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 L-nitro-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:●●●-●●●.)

Key Words: protease-nexin 1 ■ smooth muscle cells ■ aorta ■ hypertension ■ thrombin
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\textsuperscript{\textcircled{\text{-}}}\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME).

**Methods**

**Purified Proteins**

Human \(\alpha\)-thrombin (EC.3.4.21.5) was purified and iodinated as previously described. A recombinant rat PN-1, a generous gift from Prt 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 2 to 6 passages. RASMCs were identified by their characteristic hill and valley growth appearance and by immunostaining for SM \(\alpha\)-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 caring and euthanasia of the animals were in accordance with the European Community Standards (Ministère de l’Agriculture, France; authorization No. 00577).

Systolic blood pressure and heart rate were measured once a week for 4 weeks. 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 directions. Double-stranded cDNAs were synthesized and amplified as previously described. The amplification was carried out for 2 h in a PCR machine with 25 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C; and then 7 min 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’-GACCACAGTGACTTTATACT-3’; the antisense primer was 3’-GGCTGGTGCTGGAGAA GTT3’; the sense primer for GAPDH was 5’-GTTGAAAGTCGGAGTCACG3’ and the antisense primer was 3’-GTTGAAGACGGCC AGTGGACTC3’.

**Reverse Transcription and Semiquantitative 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 2 h in a PCR machine with 25 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C; and then 7 min 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’-GACCACAGTGACTTTATACT3’ and the antisense primer was 5’-GGCTGGTGCTGGAGAA GTT3’. The sense primer for GAPDH was 5’-GTTGAAAGTCGGAGTCACG3’ and the antisense primer was 3’-GTTGAAGACGGCC AGTGGACTC3’.

**Western Blot Analysis**

Proteins were extracted from aortic samples or RASMCs in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 130 mmol/L NaCl, 3 mmol/L EDTA, 0.25% TX-100) and centrifuged at 12 000g 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 \(^{125}\text{I}-\alpha\)-Thrombin-PN-1 Complexes in Aortic Extracts**

Complexes between PN-1 present in aortic extracts and \(^{125}\text{I}-\alpha\)-thrombin were formed by incubating 20 mmol/L \(^{125}\text{I}-\alpha\)-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-Argchloroethyl-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 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% Phoric acid in DMEM and then washed with Tyrode solution. Confocal images were acquired using a Zeiss LSM-510 inverted confocal microscope with a LD Achroplan ×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 t-test 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 transcribease (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 extracts, PN-1 was detected as a single band of 45 kDa. A nonidentified additional band migrating at \(\approx 110 \text{ kDa}\) 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 $\alpha$-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 $[\text{Ca}^{2+}]_i$, which is one of the earliest events measurable in thrombin-stimulated cells. The effect of exogenous PN-1 on $\alpha$-thrombin–induced calcium mobilization was examined in fluo3-AM–loaded cells. Addition of 2 nmol/L $\alpha$-thrombin to RASMCs induced a transient and important increase in $[\text{Ca}^{2+}]_i$. 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 $\alpha$-thrombin elicited a 2.5-fold increase in cell proliferation after 48 hours of stimulation compared with control (absence of added

Figure 1. PN-1 is present in RASMCs and in rat aorta. RT-PCR and Western blotting detect PN-1 from confluent RASMC cultures and from aortic extracts.

Figure 2. PN-1 inhibits thrombin-induced cell responses. A, Intracellular calcium mobilization. Cells were stimulated with 2 nmol/L $\alpha$-thrombin in the absence (•) or in the presence (∗) of 10 nmol/L, 20 nmol/L (∗), and 30 nmol/L (†) PN-1. Results are presented as the difference between the mean fluorescence before addition of thrombin ($F_0$) and during treatment ($F$) (thrombin was added after 40 seconds). The results are representative of two different experiments. B, Proliferation assay. Quiescent RASMCs were treated with 10 nmol/L $\alpha$-thrombin in the presence of increasing amounts of PN-1. Control represents nonstimulated RASMCs. Results are the mean ± SD of triplicates from 1 representative experiment out of 2.
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 corresponds with free, noncovalently bound thrombin. The 81-kDa band was identified as an equimolecular complex between 125I-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 band was observed in the autoradiography with the aortic homogenates. Because of its electrophoretic mobility and because purified antithrombin complexed with 125I-α-thrombin had the same electrophoretic profile, this 95-kDa complex represents the binding of 125I-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 125I-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. Both PPACK and hirudin blocked the formation of the complex between 125I-α-thrombin and PN-1 in the aortic homogenates, confirming the involvement of the thrombin catalytic site in the covalent linkage (Figure 3B). In contrast, the C-terminal tail of hirudin (SH54-65), which binds exclusively to thrombin exosite 1, did not affect complex formation (Figure 3B). Heparin is known to increase thrombin inhibition by serpins. In the presence of heparin, the amount of thrombin bound to both PN-1 and AT was increased, paralleling a decrease in the band corresponding to free 125I-α-thrombin (Figure 3B).

The localization of PN-1 in the aortic wall was determined by immunohistochemical staining on fixed cryosections of

![Image](http://atvb.ahajournals.org/)

**Figure 3.** Thrombin forms a specific 81-kDa covalent complex with PN-1 expressed in rat aorta. A, Autoradiography and Western blot of covalent complexes formed between 125I-α-thrombin and recombinant PN-1 or aortic extracts. 125I-α-thrombin was incubated with recombinant rat PN-1 (+ rec PN-1) or with aortic homogenates from control rats (control). 125I-α-thrombin/AT corresponds to the thrombin-antithrombin complex. B, Autoradiography of covalent complexes formed between 125I-α-thrombin and aortic extracts. Samples were also prepared in the presence of heparin (+ heparin), with a 100-fold excess of cold thrombin (+ cold α-th), in the presence of PPACK (+ PPACK), with hirudin (+ hirudin), or in the presence of the C-terminal part of hirudin (+ SH54 to 65). The last sample corresponds to 125I-α-thrombin incubated with purified AT (+ AT).
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 reported by others, a significant increase in 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±22%, 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 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 Xla in the presence of heparin, and factor Xla 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.20-27 This suggests that either Ang II or NO could be involved in PN-1 overexpression, as has been demonstrated for PAI-1.28-29 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 cells30 and in fibroblasts.31 Moreover, proinflammatory factors are known to be significantly increased in the vascular wall of rats after l-NAME administration,27 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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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>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