Natriuretic Factors and Nitric Oxide Suppress Plasminogen Activator Inhibitor-1 Expression in Vascular Smooth Muscle Cells

Role of cGMP in the Regulation of the Plasminogen System

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Abstract—Increased expression of plasminogen activator inhibitor-1 (PAI-1) has been reported in atherosclerotic and balloon-injured vessels. Little is known regarding the factors and mechanisms that may negatively regulate PAI-1 expression. In this report, the effect of cGMP-coupled vasoactive hormones, including natriuretic factors and nitric oxide, on the regulation of PAI-1 expression in vascular smooth muscle cells was examined. Atrial natriuretic factor 1–28 (ANF) and C-type natriuretic factor-22 (CNP) reduced angiotensin II (Ang II)– and platelet-derived growth factor–stimulated PAI-1 mRNA expression in rat aortic smooth muscle cells by 50% to 70%, with corresponding reductions in PAI-1 protein release. Treatment of human aortic smooth muscle cells with CNP similarly inhibited both platelet-derived growth factor–induced PAI-1 mRNA expression and PAI-1 protein release by 50%. Dose-response studies revealed that the inhibitory effects of CNP and ANF on PAI-1 expression were concentration dependent, with IC50s of ≈ 1 nmol/L for both natriuretic peptides. Ang II–stimulated PAI-1 expression was also inhibited by the nitric oxide donor S-nitroso-N-acetylpenicillamine. The membrane-permeant cGMP analogue 8-Br-cGMP reduced Ang II–stimulated PAI-1 expression by 60%, and an inhibitor of soluble guanylyl cyclase (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) significantly impaired the inhibitory effects of S-nitroso-N-acetylpenicillamine on Ang II–stimulated PAI-1 expression. Studies of PAI-1 mRNA stability in cells treated with actinomycin D showed that ANF did not alter PAI-1 mRNA half-life, suggesting that natriuretic factors reduce PAI-1 transcription. These data show that natriuretic factors and nitric oxide, via a cGMP-dependent mechanism, inhibit PAI-1 synthesis in vascular smooth muscle cells. Thus, cGMP-coupled vasoactive hormones may play an important role in suppressing vascular PAI-1 expression. (Arterioscler Thromb Vasc Biol. 1998;18:1771-1779.)

Key Words: natriuretic factors ■ nitric oxide ■ plasminogen activator inhibitor ■ vascular smooth muscle cells ■ angiotensin II

The plasminogen system plays an integral role in the regulation of vascular physiology by regulating fibrinolysis and the proteolysis of extracellular matrix (ECM).1,2 Plasminogen activator inhibitor-1 (PAI-1), the principle inhibitor of the plasminogen system, irreversibly inactivates both tissue and urokinase plasminogen activators.3 Inhibition of plasminogen activation by PAI-1 impairs fibrinolysis and thereby promotes thrombosis.4,5 In addition, PAI-1 has been shown to regulate ECM turnover and vascular smooth muscle cell (VSMC) migration,6,7 2 key processes in vascular remodeling and atherogenesis.

Circulating PAI-1 levels are increased in a variety of pathophysiological conditions, including insulin resistance, obesity, non–insulin-dependent diabetes mellitus (NIDDM), and insulin-dependent diabetes mellitus with microalbuminuria.4–10 Cross-sectional studies have identified elevated plasma PAI-1 as a risk factor for myocardial infarction in several high-risk populations.11–13 In addition, increased levels of PAI-1 also occur within the vascular wall at sites of atherosclerotic lesions,14–16 neointimal formation of failing bypass vein grafts,17 and balloon-injured vessels.18,19 Upregulation of PAI-1 within diseased or injured vascular tissue may locally diminish plasminogen activation and contribute to the accumulation of fibrin and ECM within vascular lesions. Conversely, a local increase in plasminogen activation potential within the vascular wall, associated with a relative increase in tissue plasminogen activator and urokinase plasminogen activator expression compared with that of PAI-1, has been shown at sites of abdominal aortic aneurysms.20,21 Within these sites, high levels of plasmin may locally increase proteolysis of ECM and thereby physically weaken the vascular wall.
A number of growth factors, hormones, and cytokines, including platelet-derived growth factor (PDGF), angiotensin II (Ang II), transforming growth factor-β, and tumor necrosis factor-α, induce PAI-1 expression in vascular smooth muscle cells (VSMCs). However, although the processes that upregulate PAI-1 expression have received considerable attention, much less is known regarding the factors and mechanisms that may negatively regulate PAI-1 expression in vascular cells. In this report, we have examined the effect of 2 types of vasorelaxant compounds, natriuretic factors and nitric oxide, on the regulation of PAI-1 expression in VSMCs. In addition to regulating vascular tone, atrial natriuretic factor (ANF), C-type natriuretic peptide (CNP), and nitric oxide–generating compounds inhibit VSMC growth and migration in vitro and reduce neointimal formation after balloon catheter–induced vascular injury. Nitric oxide has also been shown to provide antithrombotic activity and reduce PAI-1 released from platelets. As such, exogenous local delivery of nitric oxide synthase and CNP has been proposed as a treatment to reduce restenosis. However, little is known regarding the roles of natriuretic factors and nitric oxide in the regulation of the plasminogen system. In this report, we show that ANF, CNP, and a nitric oxide donor, acting via a cGMP-dependent process, are potent inhibitors of PAI-1 expression in VSMCs. These data suggest that natriuretic factors and nitric oxide may represent a class of cGMP-coupled vasorelaxant hormones that suppress vascular PAI-1 expression.

**Methods**

**Cell Culture**

Rat aortic smooth muscle cells (RASMCs) were isolated from Sprague-Dawley rats, cultured in Dulbecco’s modified Eagle’s medium (DMEM), 100 mg/dL D-glucose (Gibco-BRL), and 10% FBS (Gibco BRL) as described previously and used between passages 8 and 15. Human aortic smooth muscle cells (HASMCs) were isolated from aortic medial explants as described in Reference 39, cultured in DMEM with 20% FBS, and used between passages 7 and 10. Confluent monolayers of cells were deprived of serum in DMEM containing 0.1% (wt/vol) BSA for 18 hours before stimulation. Cells were stimulated with Ang II (Sigma Chemical Co) or PDGF (Upstate Biotech) in the absence or presence of a 5-minute pretreatment with atrial natriuretic factor 1–28 (ANF), C-type natriuretic peptide-22 (CNP, Peninsula), or S-nitroso-N-acetylpenicillamine (SNAP, Sigma) unless indicated otherwise. The role of cGMP in the regulation of PAI-1 was determined in cells treated with 8-bromo-cGMP (Calbiochem), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and KT5823 (Biomol).

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated by using TRI reagent (Molecular Research Center). Fifteen micrograms of RNA was separated in a 1% (wt/vol) agarose gel containing 20 mmol/L MOPS, pH 7.0; 5 mmol/L sodium acetate; 1 mmol/L EDTA; 0.76 mg/mL ethidium bromide; and 0.67% formaldehyde. The RNA was transferred to Biotrans membranes (ICN Pharmaceuticals) and cross-linked by UV. A cDNA probe against rat PAI-1 was labeled by using the Multiprime DNA labeling system (Amersham Corp) and purified with a NICK column (Pharmacia LKB Biotechnology Inc). A human PAI-1 antisense oligonucleotide (Oncogene Science Inc) was end-labeled by using T4 polynucleotide kinase and [32 P]ATP and then purified with a NAP-5 column (Pharmacia LKB). Blots were hybridized in 50 mmol/L PIPES, 100 mmol/L NaCl, 5 mmol/L NaPO₄, 1 mmol/L EDTA, 0.1% salmon sperm DNA (Biostar), and 5% SDS at 65°C. The blots were then washed with 0.5× SSC and 5% SDS at 65°C. Levels of mRNA were visualized and quantified by PhosphorImage analysis (Molecular Dynamics Inc). RNA loading was normalized to acidic ribosomal phosphoprotein PO (36B4) by using a [32 P]ATP end-labeled oligonucleotide probe.

**Western Blot Analysis**

PAI-1 protein levels were measured as described previously. Conditioned media from RASMCs or HASMCs, treated with Ang II and PDGF in the absence or presence of ANF and CNP, was collected. Equal aliquots of conditioned media were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose (Novex). Western blotting was performed with anti-rat PAI-1 antibody and anti-human PAI-1 antibody (American Diagnos-
tica, Inc) followed by detection with enhanced chemiluminescence (ECL, Amersham). PAI-1 was visualized and quantified using PhosphorImage analysis and ImageQuant software (Molecular Dynamics). PAI-1 levels were normalized to total protein from cell lysates by using the Bradford method (Bio-Rad).

cGMP Immunoassay
Confluent 6-well dishes of serum-deprived RASMCs were stimulated with 100 nmol/L ANF, CNP, or 10 μmol/L SNAP in the presence of 0.5 mmol/L 1-methyl-3-isobutylxanthine for 15 minutes. Cells were then washed with ice-cold PBS containing 1-methyl-3-isobutylxanthine and lysed in HCl. Levels of cGMP were measured by a cGMP enzyme immunoassay kit (Biomol).

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

Results
Effect of Natriuretic Peptides on PAI-1 mRNA Expression in RASMCs and HASMCs
The effects of both ANF and CNP on the regulation of PAI-1 expression in RASMCs and HASMCs were examined both alone and in the presence of the strong stimulators of PAI-1 expression, Ang II and PDGF. Control cells and cells
Ang II–stimulated PAI-1 expression was also examined. PAI-1 mRNA expression was compared in RASMCs treated with 100 nmol/L CNP at 30 minutes before, 5 minutes before, or 30 minutes after the start of Ang II stimulation (100 nmol/L, 3 hours). CNP reduced PAI-1 expression by 59% to 67% over this time interval (Figure 2, bottom left). These results revealed that pretreatment of cells with CNP was not required to inhibit Ang II–stimulated PAI-1 expression.

To determine whether natriuretic peptide could also regulate PAI-1 expression in HASMCs, the effect of CNP on PAI-1 expression in HASMCs was examined both alone and under PDGF-stimulated conditions. Northern blot analysis of human PAI-1 mRNA revealed 2 transcripts of 2.3 and 3.2 kb, as described previously. Stimulation of cells with PDGF (25 ng/mL) increased the level of both PAI-1 transcripts by 2- to 3-fold (Figure 3). Treatment of cells with 100 nmol/L CNP significantly inhibited PDGF-stimulated PAI-1 expression by 50% for the 3.2-kb transcript (P<0.01, ANOVA) and inhibited the 2.3-kb PAI-1 transcript to a similar extent. To our knowledge, this is the first description of a factor that can inhibit PAI-1 expression in human vascular cells.

**Effect of Natriuretic Peptides on the Release of PAI-1 Protein From RASMCs and HASMCs**

To determine whether the inhibitory effects of natriuretic peptides on PAI-1 mRNA resulted in changes in PAI-1 synthesis at the protein level, the effect of ANF and CNP on PAI-1 protein released from RASMCs and HASMCs was examined. RASMCs were pretreated with ANF (100 nmol/L) or CNP (100 nmol/L) for 5 minutes followed by the addition of Ang II (100 nmol/L). After an 18-hour incubation, PAI-1 protein levels in the conditioned media were measured by Western blot analysis. PAI-1 immunoblotting revealed a band at 52 kDa, the expected size of PAI-1.5 Treatment of cells with 100 nmol/L Ang II increased the level of PAI-1 protein in the conditioned medium by ~5-fold (Figure 4, left). Addition of either ANF or CNP significantly (P<0.05, ANOVA) decreased PAI-1 protein released from these cells by 80% and 60%, respectively, compared with cells stimulated with Ang II alone (Figure 4, left). The inhibitory effects of ANF and CNP on PAI-1 protein levels were not significantly different. In a similar series of experiments with HASMCs, the effects of CNP on PAI-1 protein levels from control and PDGF-stimulated cells were examined. Stimulation of HASMCs with PDGF increased PAI-1 protein in the conditioned medium by ~2-fold (Figure 4, right). Treatment of cells with 100 nmol/L CNP did not alter basal released PAI-1 levels but reduced PDGF-stimulated PAI-1 antigen by 50% (P<0.05, ANOVA). Thus, the inhibitory effects of ANF and CNP on PAI-1 mRNA expression in rat and human SMCs, described above, correspond with quantitatively similar decreases in PAI-1 protein released from these cells to the conditioned media.

**Effect of SNAP on PAI-1 Expression in RASMCs**

The effect of the nitric oxide donor SNAP on PAI-1 mRNA expression in RASMCs was also examined. Cells were pretreated with 10 μmol/L SNAP for 5 minutes followed by the addition of 100 nmol/L Ang II for 3 hours. Treatment of
cells with SNAP inhibited Ang II–stimulated PAI-1 expression in RASMCs by 60% (Figure 5). These results show that SNAP inhibits PAI-1 expression to an extent similar to that observed with natriuretic peptides. Treatment of cells simultaneously with CNP (100 nmol/L) and SNAP (10 μmol/L) was not more effective than CNP alone in inhibiting Ang II–stimulated PAI-1 mRNA (data not shown), suggesting that natriuretic factors and nitric oxide may share a common mechanistic pathway in the suppression of PAI-1.

Role of cGMP in the Regulation of PAI-1 Expression
ANF and CNP primarily signal via natriuretic peptide receptor/guanylyl cyclases A and B (NPR-A and NPR-B), respec-

![Figure 4. Effect of ANF and CNP on PAI-1 protein released from RASMCs and HASMCs. Left, RASMCs were pretreated with 100 nmol/L ANF or CNP for 5 minutes followed by the addition of Ang II (100 nmol/L) for 18 hours. Right, HASMCs were treated with CNP (100 nmol/L) for 5 minutes followed by an 18-hour incubation with PDGF (25 ng/mL). PAI-1 protein in the culture medium was determined by Western blot analysis using anti-rat PAI-1 and anti-human PAI-1 antibodies for left and right panels, respectively. Results were visualized by ECL, and representative Western blots are shown. PAI-1 protein levels were quantified using ImageQuant (Molecular Dynamics). Bar graph indicates results from 3 experiments performed in triplicate, and significant differences (P<0.05, ANOVA) are indicated as *. Con indicates control.

![Figure 5. Effect of SNAP on PAI-1 expression in RASMCs. RASMCs were pretreated with 10 μmol/L SNAP for 5 minutes followed by the addition of Ang II (100 nmol/L) for 3 hours. PAI-1 mRNA expression was determined by Northern blot analysis. Results were visualized and quantified by PhosphorImage analysis. Representative Northern blots of PAI-1 and 36B4 are shown. Bar graph represents PAI-1 mRNA levels normalized to 36B4 from 3 experiments performed in triplicate, and significant differences (P<0.05, ANOVA) are indicated as *. Con indicates control.

Con AngII SNAP SNAP/AngII

Con AngII SNAP SNAP/AngII

Con AngII SNAP SNAP/AngII

Con AngII SNAP SNAP/AngII

Con AngII SNAP SNAP/AngII
Nitric oxide activates a soluble guanylyl cyclase, which is the primary mediator of its actions in VSMCs. Because cGMP is a major second messenger for both natriuretic peptides and nitric oxide, we examined the role of cGMP in the regulation of PAI-1 expression. Intracellular cGMP was directly elevated by treatment of cells with the membrane-permeant cGMP analogue 8-Br-cGMP for 15 minutes followed by stimulation with 100 nmol/L Ang II for
PAI-1 expression by natriuretic factors and nitric oxide, this effect of cGMP does not appear to require cGPK activity.

Effect of ANF on PAI-1 mRNA Half-life
Recent studies have demonstrated that cGMP can reduce mRNA levels either by reducing transcription or by decreasing transcript stability. The effect of ANF on PAI-1 mRNA half-life was evaluated in RASMCs treated with actinomycin D (ActD), an inhibitor of transcription. Cells were stimulated for 1 hour with 100 nmol/L Ang II followed by the addition of ActD in the absence or presence of 100 nmol/L ANF. PAI-1 mRNA was measured over a time course of 2 hours, such that the total stimulation time for the cells with Ang II was 3 hours, as described in Figures 1 left and 2 left. This study demonstrated that PAI-1 mRNA levels decreased in a time-dependent manner after the addition of ActD, whereas Ang II–stimulated cells incubated without ActD continue to increase PAI-1 mRNA over this time course (Figure 7). There was no significant difference in the rate of PAI-1 mRNA decline in ActD-treated cells in the absence or presence of ANF, suggesting that ANF does not destabilize PAI-1 mRNA.

Discussion
Elevated PAI-1 levels have been strongly implicated as a risk factor for cardiovascular disease in NIDDM, insulin resistance, and obesity. Because impaired endothelium-dependent vascular relaxation occurs in the insulin resistance syndrome and NIDDM, we investigated the potential role of cGMP-coupled vasorelaxant hormones, including natriuretic factors and nitric oxide, in the regulation of PAI-1 expression in VSMCs. This study revealed that ANF and CNP and the nitric oxide donor SNAP were potent inhibitors of PAI-1 expression, at both the mRNA and protein levels. Dose-response studies demonstrated that similar low nanomolar concentrations of ANF and CNP were sufficient for the inhibition of PAI-1 expression, suggesting that physiological concentrations of these natriuretic factors may be sufficient to reduce vascular PAI-1 mRNA levels in vivo. The inhibition of PAI-1 expression by these natriuretic factors and SNAP was mimicked by the membrane-permeant cGMP analogue 8-Br-cGMP, and the inhibition of PAI-1 expression by SNAP was blocked by the soluble guanylyl cyclase inhibitor ODQ. These results provide strong evidence that the elevation of cGMP by natriuretic peptides and NO inhibits PAI-1 expression in VSMCs. To our knowledge, this is the first description of such a role for vasoactive hormones, and a general signaling pathway, in the inhibition PAI-1 expression.

Previous reports have shown that the vasoconstrictive peptide Ang II is a potent stimulator of PAI-1 expression in both vascular endothelial and SMCs, and that angiotensin-converting enzyme (ACE) inhibition reduces both vascular and plasma PAI-1 levels. These reports suggest that the renin-angiotensin system, via the actions of Ang II, can contribute to the expression of PAI-1. However, because ACE inhibitors also impair the breakdown of kinins, increased kinin-stimulated nitric oxide synthesis may contribute to the decreases in PAI-1 expression associated with ACE inhibition. Thus, it will be important to determine whether...
Ang II receptor antagonists are as effective as ACE inhibitors in reducing PAI-1 levels in vivo.

One remarkable feature of the inhibition of PAI-1 expression by ANF, CNP, nitric oxide, and 8-Br-cGMP was that the maximal inhibitory effectiveness of these agents was similar, ranging from 50% to 70% inhibition. This finding was consistently observed over a range of Ang II and PDGF concentrations (Figure 1, left and right) and over a time course of natriuretic treatment (Figure 2, bottom right). The finding that ANF did not alter PAI-1 mRNA half-life (Figure 7) suggests that natriuretic factors suppress PAI-1 mRNA levels by inhibiting transcription. This finding is consistent with other reports that have shown that ANF can inhibit transcription of its receptor (NPR-A) and type I cGMP–dependent protein kinase. Moreover, the autoregulation of NPR-A may provide a negative feedback on ANF’s inhibitory effect on PAI-1 under conditions of chronic ANF stimulation. The observation that natriuretic factors, SNAP, and 8-Br-cGMP did not completely inhibit PAI-1 expression may suggest either that these factors can block only a subset of the PDGF- and Ang II–signaling pathways that induce PAI-1 expression or that cGMP reduces PAI-1 transcription independently of these hormone-signaling pathways. Studies are currently underway to identify the specific mechanism(s) responsible for the inhibitory effects of cGMP on PAI-1 expression.

The finding that natriuretic factors and nitric oxide inhibit PAI-1 expression in VSMCs may have important implications related to the regulation of fibrinolysis and vascular disease. Exogenous delivery of nitric oxide, by administration of a nitric oxide donor, has been shown to reduce thrombus formation. The suppression of PAI-1 released by platelets and by VSMCs (current report) may contribute to this nitric oxide effect by promoting fibrinolysis. Consistent with this hypothesis, nitric oxide synthase inhibition has been reported to increase plasma PAI activity in environmentally stressed rats. Moreover, the suppressive effect of nitric oxide on PAI-1 may be diminished in certain pathophysiological states, such as obesity, insulin resistance, and NIDDM, which display both reduced endothelium-dependent vasodilation and elevated PAI-1 levels.

The plasminogen activator/inhibitor system has been shown to modulate the development of vascular lesions in injured and atherosclerotic vessels. Studies using gene knockout mice have shown that plasminogen deficiency impairs and PAI-1 deficiency enhances neointimal development after arterial injury. These changes in neointimal formation may reflect an important role of plasmin-mediated proteolysis of the ECM in releasing constraints that interfere with VSMC migration. In addition, PAI-1 can also directly impair VSMC migration by binding to vitronectin and thereby interfere with its interactions with the cellular vitronectin receptor αvβ3 that facilitates integrin-mediated cell migration. These studies suggest that PAI-1 may reduce the stage of neointimal development that is dependent on VSMC migration. However, although natriuretic factors and nitric oxide suppress PAI-1 expression, these vasoactive factors also directly inhibit VSMC migration. Therefore, it will be important to determine whether inhibition of PAI-1 expression by these cGMP-coupled hormones modulates their effects on cellular migration. In contrast, plasminogen deficiency accelerates lesion development in atherosclerosis-prone apolipoprotein E–deficient mice. Thus, plasmin appears to provide protective effects against atherosclerotic lesion formation that may be diminished by inhibitors of plasminogen activation, such as PAI-1. Inhibition of vascular PAI-1 expression by natriuretic factors and nitric oxide may promote fibrinolysis and turnover of the ECM, thereby reducing advanced stages of lesion formation.

In summary, we report that natriuretic factors, including CNP and ANF, and nitric oxide, acting via a cGMP-dependent mechanism, are potent inhibitors of PAI-1 expression in VSMCs. The suppression of PAI-1 expression by these vasorelaxation factors antagonizes the induction of PAI-1 by the vasopressive hormone Ang II, suggesting that the combined effects of vasopressor and vasorelaxant hormone action may influence the fibrinolytic balance.

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