The Serpin Protease-Nexin 1 Is Present in Rat Aortic Smooth Muscle Cells and Is Upregulated in L-NAME Hypertensive Rats

Marie-Christine Bouton, Benjamin Richard, Patrick Rossignol, Monique Philippe, Marie-Claude Guillin, Jean-Baptiste Michel, Martine Jandrot-Perrus

Objective—Protease-nexin 1 (PN-1) belongs to the serpin superfamily and behaves as a specific thrombin inhibitor in the pericellular environment. Little is known about PN-1 expression and its regulation in the vascular system. In this study, we examined the expression of functionally active PN-1 in vitro in rat aortic smooth muscle cells and in vivo in rat arterial media and its regulation in hypertensive rats.

Methods and Results—The vascular PN-1 formed specific covalent complexes with thrombin involving the catalytic site of the protease, and heparin increased the formation of these complexes. We also demonstrated PN-1 in rat arterial media by immunohistochemical staining. Moreover, we examined in vivo vascular expression of PN-1 in a model of chronic hypertension induced by long-term administration of Nω-nitro-L-arginine methyl ester (L-NAME). Marked increases in PN-1 mRNA (3-fold) and protein (2-fold) were observed after 2 months of hypertension. Increased expression of PN-1 in the vascular wall was associated with an increase in the formation of complexes between radiolabeled-thrombin and PN-1, indicating that PN-1 was functional.

Conclusions—PN-1 may thus participate in the mechanisms that regulate thrombin activity in the vessel wall. (Arterioscler Thromb Vasc Biol. 2003;23:142-147.)

Key Words: protease-nexin 1 □ smooth muscle cells □ aorta □ hypertension □ thrombin

Protease nexin 1 (PN-1) is a 43- to 45-kDa protease inhibitor that belongs to the serpin superfamily. PN-1 is secreted by a wide range of cultured cells, including glial cells and neurons, human foreskin fibroblasts, and human skeletal muscle myotubes, and is present in platelets but is barely detectable in plasma. In cell cultures, PN-1 rapidly inhibits its target proteases (trypsin, α-thrombin, plasminogen activators, and plasmin), forming SDS-stable equimolecular complexes that are rapidly internalized and degraded. Secreted PN-1 binds tightly to the extracellular matrix (ECM). Binding occurs mostly via the heparan-sulfates and regulates both PN-1 activity and specificity. PN-1 interaction with heparan sulfates accelerates thrombin inhibition, the protease becoming thus the preferred target protease. In addition, it has been reported that PN-1 also interacts with collagen IV, which decreases the rate of inhibition of urokinase and plasmin without affecting the rate of thrombin inhibition by PN-1. PN-1 is therefore considered to be a specific thrombin inhibitor in the pericellular environment.

Thrombin exerts several direct effects on vascular cells. The specific roles of thrombin in modulating the responses to vascular injury are relatively complex. Indeed, thrombin has been shown to elicit an inflammatory response, to induce smooth muscle cells (SMCs) to synthesize collagen that may contribute to ECM accumulation, and to stimulate SMC contraction and proliferation. Moreover, a growing body of evidence from a broad range of animal models indicates the importance of thrombin-promoted neointima formation in arterial lesions. Thus, thrombin probably plays a significant role in the vasculoproliferative response to injury. A critical question raised by these observations concerns the regulation of thrombin within the vessel wall. Although both thrombin and the plasma thrombin inhibitor antithrombin have been detected in human aortic intima, thrombin-antithrombin complexes have not been observed in atherosclerotic plaques, indicating that thrombin activity is not regulated by antithrombin within the atherosclerotic arteries. Colocalization of PAI-1, vitronectin, and thrombin in the neointima of human atherosclerotic arteries has been observed, but the existence of a mechanism of thrombin regulation by PAI-1/vitronectin complexes in the vessel wall remains to be demonstrated. PN-1 is clearly another potential candidate for thrombin inhibition in the vascular wall, but no direct evidence for its presence in the vessels has yet been provided. The aim of this study was to investigate whether PN-1 is produced by vascular smooth muscle cells. Our results show that PN-1 is
expressed both in vitro in rat vascular smooth muscle cells in culture and in vivo in rat arterial media. In addition, our data indicate that the vascular expression of PN-1 is upregulated in an in vivo rat model of chronic hypertension induced by long-term administration of the orally active nitric oxide synthase (NOS) inhibitor, $N^\omega$-nitro-$\omega$-arginine methyl ester (L-NAME).

### Methods

#### Purified Proteins

Human $\alpha$-thrombin (EC.3.4.21.5) was purified and iodinated as previously described. Recombinant rat PN-1, a generous gift from Pr D. Monard, (FMI, Basel, Switzerland) was produced in yeast as previously reported.

#### Cell Culture

Rat aortic smooth muscle cells (RASMCs) were isolated from 180 to 200 g male Wistar rats and cultured in DMEM (Life Technologies) with 10% FCS, as previously described, and cells were used for experimentation after 3 to 5 passages. RASMCs were identified by their characteristic hill and valley growth appearance and by immunostaining for SMC $\alpha$-actin. When RASMCs reached confluence, the culture medium was replaced with serum-free medium for 1 day before treatment with the different agonists.

#### Animals

Male Wistar rats (120 to 130 g, IFFA CREDO, Lyon, France) were divided into the following 3 groups: (1) a control group (n=14); (2) a group treated with L-NAME (n=19, 50 mg/kg per day in the drinking water, Sigma Chemical Co); and (3) a group treated with L-NAME supplemented with the angiotensin-converting enzyme (ACE) inhibitor zofenopril (n=14, 15 mg/kg per day in food intake, Firenze). The procedures used for the care and euthanasia of the animals were in accordance with the European Community Standards (Ministère de l’Agriculture, France; authorization No. 00577).

#### Reverse Transcription and Semi-quantitative Polymerase Chain Reaction

Total cellular RNA was isolated from RASMCs and aortic samples with the Trizol reagent (Invitrogen), according to the manufacturer’s directions. Double-stranded cDNAs were synthesized and amplified as previously described. The amplification was carried out for PN-1 with an initial 5 minutes of denaturation at 95°C, 29 cycles of 1 minute at 95°C, 1 minute at 50°C and 1 minute at 72°C, and then 7 minutes at 72°C. For the housekeeping gene GAPDH, the polymerase chain reaction (PCR) was performed for 27 cycles, and the annealing temperature was 56°C. The sense primer for PN-1 was 5’GACCAACAGTGTCCATTATCT3’ and the antisense primer was 5’GGCTGGTGCCTGGAAA GTT3’; the sense primer for GAPDH was 5’GTGAAGGCTGAGTCAAGC3’ and the antisense primer was 5’GGTGAAAGACGCC AGTGGACTC3’. PN-1 mRNA levels were normalized to GAPDH mRNA.

#### Western Blot Analysis

Proteins were extracted from aortic samples or RASMCs in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 3 mmol/L EDTA, 0.25% Tx-100) and centrifuged at 12 000 g at 4°C for 30 minutes. Protein concentrations were measured using a Bio-Rad protein assay. For immunoblotting of PN-1, the monoclonal 4B3 anti-PN-1 antibody (750 μg/mL, dilution 1:700, a kind gift from Pr D. Monard) was used, followed by peroxidase-conjugated anti-mouse IgG (dilution 1:8000) (Amersham) and the chemiluminescence system ECL (Amersham).

#### Detection of $^{125}$I-$\alpha$-Thrombin-PN-1 Complexes in Aortic Extracts

Complexes between PN-1 present in aortic extracts and $^{125}$I-$\alpha$-thrombin were formed by incubating 20 mmol/L $^{125}$I-$\alpha$-thrombin with 100 μg protein extracts derived from aortas at 37°C for 15 minutes in 20 mmol/L NaH$_2$PO$_4$, pH 7.4, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% polyethylene-glycol 6000. Some experiments were conducted with different $\alpha$-thrombin ligands: 10 IU/mL hirudin (Serbio), its sulfated C-terminal peptide 54 to 65 (27 μmol/L SH 54 to 65) (Bachem), or 150 IU/mL heparin (Choay). Other experiments were performed in the presence of the unlabelled $\alpha$-thrombin in up to 100-fold excess or in the presence of 20 mmol/L Pro-Phe-Arg-chloroethyl-ketone (PPACK) (Calbiochem). Samples were boiled for 5 minutes in the presence of 2% SDS. Radiolabeled bands were detected after separation of the proteins by 10% SDS-PAGE and autoradiographed.

#### Immunohistochemical Detection of PN-1

Immunohistochemical detection of PN-1 in frozen-fixed sections (5 μm) from rat aorta and in RASMCs was performed with the use of the PN-1 monoclonal antibody 4B3 (750 μg/mL, dilution 1:50), as previously described. For control staining, the first antibody was either omitted or replaced by an isotype-matched antibody directed against ACE.

#### Intracellular Calcium Mobilization

RASMCs were loaded for 1 hour at room temperature with 5 μmol/L Fluo3-AM and 0.02% Phloronic acid in DMEM and then washed with Tyrode solution. Confocal images were acquired using a Zeiss LSM-510 inverted confocal microscope with a LD Achromplan ×40 objective (Zeiss) (numeric aperture, 0.6). Fluo3-AM was excited by the 488-nm line of an argon laser, and fluorescence was measured at >505 nm. Zeiss confocal software Windows NT controlled the scanner module and performed images analysis.

#### Proliferation Assay

RASMCs were plated in a 96-well plate and grown to 80% confluence. Proliferation was measured using a colorimetric assay based on the measurement of BrDU incorporation during DNA synthesis (Roche), according to the manufacturer’s procedure.

#### Statistical Analysis

Data are expressed as mean±SEM. Statistical significance was estimated between groups by 1-way ANOVA followed by Bonferroni analysis. Differences were considered significant at $P<0.05$.

#### Results

#### PN-1 Expression In Vitro in RASMCs

As shown in Figure 1, PN-1 mRNA was detected by reverse transcriptase (RT)-PCR in cultured RASMCs at confluence, and Western blot analysis demonstrated the presence of the protein in the cell extracts. In these experiments (Figure 1), recombinant rat PN-1 appeared as a double band with a major band migrating at 41 kDa; the double-band character of the protein has been previously explained by a differential glycosylation pattern by yeast cells. In the RASM E extracts, PN-1 was detected as a single band of 45 kDa. A nonidentified additional band migrating at $\approx$110 kDa was observed.

The distribution of PN-1 antigen in RASMCs was analyzed by immunostaining with the monoclonal antibody anti-PN-1. PN-1 immunoreactivity in nonpermeabilized RASMCs was intense and uniform, indicating the presence of PN-1 at the
cell surface (see online Figure I, available at http://atvb.ahajournals.org).

**Regulation of α-Thrombin Effects on RASMCs by Exogenous PN-1**

To determine to what extent variations in the extracellular PN-1 concentration may regulate the response of RASMCs to thrombin, we measured the influence of recombinant PN-1 addition on thrombin-induced intracellular calcium mobilization and cell proliferation.

Thrombin acts on RASMCs via the activation of its G-protein–coupled receptor PAR-1, inducing an increase in 
\[ \text{Ca}^{2+} \]. This is one of the earliest events measurable in thrombin-stimulated cells. The effect of exogenous PN-1 on α-thrombin–induced calcium mobilization was examined in fluo3-AM–loaded cells. Addition of 2 nmol/L α-thrombin to RASMCs induced a transient and important increase in 
\[ \text{Ca}^{2+} \]. PN-1 dose-dependently blocked the thrombin-induced calcium signal (Figure 2A). When norepinephrine, another agonist that induces a calcium signal, was used, PN-1 had no effect on the signal, confirming the specific effect of PN-1 on thrombin (data not shown).

Thrombin is well-known to be a potent mitogen for RASMCs. As shown in Figure 2B, 10 nmol/L α-thrombin elicited a 2.5-fold increase in cell proliferation after 48 hours of stimulation compared with control (absence of added thrombin). In the presence of PN-1, thrombin-induced cell proliferation was inhibited in a dose-dependent manner.

**Presence of PN-1 in the Rat Aortic Wall**

The presence of the PN-1 transcript in aortas was observed by RT-PCR analysis (Figure 1). PN-1 protein was also detected in aortas by Western blotting as a single band of 45 kDa. In contrast to RASMCs, no additional high-molecular-mass band was observed. Similar results were observed with the samples of aorta devoid of adventitia and endothelium, indicating the presence of PN-1 in the media.

To determine whether the PN-1 present in the aortic wall was functional, we analyzed the covalent complexes formed during incubation of aortic homogenates with radiolabeled α-thrombin by SDS-PAGE followed by autoradiography. As shown in Figure 3A, a major 36.5-kDa band was present, which correlates with free, noncovalently bound thrombin. The 81-kDa band was identified as an equimolecular complex between \(^{125}\)I-α-thrombin and PN-1 present in the aortic extracts for the following reasons: (1) the migration rates of this band and the complex formed between labeled α-thrombin and recombinant rat PN-1 were similar; and (2) the presence of PN-1 in the 81-kDa complex was confirmed by Western blotting with the monoclonal antibody anti-PN-1 (Figure 3A). This result indicates that PN-1 associated with aortic extracts was functional and confirmed that the complexes were covalent, because they were resistant to dissociation in boiling SDS. An additional major 95-kDa complex represents the binding of \(^{125}\)I-α-thrombin to antithrombin originating from blood contamination present in the aortic extracts. Specificity of the thrombin linkage in the complexes formed with the aortic extracts was also investigated by adding simultaneously \(^{125}\)I-α-thrombin and a 100-fold molar excess of unlabelled α-thrombin; the formation of the complex was prevented, whereas the intensity of the band corresponding to unbound labeled α-thrombin was increased (Figure 3B). The effect of different thrombin inhibitors on complex formation
was also studied. PPACK is a direct active site inhibitor, whereas hirudin, a thrombin-specific inhibitor from the leech, binds both to the thrombin macromolecular recognition site called exosite 1 and to the thrombin catalytic site. PPACK and hirudin blocked the formation of the complex between $^{125}$I-$\alpha$-thrombin and PN-1 in the aortic homogenates. The last sample corresponds to $^{125}$I-$\alpha$-thrombin incubated with purified AT (+AT).

The localization of PN-1 in the aortic wall was determined by immunohistochemical staining on fixed cryosections of aortic tissue. The labeling revealed that PN-1 was present in the medial smooth muscle cells (Figure 4).

**Regulation of Arterial PN-1 Expression by Chronic $L$-NAME Administration**

Long-term inhibition of NOS is known to induce hypertension and perivascular fibrosis. Recent evidence also suggests that chronic blockade of NO production induces expression of a member of the serpin superfamily, PAI-1, in vascular tissues, both the upregulation of this PAI-1 expression and the structural changes being prevented by ACE inhibition. We hypothesized that PN-1, which belongs to the same superfamily of serine protease inhibitors, could also be regulated at the gene level by long-term NOS inhibition.

No significant differences in body weight (Table I, available online at http://atvb.ahajournals.org) were observed between the 3 groups of rats. As previously described, the systolic blood pressure increased compared with the control rats during the first 3 weeks of administration of $L$-NAME and remained constant during the following 5 weeks. In $L$-NAME plus ACE inhibitor–treated rats, systolic blood pressure was not different from control levels.

As previously reported by others, a significant increase in PAI-1 mRNA levels in the aorta was observed after 8 weeks of $L$-NAME treatment, and the increase was prevented by simultaneous administration of the ACE inhibitor zofenopril (not shown). We also observed a marked increase in PN-1 mRNA (3.5-fold) and protein (2-fold) levels (see Figure II, available online at http://atvb.ahajournals.org), which were prevented by zofenopril administration. Because the structural changes induced by chronic NOS inhibition mainly involve a biological response of the media, similar analyses were performed in the aorta devoid of its adventitia and endothelium. A 2-fold upregulation of PN-1 mRNA and protein levels was observed in the media extracts from $L$-NAME rats compared with control rats (see Figure II, available online at http://www.ahajournals.org).

**Effect of Angiotensin II and NO Donors on PN-1 Expression in RASMCs**

Because suppression of NOS activity is linked to the enhancement of blood pressure via the potentiation of angiotensin II (Ang II) signalization, we tested the ability of Ang II to modify PN-1 expression in RASMCs. RASMCs were incubated with 100 nmol/L Ang II. The relative PN-1 mRNA levels, expressed as a percentage of the control (mean±SD of triplicates from 1 representative experiment out of 3), were $101±2\%$, $100±19\%$, $104±3\%$, and $84±2\%$ after 3, 6, 18, and 24 hours of incubation with Ang II, respectively, indicating that Ang II did not significantly modify PN-1 mRNA levels.
expression. The effect of an NO donor (100 μmol/L nitroprusside) was tested in parallel. The relative PN-1 mRNA levels expressed as a percentage of the control (mean ± SD of triplicates from 1 representative experiment out of 2) were 103±12%, 115±10%, 98±19%, and 112±10% after 30 minutes and 3, 6, and 12 hours of incubation with the NO donor, respectively, indicating that nitroprusside did not modulate PN-1 mRNA expression in RASMCs.

Discussion
Much has been reported about PN-1 expression in foreskin fibroblasts, but these are the first studies showing PN-1 expression in cultured RASMCs and in the rat aortic wall. We found that both the messenger and the protein corresponding to this serpin were significantly expressed in cultured RASMCs. An additional immunoreactive band migrating at ≈110 kDa was also detected; its nature is yet unknown, and the possibility that it might correspond to PN-1 complexed with a protease present in cultured RASMCs should be tested. A similar band has also been detected in mouse skeletal muscle extracts. PN-1 expression by RASMCs was not limited to in vitro conditions, because PN-1 transcripts and the protein were also detected in aortic homogenates. Moreover, the PN-1 expressed by the vasculature fulfilled all the criteria of a functionally active serpin: it formed specific covalent complexes with thrombin involving the catalytic site of the protease, and heparin increased the formation of these complexes. We observed the presence of PN-1 at the RASMC surface, supporting the existence of constitutive PN-1 secretion by SMCs. Whether the amount of PN-1 produced locally is sufficient to modulate the response of SMCs to thrombin remains to be demonstrated. Nevertheless, the experiments presented here indicate that the cellular effects of thrombin can be inhibited dose-dependently by PN-1. Moreover, PN-1 has been reported to be a potent inhibitor of factor XIIa in the presence of heparin, and factor XIIa plays a role in the formation of thrombin via the contact activation blood-clotting pathway. This raises the interesting question of the possible involvement of PN1 in pathological conditions.

Vessels respond to chronic hypertension by adaptive mechanisms, including SMC hypertrophy. Under these conditions, the expression of several genes, including antiproteases, are modified. We have thus studied PN-1 expression in a well-characterized model of hypertension induced by the chronic administration of L-NAME to rats and demonstrate for the first time that long-term NOS inhibition upregulates the expression of functionally active PN-1 in the aortic wall. PN-1 mRNA levels were increased in vessels devoid of adventitia and endothelium, suggesting that PN-1 upregulation mainly depends on SMCs. Whether PN-1 may contribute to the vascular pathology that develops during long-term NOS inhibition could be investigated by studying mice that are deficient in PN-1.

PN-1 expression was normalized in L-NAME rats treated with the ACE inhibitor. It has already been shown that ACE inhibition reverses hypertension induced by L-NAME and all of the accompanying modifications of the gene expression pattern in the arterial wall. This suggests that either Ang II or NO could be involved in PN-1 overexpression, as has been demonstrated for PAI-1. However, neither Ang II nor NO donors had a direct effect on PN-1 expression in RASMCs, indicating that the molecular mechanisms responsible for the induction of PN-1 and PAI-1 expression differ. Several injury-induced related factors have been shown to stimulate PN-1 expression in cultured brain cells and in fibroblasts. Moreover, proinflammatory factors are known to be significantly increased in the vascular wall of rats after L-NAME administration, so they may be involved in PN-1 overexpression. The effect of proinflammatory factors on PN-1 expression in RASMC cultures is presently under investigation.

In conclusion, we show in the present study that a potent thrombin inhibitor, PN-1, is expressed by RASMCs in culture and is present in the media of rat aorta and that its expression is upregulated in an animal model of chronic hypertension. These observations suggest that PN-1 could be involved in the processes of protection of the vasculature against the effects of thrombin. The potential role of PN-1 as a molecular effector of the vascular response to hypertension clearly warrants additional investigation. The question is of importance, because thrombin receptors have been shown to be functionally increased in the vascular smooth muscle layer of hypertensive rat aortas, suggesting a role for thrombin in modulating aortic tone and stiffness in hypertensive rats.

Acknowledgments
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References
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PN-1 Expression and Regulation During Hypertension


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the serpin protease-nexin 1 is present in rat aortic smooth muscle cells and is up-regulated in L-NAME hypertensive rats.

Submission Type: Original Contribution
TABLE 1. General parameters of treated and untreated rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=14)</th>
<th>L-NAME (n=19)</th>
<th>LNAME+ACEI (n=14)</th>
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<td>461±1</td>
<td>450±1</td>
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<td>Systolic BP, mmHg</td>
<td>157±3</td>
<td>228±4*</td>
<td>157±5†</td>
</tr>
</tbody>
</table>

Values are mean ± SEM

*P<0.05 compared with control.
†P<0.05 compared with L-NAME.
Legend to figures

Figure I: Immunolocalisation of PN-1 in RASMCs.

Nonpermeabilized RASMCs were immunostained with the anti-PN-1 monoclonal antibody 4B3 (A). No signal was obtained in the absence of the first antibody (C). B and D represents Hoechst staining of the panels A and C respectively. Hoechst is a DNA stain that fluoresces bright blue upon binding to DNA.

Figure II: Experimental hypertension increases PN-1 expression in the total aorta (A) and in the isolated media (B).

Results are expressed as the ratio of the radioactivity corresponding to PN-1 RT-PCR products to that corresponding to GAPDH RT-PCR products. Control values in each blot were designated as 100 %. n = 4 to 8 per group.

*P<0.05, ***P<0.001 versus control, †P<0.05, ††P<0.01 versus L-NAME treated rats.
Fig. II