Angiotsin II and Its Metabolites Stimulate PAI-1 Protein Release From Human Adipocytes in Primary Culture

Thomas Skurk, Yu-Mi Lee, Hans Hauner

Abstract—Plasminogen activator inhibitor (PAI)-1 is the main inhibitor of the fibrinolytic system and was recently shown to be produced by adipose cells. Obesity is associated with an increased production and release of PAI-1 protein. The aim of this study was to investigate the role of angiotensin (Ang) II and its degradation products for PAI-1 release from human adipose cells. For this purpose, we used the model of in vitro differentiated human adipocytes in primary culture. Exposure of human adipocytes to Ang II resulted in a dose- and time-dependent stimulation of PAI-1 release into the culture medium. The maximum effect of Ang II was found at a concentration of 10⁻⁵ mol/L for 48 hours, increasing PAI-1 release by 276±53% compared with control cultures (P<0.05). This stimulation was preceded by an increase in specific PAI-1 mRNA copies by 65±12% (P<0.05), with a maximum after 6 hours. Incubation of adipocytes with 10⁻⁵ mol/L Ang III and Ang IV, respectively, also resulted in a stimulation of PAI-1 release into the medium by 195±60% (P<0.05) and 142±24% (P<0.05), respectively, compared with control cultures. Addition of the angiotensin-receptor subtype 1 (AT₁) blocker candesartan abolished the stimulatory action of Ang II and its metabolites, indicating that this effect is mediated by AT₁. Addition of the AT₂ blocker alone to unstimulated cultures reduced PAI-1 release by 41±25% (P<0.05), suggesting that endogenous Ang II synthesis contributes to PAI-1 secretion from adipose tissue in an autocrine/paracrine manner. In conclusion, Ang II and its metabolites promote PAI-1 production and release by human fat cells and may contribute to the impairment of the fibrinolytic system typical for obesity. AT₁ receptor blockade reduces basal and abolishes Ang II–stimulated PAI-1 release from human adipocytes. (Hypertension. 2001; 37:1336-1340.)

Key Words: plasminogen ■ adipose ■ obesity ■ angiotensin ■ receptors, angiotensin

Plasminogen activator inhibitor (PAI)-1 is the main inhibitor of the fibrinolytic system. Elevated PAI-1 concentrations were found to be associated with an increased occurrence of coronary heart disease, deep-vein thrombosis, and thrombotic events in malignancies.¹,² It was reported recently that adipocyte precursor cells and mature fat cells are an important source of PAI-1.³–⁷ These and other studies also indicated that PAI-1 levels are positively correlated with the visceral fat mass. It is well known that human adipose expression and release of PAI-1 are subject to complex hormonal regulation.⁴⁻⁷⁹

Studies in other cell types originally suggested that angiotensin (Ang) II is a positive regulator of PAI-1 synthesis. In cultured endothelial cells as well as in smooth muscle cells, Ang II was reported to stimulate PAI-1 release.¹⁰ Moreover, in a recent clinical study in healthy volunteers, infusion of Ang II resulted in a significant increase of PAI-1 antigen in the circulation.¹¹ Recent studies have also established that adipose tissue expresses angiotensigen and all other components of the renin-angiotensin system (RAS) required to produce Ang II.¹² It is therefore tempting to speculate that local Ang II in addition to the circulating Ang II from other sources may be involved in the regulation of PAI-1 production in human adipose tissue. To test this hypothesis, we studied the effect of Ang II and its degradation products Ang III and Ang IV on PAI-1 synthesis and release from human adipocytes in culture. In addition, we used 2 specific AT₁ receptor blockers to examine the role of the angiotensin-receptor type 1 (AT₁) in this context.

Methods

Materials

Human insulin and cortisol were kindly donated by Hoechst; candesartan was generously provided by AstraZeneca and valsartan by Novartis. Collagenase CLS type 1 was obtained from Worthington. Ang II, III, and IV were purchased from Sigma. Culture media, superscript RT, and deoxy-nucleotides were obtained from Gibco/BRL. For sequencing, we used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer. The Light-Cycler DNA Master SYBR Green I kit was purchased from Roche.

Subjects

Subcutaneous adipose tissue samples were obtained from young normal-weight women (body mass index <26 kg/m²; age range, 18...
to 45 years) undergoing elective mammary reduction. All subjects were white and did not have acute infection, malignancies, or any other consuming disease. Informed consent was obtained from all subjects. Tissue collection was approved by the ethics committee of Heinrich-Heine-Universität Düsseldorf.

Culture of Human Preadipocytes
Adipocyte precursor cells were isolated and cultured as described previously.13 Briefly, human adipose tissue samples were cut into small pieces and digested in PBS buffer (pH 7.4) containing 600 U collagenase/g wet tissue (Biochrom; specific activity, 172 U/mg). After disaggregation, cells were passed through a 150-μm nylon mesh to remove undigested tissue. The isolated sedimented cell fraction was incubated with an erythrocyte lysing buffer (pH 7.3) for 10 minutes. After centrifuging (200g for 10 minutes), the pellet was resuspended in a small volume of DMEM/F-12 medium supplemented with 10% FCS and 50 μg/mL gentamycin (Life Technologies) and filtered through a 70-μm nylon mesh. Stromal cells were seeded at a density of 30 000 cells/cm² and maintained in an incubator at 37°C, 5% CO₂ for 20 hours until cells were attached. To induce adipose differentiation, cells were refeed with a serum-free medium consisting of DMEM/F-12 medium supplemented with 10⁻⁹ mol/L cortisol, 10⁻⁶ mol/L triiodothyronine, 6.6×10⁻⁷ mol/L insulin, and, for the first 3 days, 1 μg/mL troglitazone and 0.5 mmol/L isobutyl-methylxanthine. The medium was changed every other day. Experiments were performed on day 16, when most cells had acquired the adipocyte phenotype. For this purpose, Ang II and its metabolites were freshly diluted in PBS to obtain a stock solution and added to the incubation medium on the day of the experiment to achieve the final concentrations indicated.

Assessment of Differentiation
Differentiation was assessed by measuring the activity of glycerol-3-phosphate dehydrogenase (GPDH), a lipogenic marker enzyme, with an established procedure.14 Cells were washed with cold PBS (pH 7.4) scraped from the well and collected in 400-μL harvesting buffer (50 mmol/L Tris) supplemented with 1 mmol/L EDTA and 1 mmol/L mercaptoethanol (pH 7.4). After homogenization by sonication, samples were stored at −20°C until measurement. Activity of GPDH (EC 1.1.1.8) was expressed in milliunits per milligram of cellular protein, with 1 mU being equal to the oxidation of 1 mmol/L NADH per minute. In addition, the rate of differentiation was estimated by microscopically assessing the percentage of differentiated cells.

Determination of Protein Content
The protein content of the cultures was measured with a commercially available test kit, based on bicinchoninic acid for the colorimetric detection and quantification of total protein.

RNA Isolation, Probes, and Amplification
Total RNA was isolated by the Qiagen RNeasy isolation kit, including DNA digestion. The total amount of RNA was determined spectrophotometrically at a wavelength of 260 nm. Purity was assessed by the quotient 260/280 nm and integrity was confirmed by denaturing agarose gel analysis. For reverse transcription, 1 μg RNA was transcribed with Omniscript RT; the amplification was then performed with Light-Cycler technology (Roche Diagnostics). A polymerase chain reaction (PCR) product of the PAI-1 mRNA was cloned into a T-easy vector (Promega). The standard curve was established with copy numbers ranging from 200 to 20,000. The conditions for each run were 1 second at 95°C for denaturation, 5 seconds at 65°C for annealing, and 12 seconds at 72°C for elongation. The amount of amplification products was measured after each cycle by SYBR-green I (Eurogene) fluorescence at 530 nm. The efficacy of the PCR was >1.9 and the error <0.05. A melting curve was performed after each run to determine specific amplification ranging from 45°C to 98°C. The specific melting temperature for the PAI-1 PCR product was 85.4°C. The primers used were 5'-GGT TTT CAG CAG GTG GCG C and 3'-GTT CAG GCC CAG CCT CAG GAA GA A.

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Figure 1. Effect of adipose differentiation and addition of Ang II on PAI-1 protein release into culture medium from human preadipose and adipose cells in primary culture after 24-hour incubation period. Ang II was added on day 16, when cells had acquired adipocyte phenotype. Results represent mean±SEM of 4 independent experiments in triplicate (undiff indicates undifferentiated; diff, differentiated). *P<0.05.

GAA CCG of a highly purified grade amplifying a 299-bp product. The sequence of the transcript obtained was confirmed by use of the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the instructions of the manufacturer. As an internal standard to normalize for the relative abundance of PAI-1 transcripts, the ubiquitously expressed transcription factor Sp1 was used under the same PCR conditions as above. The primer sequences for Sp1 were 5'-GGT GAG AGT GCC TCA CAG CCT GTC and 3'-GGT CAG AGC ATC AGA CCC CTC. The coefficient of variation was ~6% when a given cDNA was measured several times with the same standard curve.

Measurement of PAI-1 Protein
For quantification of PAI-1 protein in the culture medium, we used an enzyme-linked immunosorbent assay, which measures both free and complexed human PAI-1 antigen (Technoclone). The correlation coefficient for the standard curve was >0.99. Samples were diluted depending on the state of differentiation. The interassay and intra-assay coefficient of variation was <10% and 5%, respectively.

Statistical Analysis
Statistical analysis was performed with the Student’s t test for paired data. Values are expressed as mean±SEM. Differences were considered significant at a value of P<0.05.

Effect of Ang II on PAI-1 Release From Human Adipose Cells
Under adipogenic culture conditions, on average >50% of the stromal cells developed the adipocyte phenotype, resulting in GPDH activities >600 mU/mg protein (data not shown). Undifferentiated control cells cultured in the same serum-free medium but without troglitazone and isobutylmethylxanthine were found to produce little PAI-1 on day 16 compared with fully differentiated adipocytes (12±6% of completely differentiated cultures), indicating that differentiated adipocytes are the main source of PAI-1 protein (Figure 1). When Ang II was added to fully differentiated human adipocytes on day 16, PAI-1 protein release into the culture medium was stimulated in a dose-dependent manner. The maximum response to Ang II was observed at a concentration of 10⁻⁶ mol/L. A complete dose response is shown in Figure 1 (undiff indicates undifferentiated; diff, differentiated). *P<0.05.
of $10^{-5}$ mol/L (Figure 1). Compared with control cultures with a PAI-1 concentration of $28\pm4$ ng/mL medium, a 24-hour incubation with $10^{-5}$ mol/L Ang II induced an increase in PAI-1 release to $50\pm6$ ng/mL medium ($P<0.05$). Figure 2 shows the time course of the effect of $10^{-5}$ mol/L Ang II on PAI-1 release. Peak values were reached after an incubation time of 48 hours. To address the question of whether the stimulatory effect of Ang II is associated with changes at the transcriptional level, we also measured steady-state mRNA levels of PAI-1 in the absence and presence of Ang II. As presented in Figure 3, $10^{-5}$ mol/L Ang II induced a time-dependent increase in PAI-1 mRNA, with a maximum peak after 6 hours, corresponding to an increase by $65\pm12\%$ ($P<0.05$).

**PAI-1 Release From Human Adipose Cells Exposed to Ang III and Ang IV**

Because distinct actions of the two Ang II–dependent degradation products on fibrinolysis are well known from other organs,\(^5\) we also studied the effect of Ang III and Ang IV on PAI-1 secretion from human adipose cells. At a concentration of $10^{-5}$ mol/L, Ang III stimulated PAI-1 release by $195\pm60\%$ after a 48-hour incubation period compared with control cells ($P<0.05$) (Figure 4). In addition, $10^{-2}$ mol/L Ang IV induced a time-dependent release of PAI-1 protein, with a maximum and statistically significant elevation by $142\pm24\%$ ($P<0.05$) (Figure 4). At direct comparison, Ang II proved to be the most potent peptide in terms of PAI-1 release. Ang I had only a weak, not significant stimulatory effect on PAI-1 release, increasing PAI-1 protein concentration in the culture medium by $84\pm37\%$ compared with control cells (Figure 4).

**Effect of Selective AT\(_1\)-Receptor Blockers on PAI-1 Release From Human Adipose Cells**

To study the role of the AT\(_1\)-receptor subtype, experiments were performed with 2 selective blocking agents. Exposure of differentiated adipose cells to candesartan alone resulted in a concentration-dependent reduction of PAI-1 release into the culture medium, with a maximum effect at $10^{-4}$ mol/L. At a concentration of $10^{-4}$ mol/L, basal PAI-1 release from adipocytes was reduced by $41\pm25\%$ after a 24-hour incubation ($P<0.05$) (Figure 5). Valsartan, another selective AT\(_1\)-receptor blocker, was equally effective at a concentration of $10^{-4}$ mol/L, reducing basal PAI-1 secretion by $38\pm18\%$ ($P<0.05$). When $10^{-5}$ mol/L Ang II was added to the culture medium to stimulate PAI-1 production, $10^{-4}$ mol/L candesartan was found to completely abolish the Ang II–mediated increase in PAI-1 release to $89\pm42\%$ of control values ($P<0.01$), indicating that the stimulatory action of Ang II is exclusively mediated by AT\(_1\) (Figure 5).

In addition, we examined the question of whether candesartan is also able to block the stimulatory action of the two metabolites Ang III and Ang IV. The AT\(_1\)-receptor blocker was added 1 hour before the addition of the angiotensins at a concentration of $10^{-5}$ mol/L. After a 24-hour coincubation with either Ang III or Ang IV, both at a concentration of $10^{-5}$ mol/L, PAI levels were $78\pm31\%$ of control cultures in the case of Ang III and $71\pm28\%$ of controls in the case of Ang IV. Finally, we studied the effect of the ACE inhibitor
Among the many factors that may influence PAI-1 release from different tissues and potentially favor thromboembolic complications, Ang II has received increasing attention after studies have shown that it stimulates PAI-1 secretion from endothelial and juxtaglomerular cells. This finding was confirmed in a clinical study in which intravenous infusion of Ang II in healthy volunteers resulted in an increase of circulating PAI-1 protein.

It is noteworthy that human adipocytes express angiotensinogen and all other components of the RAS required for the local production of Ang II and that activation of the RAS causes PAI-1 elevation of plasma levels. Our results now clearly suggest that locally produced Ang II may be involved in the production of PAI-1 by adipocytes. This is particularly supported by those experiments in which exposure of adipocytes to AT1-receptor antagonists alone was followed by a significant reduction of PAI-1 release by ~40%.

The concentrations to elicit an increase in PAI-1 secretion were in the micromolar range, which is far above the nanomolar binding affinity of Ang II to its receptor. However, in other studies on the biological effects of Ang II, similar concentrations were required. For example, induction of cyclin D1 expression by Ang II in cultured human preadipocytes was also observed only in the micromolar range.

In vitro studies in rat endothelial cells originally established that Ang III and Ang IV are also able to stimulate PAI-1 release. In the present study, we also looked at the effect of the Ang II metabolites Ang III and Ang IV on PAI-1 secretion. There was a clear-cut stimulatory action of both metabolites on PAI-1 release from human adipocytes. Thus, our results confirm previous reports addressing this aspect in endothelial tissue and extend this observation to adipocytes as another important source of PAI-1 production.

An interesting question in this context was which Ang II receptor subtype is mediating this action. To date, only the presence of the AT1-receptor subtype has been clearly demonstrated in adipose tissue. In a recent study, Crandall et al were not able to show the presence of AT2 receptors in human adipose cells. At present, there is no study dealing with the other AT-receptor subtypes, particularly the AT4-receptor, which is thought to be ubiquitously distributed. However, it is important to note that incubation with candesartan prevented the stimulatory effect of Ang III and Ang IV, again suggesting that the biological actions of the two degradation products are also exerted by the AT1-receptor.

Clinical data also support the hypothesis of a physiological role of Ang II in fibrinolysis. In the HEART study, PAI-1 levels were significantly lower under administration of ramipril, which may also suppress Ang II generation in adipose tissue. In another study, acute administration of captopril reduced PAI-1 protein and activity in subjects after acute myocardial infarction, whereas long-term treatment with trandolapril in a similar group of patients with previous myocardial infarction did not. The latter finding would also fit with our results because captopril proved to be ineffective to reduce both basal and Ang II–induced PAI-1 release from human fat cells.

Conclusions
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**Discussion**

This study demonstrates for the first time that Ang II and its metabolites are able to stimulate PAI-1 expression and release in human adipocytes. With the use of specific antagonists to the AT1-receptor subtype, the data also strongly indicate that this stimulatory effect of Ang II is mediated by the AT1-receptor. In addition, AT1-receptor antagonists alone also reduced PAI-1 levels in the absence of Ang II, suggesting that endogenously produced Ang II contributes to the release of PAI-1, at least under in vitro conditions.

Figure 5. Effect of AT1-receptor antagonists valsartan and candesartan on basal and Ang II–stimulated PAI-1 release from in vitro differentiated human adipocytes. AT1-receptor antagonists were added at concentration of 10·5 mol/L 1 hour before 24-hour incubation with 10·5 mol/L Ang II. Data are given as mean±SD of 3 separate experiments. *P<0.05 compared with control cultures, ***P<0.001 compared with Ang II–stimulated cultures, respectively.

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**Figure 6.** Effect of ACE inhibitor captopril on basal and Ang II–stimulated PAI-1 release from in vitro differentiated human adipocytes. Captopril was added at final concentration of 10·4 mol/L 1 hour before 24-hour incubation with 10·5 mol/L Ang II. Data are given as mean±SD of 3 separate experiments.
potently increase PAI-1 production and release by human adipocytes, further supporting the concept that the RAS and the fibrinolytic system are closely linked. In addition, our data also show that this action of Ang II can be blocked by specific antagonists of the AT$_1$-receptor, indicating that the stimulation of PAI-1 release is mainly if not exclusively mediated by this receptor subtype. Our findings may have important clinical implications because they suggest that AT$_1$-receptor blockers are not only effective blood pressure-lowering compounds but may also have a beneficial effect on the fibrinolytic system. Further studies are required to examine this hypothesis in clinical settings.

Acknowledgments

We wish to thank Prof Dr Rolf-Rüdiger Olbrisch and his team from the Department of Plastic Surgery at the Florence Nightingale Hospital Düsseldorf-Kaiserswerth and Prof Dr Bernhard Husemann and his team from the Surgical Department of the Dominikus-Krankenhaus, Düsseldorf-Heerdt, for their support in obtaining adipose tissue samples.

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Hypertension. 2001;37:1336-1340
doi: 10.1161/01.HYP.37.5.1336

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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