was quantified using transmission densitometry. Experiments were performed in triplicate and means±SD are shown.

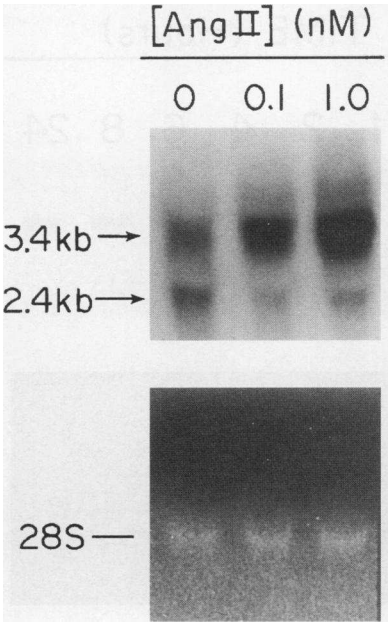

Figure 6. Induction of PAI-1 mRNA expression in HUVECs. Northern blot analysis showing relative amounts of PAI-1 transcript from HUVECs treated with Ang II. Cells were incubated for 6 h in fresh DME supplemented with 5% FBS alone or in the presence of the indicated concentrations of Ang II. Total RNA was extracted and analyzed by Northern blotting as described. A representative autoradiogram is shown, with relative amounts of RNA loaded in each lane visualized in the lower panels by staining with ethidium bromide.

probe (22) against the bovine AT_1 (data not shown). Furthermore, given the relatively low potency of Dup 753 and [Sar^1, Ile^8]-Ang II in blocking the binding of ^{125}I–Ang II to BAECs, it is not particularly surprising that neither agent blunts the induction of PAI-1 mRNA in cells exposed to Ang II.

The effect of Ang II in inducing PAI-1 mRNA expression is not unique to BAECs. Cells from three different species of mammals have now been shown to exhibit this response, including HUVECs in this study and the previous studies of Olson et al. describing a similar effect in cultured murine astrocytes (30). There are slight differences in the pattern of mRNA expressed by the BAECs and HUVECs: only a single RNA species is seen in the Northern blots from BAECs, whereas two species are seen in the experiments with HUVECs. In previous studies dealing with the regulation of the expression of PAI-1 mRNA, the presence of two PAI-1 transcripts is often reported, with the larger species containing additional 3′ untranslated sequence that is not present in the smaller transcript (14). The present findings may reflect the fact that the probe used in these studies was human in origin and does not hybridize well with the smaller RNA species from BAECs. This factor may have been compounded by the relatively stringent conditions that were used in the Northern blot experiments. Additionally, the PAI-1 cDNA probe used in these experiments includes a significant portion of sequence from the 3′ untranslated region of PAI-1—sequence that is present to a much greater extent in the larger (i.e., 3.3-kb) polyadenylated PAI-1 transcript (14).

Although there was a clear dose–response relationship between Ang II and PAI-1 secretion in these studies using BAECs, the EC_{50} for this response was at least an order of magnitude higher than the K_d for the interaction. This finding may be explained as merely a result of the conditions under which the experiments were performed (and therefore artifactual), or it may be indicative of the fact that the transcriptional and translational/secretory mechanisms are independently controlled. Since we have previously shown that physiological concentrations of Ang II can result in a rapid increase in plasma PAI-1 levels (17), this suggests that the former explanation may be correct. It is also possible that some of the observed effect of Ang II on PAI-1 secretion could be due to a contaminating effect of endotoxin. The Ang II preparations used in these studies had a LPS content of $\leq$ 1.25 pg per μg of peptide, which corresponds to a LPS concentration of $\sim$ 1 pg/ml in the presence of an Ang II concentration of 1.0 μM. Although this level of LPS has previously been shown to have little or no effect on PAI-1 expression (14), some additive effect of endotoxin at the highest Ang II concentrations cannot be entirely excluded. Such an effect may have in fact contributed to the biphasic contour of the dose–response curve shown in Fig. 4.

A relationship between Ang II and endothelial cell production of PAI-1 is particularly interesting when considered in light of two large, recent clinical studies independently demonstrating that the administration of angiotensin converting enzyme (ACE) inhibitors to patients with left ventricular dysfunction reduces the incidence of recurrent myocardial infarction and unstable angina pectoris by $>$ 20% (44, 45). The mechanism of this newly recognized anti-ischemic effect of ACE inhibitors is unknown, but given previously published data correlating elevated PAI-1 levels with an increased risk of reinfarction in survivors of myocardial infarction (6), we speculate that ACE

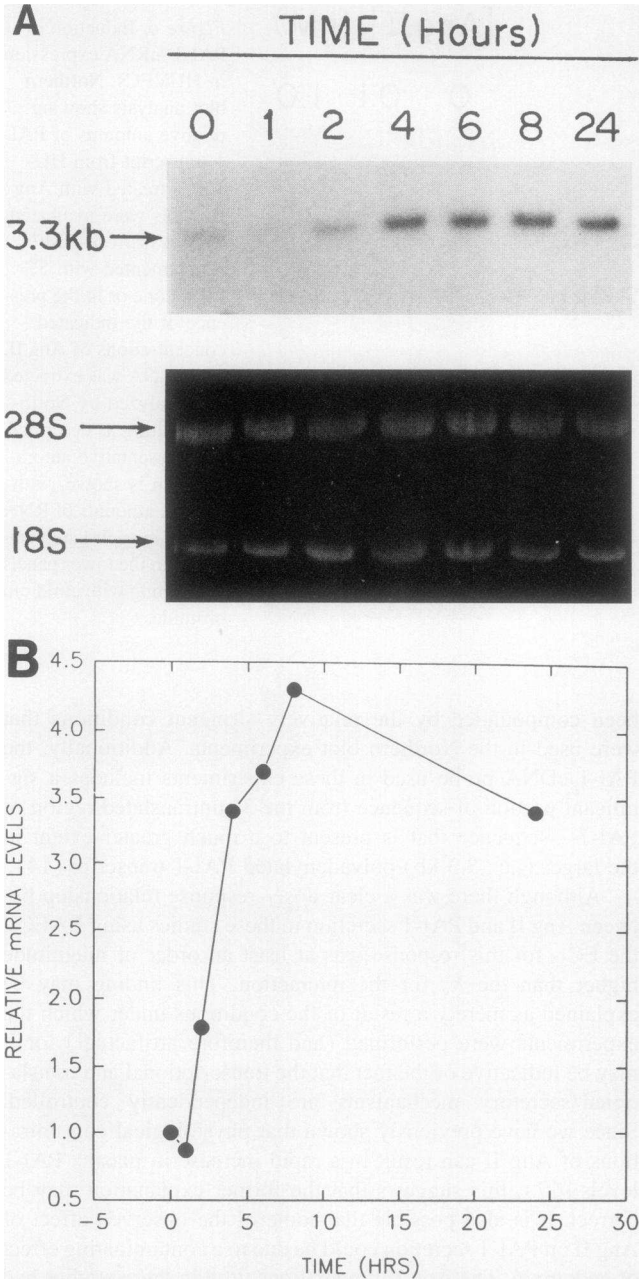

Figure 7. Time course of induction of PAI-1 mRNA by Ang II. (*A*) Confluent cultures of BAECs were incubated with Ang II (20 nM) in serum-free DME. Total cytoplasmic RNA was extracted at the indicated times and analyzed by Northern blotting. A representative autoradiogram is shown. (*B*) Graphical representation of the time course of PAI-1 mRNA expression. The results corresponding to the 3.3-kb PAI-1 mRNA were quantified by transmission densitometry. To control for variability in gel loading, relative amounts of RNA loaded in each lane were visualized by staining with ethidium bromide, photographed, and quantified via reflectance densitometry. Normalized data are plotted in the graph.

inhibitors, which lower plasma Ang II levels, promote a decrease in PAI-1 production and a net improvement in vascular fibrinolytic balance. This hypothesis is supported by a recent study demonstrating that the administration of an ACE inhibitor to patients does in fact lower plasma PAI-1 levels (46). Induction of PAI-1 production by Ang II may also contribute to the

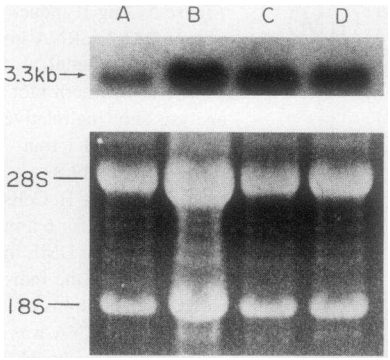

Figure 8. Effect of competitive receptor antagonists on PAI-1 induction by Ang II in BAECs. Confluent cultures of BAECs were incubated for 6 h with Ang II (20 nM) in the presence or absence high affinity ligands for the AT_1 and AT_2 receptors. Antagonists were added 30 min before the addition of Ang II. Total RNA was extracted and analyzed by Northern blotting, with 10 μg of total RNA was loaded in each lane unless otherwise indicated. A representative autoradiogram is shown, with relative amounts of RNA loaded in each lane visualized in the lower panels by staining with ethidium bromide. Lane *1*, vehicle control; lane *2*, Ang II alone (20 μg RNA); lane *3*, [Sar^1, Ile^8]-Ang I (1 μM); and lane *4*, Dup 753 (1 μM).

increased risk of myocardial infarction in individuals with a deletion polymorphism in the ACE gene, which is associated with higher levels of ACE in plasma (47). Since there is no correlation between this ACE polymorphism and blood pressure, it has been proposed that focal mitogenic and/or vasoconstrictive effects of Ang II on the coronary arteries may play a role in the pathogenesis of myocardial infarction in these patients (47). Increased ACE activity could conceivably make a significant impact upon fibrinolytic balance by increasing PAI-1 synthesis and secretion as a result of enhanced production of Ang II. Alternatively, the enhanced degradation of bradykinin by ACE may lead to decreased endothelial t-PA secretion, since bradykinin has been shown to be a potent stimulus for t-PA release in vivo (48). Both of these mechanisms merit investigation for contributing to the increased cardiovascular risk in patients with the ACE/DD genotype and the decreased risk of myocardial infarction in patients treated with ACE inhibitors.

The present results are consistent with the possibility that elevated levels of Ang II promote the increased synthesis of PAI-1 by the endothelium, resulting in the attenuation of fibrinolytic activity in plasma or at specific sites within the vasculature. This new information complements previous data demonstrating that the infusion of physiological concentrations of Ang II promotes increased plasma PAI-1 levels in humans (17). Taken together, these data suggest that the apparent clinical beneficial effective ACE inhibitors in reducing the incidence of myocardial infarction in selected populations (44, 45) may be mediated, at least in part, by an effect on fibrinolytic balance as well. Additional studies of the role of the renin–angiotensin system in the regulation of fibrinolytic activity will be required to define more completely the significance of this interaction.

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