Endothelial Protease Nexin-1 Is a Novel Regulator of A Disintegrin and Metalloproteinase 17 Maturation and Endothelial Protein C Receptor Shedding via Furin Inhibition

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Objective—Human protein C is a plasma serine protease that plays a key role in hemostasis, and activated protein C (aPC) is known to elicit protective responses in vascular endothelial cells. This cytoprotective activity requires the interaction of the protease with its cell membrane receptor, endothelial protein C receptor. However, the mechanisms regulating the beneficial cellular effects of aPC are not well known. We aimed to determine whether a serine protease inhibitor called protease nexin-I (PN-1) or serpinE2, expressed by vascular cells, can modulate the effect of aPC on endothelial cells.

Approach and Results—We found that vascular barrier protective and antiapoptotic activities of aPC were reduced both in endothelial cells underexpressing PN-1 and in endothelial cells whose PN-1 function was blocked by a neutralizing antibody. Our in vitro data were further confirmed in vivo. Indeed, we found that vascular endothelial growth factor–mediated hyperpermeability in the skin of mice was markedly reduced by local intradermal injection of aPC in wild-type mice but not in PN-1–deficient mice. Furthermore, we demonstrated a previously unknown protective role of endothelial PN-1 on endothelial protein C receptor shedding. We provided evidence that PN-1 inhibits furin, a serine protease that activates a disintegrin and metalloproteinase 17 involved in the shedding of endothelial protein C receptor. We indeed evidenced a direct interaction between PN-1 and furin in endothelial cells.

Conclusions—Our results thus demonstrate an original role of PN-1 as a furin convertase inhibitor, providing new insights for understanding the regulation of endothelial protein C receptor–dependent aPC endothelial protective effects.

Key Words: endothelial cell protein C receptor ■ furin ■ protease nexin I ■ protein C ■ serpinE2

Activating protein C (aPC) is a serine protease derived from its inactive zymogen, protein C. Endothelial protein C receptor (EPCR) is important for aPC activities. EPCR binds aPC and participates in the multiple protective responses of aPC mediated by signaling through the thrombin receptor, protease-activated receptor-1 (PAR-1), on cultured vascular endothelial cells.1 Indeed, aPC exhibits significant anti-inflammatory activity2 and can inhibit both staurosporin-induced apoptosis3 and thrombin-induced endothelial permeability.4 The regulation of aPC activity on endothelial cells is thus critical. Despite the importance of the beneficial cellular effects of aPC, the molecular mechanisms through which aPC mediates these effects remain incompletely understood. Protease nexin-I (PN-1) or serpinE2, a serine protease inhibitor belonging to the serpin superfamily, is expressed in many different cells5–7 and inhibits numerous proteases, playing key roles in hemostasis and thrombosis, including aPC.8 We have shown that PN-1 expressed in vascular endothelial cells is able to regulate thrombin activity.8 We thus investigated whether endothelial PN-1 could also modulate aPC activity. For this purpose, we used EAhy926 cells, an immortalized cell line produced by hybridization of human umbilical vein endothelial cells with lung carcinoma cells, A549.9 This cell line has been largely used and reported to express the typical endothelial proteins over successive passages.10

Esmon’s group has demonstrated that EPCR is shed by a disintegrin and metalloproteinase (ADAM) named ADAM17 or tumor necrosis factor–α–converting enzyme.12 ADAM17 is an important sheddase involved in the proteolysis of many membrane proteins, including tumor necrosis factor-α and its receptors.13,14 Importantly, a maturation process is required to render ADAM17 active. This essential step for ADAM17 activation is the proteolytic processing by a chymotrypsin–like serine protease, named furin, which belongs to the prohormone convertase family.15,16 Furin is involved in the activation and function of many proteins in almost all mammalian cell
Proteolytic activation of proteases mediated by furin can take place in the trans-Golgi network, in endosomes, and also at the cell surface. Furin has indeed been assigned to the Golgi apparatus, the endosomes, and the plasma membrane of endothelial cells. Because both furin and PN-1 are present in endothelial cells, we investigated whether PN-1 could be a potential inhibitor of furin and demonstrated that formation of complexes between PN-1 and furin can indeed occur in endothelial cells, leading to an inhibition of ADAM17, consequently resulting in the protection of EPCR from shedding by the metalloproteinase. Taken together, our data bring to light an original PN-1-dependent mechanism by which EPCR shedding can be regulated.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
PN-1 Facilitates the Antiapoptotic Effect of aPC
aPC is known to mediate different cytoprotective effects, including antiapoptotic activity. These cytoprotective activities of aPC require binding to EPCR and are mediated by signaling through the thrombin receptor PAR-1 or other PARs in some cases. EAhy926 is the most studied and characterized permanent human vascular endothelial cell line, which is known to express EPCR and PAR-1. Stauroporin, an ATP analog and inhibitor of protein kinase C, is a well-known and potent inducer of apoptosis. As estimated here by terminal deoxynucleotidyl transferase dUTP nick end labeling staining and in agreement with previous results, the treatment of EAhy926 cells with aPC inhibited staurosporin-induced cell death (Figure 1). We examined whether endothelial PN-1 can modulate such an effect. The cytoprotective effect of aPC was significantly inhibited when EAhy926 cells were incubated with a blocking anti–PN-1 antibody, whereas no change was observed with an irrelevant IgG (Figure 1). The blocking anti–PN-1 antibody alone had no effect on staurosporin-induced apoptosis, indicating that blockade of PN-1 by itself had no effect on cell death.

PN-1 Facilitates the Endothelial Barrier Function Mediated by aPC
The cytoprotective activity of aPC was also analyzed on endothelial barrier integrity in a dual-chamber system. As previously described in this system, thrombin induced an increase in cell permeability, which could be blocked by preincubation of cells with aPC. We showed that the capacity of aPC to enhance the barrier function in EAhy926 endothelial cells was inhibited when the cells were incubated with a blocking PN-1 antibody (Figure 2A) or when endothelial PN-1 expression was knocked down with a PN-1-small interfering RNA (siRNA; Figure 2B). Neither the blocking anti–PN-1 IgG alone nor the irrelevant IgG alone changed barrier disruption induced by thrombin, in the absence of aPC, under our experimental conditions (OD650 = 0.24±0.06 for anti–PN-1 IgG alone and 0.19±0.03 for irrelevant IgG alone versus 0.21±0.03 for the positive control). Our data showed for the first time that endothelial PN-1 facilitates the endothelial barrier function mediated by aPC.

We substituted factor Xa for aPC because factor Xa is also known to exhibit endothelial barrier protective function via the activation of PARs. Interestingly, we observed that endothelial barrier activity remained protected when factor Xa was used to...
induce the endothelial barrier protection, either in cells preincubated with a blocking anti–PN-1 antibody (Figure 2C) or in cells transfected with a PN-1-siRNA (Figure 2D). The PN-1 effect is thus specific to aPC activity and may therefore act on the interaction of aPC with its endothelial receptor EPCR.

To demonstrate such a potential link between PN-1 and EPCR and because EPCR is known to be strongly expressed in the large vessels of the lung,25 the expression of EPCR in lung homogenates from mice was analyzed by immunoblotting. Interestingly, a significant reduction in EPCR expression was clearly observed in lung extracts from PN-1–deficient mice (PN-1−/−) compared with wild-type (WT) mice (Figure 3A), indicating that PN-1 plays a significant role in the regulation of EPCR expression.

To further define in vivo the potential role of the endogenous PN-1 in the endothelial barrier protection induced by aPC, dermal vascular hyperpermeability was measured in mice by the leakage of Evans blue in response to local intradermal injection of vascular endothelial growth factor (VEGF; modified Miles assay). Evans blue binds to plasma proteins and therefore extravasates along with them at sites of increased permeability.26 As shown in Figure 3B, PN-1−/− mice and their WT littermate showed similar leakage of Evans blue into sites injected with VEGF. As expected, local intradermal injection of aPC suppressed VEGF-mediated Evans blue dye extravasation in WT mice, confirming the endothelial barrier protective activity of aPC. This protective effect of aPC was remarkably abolished in PN-1−/− mice. Indeed, in PN-1−/− mice, the local injections of aPC had no significant effect on VEGF-induced Evans blue extravasation (Figure 3B). Taken together, our data point out the impact of vascular PN-1 on the protective effect of aPC.

**Endothelial PN-1 Protects EPCR Shedding Induced by ADAM17**

To investigate the mechanism by which endothelial PN-1 potentiates the activity of aPC, we analyzed EPCR expression in EAhy926 cells. Interestingly, we observed that PN-1-siRNA transfection in EAhy926 cells induced not only a strong reduction in PN-1 expression (Figure 4A and 4B) but also a significant reduction in EPCR at the protein level (Figure 4B), without affecting EPCR mRNA levels (Figure 4A).

Furthermore, as shown in Figure 4C and in the densitometric analysis of the corresponding results (Figure 4D), our data revealed that the reduction of EPCR observed in cells incubated with a neutralizing anti–PN-1 antibody or in cells transfected with PN-1-siRNA was accompanied by the presence of soluble EPCR in the conditioned medium of cells devoid of functional PN-1. Our data thus indicate that the presence of PN-1 on endothelial cells protects EPCR from shedding.

EPCR shedding is known to be mediated in endothelial cells by an important member of ADAM family, ADAM17.
also named tumor necrosis factor-α–converting enzyme. We tested the effect of an inhibitor of ADAM17 on EPCR shedding in control cells and in cells devoid of functional PN-1. As shown in Figure 4E and in the densitometric analysis of the corresponding results (Figure 4F), shedding of EPCR was prevented if cells underexpressing PN-1 were treated with a synthetic inhibitor of ADAM17. Our data thus demonstrate that the presence of PN-1 in endothelial cells prevents the shedding of EPCR by ADAM17.

**PN-1 Inhibits the Proteolytic Processing of ADAM17 by Furin**

It is noteworthy that PN-1 belongs to the serpin family and has thus no direct inhibitory effect on ADAM such as ADAM17. ADAM17 is a membrane-anchored metalloproteinase that needs to be proteolytically processed to become active. We have shown by immunoblotting with an anti-ADAM17 antibody (Figure 5A and 5B) that incubation of cells with a blocking anti–PN-1 antibody or transfection of cells with siRNA targeting PN-1 resulted in a significant decrease in the level of zymogen pro-ADAM17 compared with the data obtained for the respective control cells. Consistent with these in vitro observations, we observed by Western blots of mice lung extracts a decrease in the pro-ADAM17 band in PN-1−/− mice compared with WT (Figure I in the online-only Data Supplement). These data indicate that pro-ADAM17 maturation was increased in cells devoid of PN-1 and thus demonstrate that endothelial PN-1 acts to limit ADAM17 maturation.

ADAM proteinases are activated by proconvertases, including furin that was the first mammalian proconvertase identified. Furin is expressed ubiquitously and has been shown to be involved in the maturation of pro-ADAM17. Because furin is a serine protease, we hypothesized that PN-1 may regulate the proteolytic processing of pro-ADAM17 in EAhy926 cells by inhibiting endothelial furin. To verify this hypothesis, we tested the effect of an inhibitor of furin on pro-ADAM17 maturation in control cells and in cells devoid of functional PN-1. As shown in Figure 5A and in the densitometric analysis of the corresponding results (Figure 5B), the proteolytic processing of pro-ADAM17 observed in cells devoid of functional PN-1 was prevented when these cells were treated with the inhibitor of furin. Altogether, our data strongly suggest that PN-1 and furin are both involved in the maturation of pro-ADAM17 in endothelial cells. To consolidate the link between PN-1, furin, and EPCR shedding, we analyzed what happens to EPCR in the presence of furin inhibitor in control cells and in cells devoid of functional PN-1. As shown in Figure 5C and 5D, EPCR shedding observed in cells devoid of functional PN-1 is prevented when these cells were treated with furin inhibitor. Our data thus indicate that the presence of PN-1 in endothelial cells prevents the shedding of EPCR via a furin-dependent mechanism.

**In Vitro Inhibition of Furin by PN-1**

We demonstrated in vitro, by immunoblotting with anti-furin and anti–PN-1 antibodies, the formation of a covalent SDS-stable complex of ∼110 kDa during incubation of recombinant PN-1 with recombinant furin (Figure 6A). The formation of the complex was prevented in the presence of furin inhibitor, indicating that furin activity was necessary for complex formation. We confirmed that recombinant PN-1 was able to block recombinant furin by measuring the catalytic activity of furin toward
its fluorogenic substrate in the presence of increasing concentrations of PN-1. The catalytic activity of furin was decreased ≈2-fold in the presence of an equimolar concentration of PN-1 (Figure 6B). These data clearly indicate that furin is a potential target of PN-1.

**Discussion**

Our results demonstrate a novel mechanism involving the serpin PN-1 and regulating the protection of the receptor EPCR from proteolytic cleavage. EPCR has been shown to be cleaved by neutrophil proteinase 3, which involves vascular injury and binding of activated neutrophils to endothelial cells.28 EPCR has also been shown to be cleaved by ADAM17, in particular, in human umbilical vein endothelial cells because EPCR shedding was drastically reduced after knocking down ADAM17 expression in these cells.12 Our data are the first to demonstrate that the shedding of EPCR can be inhibited by PN-1.
regulated at the posttranslational level, upstream of ADAM17 action, by the cellular serpin PN-1. It cannot be excluded that factors other than ADAM17 may also account for PN-1-regulated shedding of EPCR. However, it is quite clear that PN-1 downexpression was accompanied by a marked downregulation of cell EPCR.

PN-1 is a serpin that is barely detectable in plasma but is produced by most cell types and, in particular, by vascular cells. PN-1 is a powerful inhibitor of thrombin and plasminogen activators and has been revealed during this last decade to be an important regulator in the vascular system.29 We demonstrated that PN-1 expressed by vascular cells regulates cell susceptibility to proteases of the coagulation and fibrinolytic systems. The importance of endothelial PN-1 was illustrated by the observation that PN-1 could inhibit thrombin activity on endothelial cells.9 We thus analyzed the effect of endothelial cells. PN-1 downexpression was accompanied by a marked downregulation of cell EPCR.

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Figure 5. Endothelial protease nexin-1 (PN-1) prevents pro-a disintegrin and metalloproteinase 17 (ADAM17) maturation induced by furin. A, EAhy926 cells, either preincubated with the blocking anti–PN-1 IgG or the irrelevant IgG or transfected with small interfering RNA (siRNA) for PN-1 or irrelevant siRNA, were incubated or not with the furin inhibitor before immunoblotting with anti-ADAM17. A representative immunoblot of 3 is shown, and (B) results are expressed as the mean of densitometric quantification relative to the irrelevant control in the absence of furin inhibitor (mean±SE; n=3; *P<0.05 and §P<0.005). C, The same samples as in A were also immunoblotted with anti-endothelial protein C receptor (EPCR). A representative immunoblot is shown, and (B) results are expressed as the mean of densitometric quantification relative to the irrelevant control in the absence of furin inhibitor (mean±SE; n=3; *P<0.05 and §P<0.005). NS indicates nonsignificant.

zymogen maturation by the proconvertase furin. To date, very few naturally occurring inhibitors of the proprotein conver-
tase family have been reported in mammals. Those identified include 2 members of the serpin superfamily, an endogenous furin inhibitor released from human platelets named PIB830 and the plasminogen activator inhibitor-1 (PAI-1) in the trans-
ferred human carcinoma LoVo and Hela cell lines.31 We found here that PN-1 is also able to inhibit furin by forming SDS-
stable complexes with endogenous furin in endothelial cells. PAI-1 has also been shown to form an SDS-stable complex with furin, leading to a reduction in furin-dependent matura-
tion and activity of the insulin receptor and pro-ADAM17.31 However, in contrast to the present data, where overexpression of PN-1 was not required to demonstrate its effect on furin-
dependent pro-ADAM17 maturation in endothelial cells, such an effect of PAI-1 has been shown in a heterologous expression system requiring transfection of LoVo cells with expression vectors containing both furin and PAI-1 coding sequences. Based on our results, we propose a model illustrated in Figure II in the online-only Data Supplement in which endothelial PN-1 favors aPC cellular activities by preventing endogenous EPCR shedding.

Furin belongs to the group of specific mammalian proconvertases and cleaves various proteins. Interestingly, its catalytic triad aligns with that of thrombin in the chymotrypsin family of proteases. The crystal structures of PN-1 in complex with thrombin have been recently determined.32 When looking at these crystal structures, it can be hypothesized that Arg346 present in the reactive center loop of PN-1 is the P1 residue for furin interaction, and Arg269 of PN-1 constitutes the other
principal exosite contact interacting with furin. However, the tridimensional structure of PN-1 complexed with furin has to be established to verify the structural basis of furin recognition by PN-1. The fact that recombinant PN-1 could significantly inhibit the catalytic activity of recombinant furin, by forming SDS-stable complexes, is a direct proof that furin is a potential target for this serpin. Furthermore, PAI-1, which has the highest amino acid sequence homology with PN-1 among proteins of the serpin superfamily, has also been shown to inhibit furin.31

Importantly, the fact that aPC requires vascular PN-1 to fully exert its cellular activities is physiologically relevant. Indeed, the impact of PN-1 on the cytoprotective activity of aPC has not only been shown in vitro on endothelial cells but also in vivo in mice because we demonstrated that the protective effect of aPC against VEGF-induced vascular hyperpermeability was drastically reduced in PN-1–deficient mice compared with the WT mice. Interestingly, in contrast, no difference was observed between the 2 types of mice concerning the basic hyperpermeability induced by VEGF, which clearly indicates that PN-1 does not act, under these experimental conditions, on the signaling induced by VEGF itself, but on the effect of aPC.

Our data show for the first time that endothelial PN-1 attenuates EPCR shedding and is thus a fundamental factor in the regulation of EPCR bioavailability. Therefore, PN-1 seems to be a new regulator in protease shedding processes. Because PN-1 is expressed in numerous cell types, its ability to regulate the shedding of various cell receptors may thus be of significance in many different fields. By such a capacity to control the expression of surface molecules, PN-1 seems a new factor in the regulatory step of receptor expression. In the field of hemostasis, such a role makes endothelial PN-1 an unexpected positive regulator of aPC biological responses. Because aPC and its receptor EPCR constitute a major physiological regulatory system controlling vascular wall responses not only during thrombosis but also during inflammation, our results present potential importance and possible relevance in strategies to improve the therapeutic efficacy of the aPC system in inflammatory diseases.
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Disclosures
None.

References

Significance
Our study revealed that a serine protease inhibitor called protease nexin-1 (PN-1), or serpinE2, expressed by vascular cells, can be considered as a new player in the cytoprotective responses of activated protein C. Because activated protein C and its receptor endothelial protein C receptor constitute a major physiological regulatory system to control vascular wall responses to injury, the fact that vascular PN-1 favors activated protein C to exert its cellular activities is an original key finding, physiologically relevant. We also provided important very new insights regarding the mechanisms by which endothelial PN-1 modulates the cytoprotective effects of activated protein C. We indeed demonstrated that PN-1 can downregulate endothelial protein C receptor shedding by directly inhibiting the furin convertase present in endothelial cells. Because furin represents a processing enzyme of various cell surface receptors and growth factors, its inhibition by PN-1 opens new fields of investigation in many different disciplines.
Supplemental Figure I: Impact of PN-1 deficiency on proADAM17 expression in lung. 5 μg of lung homogenates (n = 3 for WT and PN-1-deficient mice) were subjected to 10% SDS-PAGE and immunoblotted with the anti-ADAM17, as described in the methods.
Supplemental Figure II: Schematic model of PN-1 impact on aPC’s protective responses. We propose a model where EPCR shedding by ADAM17 on endothelial cells is limited when PN-1 is present in sufficient amount to inhibit furin activity and therefore to limit ADAM17 maturation. When PN-1 is absent or too low, furin activates efficiently ADAM17 which induces the shedding of EPCR and consequently results in a decrease of aPC-induced protective responses.
Reagents. Purification of human α-thrombin \(^1\) and production of recombinant human PN-1 \(^2\) were as previously described. Recombinant human aPC and bovine factor Xa were purchased from Enzyme Research Laboratory. Recombinant human furin was obtained from R&D Systems and murine VEGF from PeproTech. The ADAM inhibitor GM6001 was from Chemicon and the furin inhibitor (Decanoyl-RVVR-chloromethylketone) from Bachem Bioscience. The fluorogenic substrate for furin (Boc-RVRR-AMC) and the monoclonal anti-furin IgG (clone MON-152) were from Enzo Life Sciences. Polyclonal rabbit anti-furin IgG (clone H-220) was from Santa Cruz Biotechnology, monoclonal anti-GAPDH IgG from Abcam, polyclonal anti-ADAM17 from Millipore (clone AB19027) and monoclonal anti-EPCR from R&D Systems. Monoclonal anti-β-actin was from Sigma. The polyclonal blocking anti-PN-1 IgG and the monoclonal anti-PN-1 IgG have been described earlier \(^3\). The secondary goat anti-mouse IgG and goat anti-rabbit IgG conjugated with peroxidase were from Jackson Immunoresearch.

Cell culture. The endothelial-like hybridoma cells EAhy926 were purchased from LGC Standards \(^4\). Cells were grown to confluency in Dulbecco’s Modified Eagle medium supplemented with HAT (Hypoxanthine, Aminopterin and Thymidine) (Life Technologies) and 10 % fetal bovine serum. These cells express EPCR and have been largely used to study the effects of aPC on endothelial cell apoptosis \(^5\) and permeability \(^6\).

Silencing of PN-1 and cell transfection. The following pre-designed annealed siRNA were chosen for PN-1 silencing: sense sequence 5’-GGUUUUCAAUCAGAUUGAGtt-3’ and antisense sequence 5’-CACAAUCUGAUUGAAAACCtt-3’. The pre-designed annealed irrelevant siRNA from Eurogentec was used as negative control. The duplexes were introduced into confluent cultured EAhy926 cells using lipofectamine reagents according to the manufacturer’s instructions (Life Technologies). Cells were assayed after 72 h of transfection. PN-1 extinction was verified at the RNA and protein levels.

TUNEL (TdT-mediated dUTP nick end labelled) staining of EAhy926 endothelial cells. EAhy926 cells were grown on gelatin-coated 8-well labteks and pre- incubated for 30 minutes with the blocking anti-PN-1 IgG (10 µg/mL), or the irrelevant IgG (10 µg/mL) (Jackson Immunoresearch) before overnight incubation with aPC (50 nmol/L). After preincubation with the different antibodies and aPC, apoptosis was induced by staurosporin (10 µmol/L)
(Sigma) for 2 h at 37°C. Hirudin (4 units/mL) was added to aPC before its use to inhibit potential trace amounts of thrombin in the commercial preparation of recombinant aPC. The TUNEL reaction was then carried out on fixed cells using the In situ Cell Death Detection kit-fluorescein (Roche Diagnostics) following the manufacturer’s instructions. After two washes with PBS, pH 7.4, the cells were incubated at room temperature with Hoechst 33342 (Sigma) for 10 min. The number of apoptotic cells was expressed as the percentage of the total number of nuclei detected by Hoechst staining which are TUNEL-positive.

**Endothelial barrier permeability in vitro assay.** Permeability was quantified by spectrophotometric measurement of the flux of Evans blue-labeled bovine serum albumin (BSA) across EAhy926 cell monolayers using a dual-chamber as previously described. Briefly, EAhy926 endothelial cells were plated at 250 000 cells/well on gelatin-coated transwell polycarbonate membranes. Cells were then either transfected with PN-1-siRNA or Irrelevant siRNA, or pre-treated 30 minutes with the blocking anti-PN-1 IgG (20 µg/mL), or the irrelevant IgG (20 µg/mL) before incubation with aPC (20 nmol/L) or factor Xa (20 nmol/L) for 2.5 h followed by permeability induction by thrombin (5 nmol/L) for 3 min as described. Permeability was assayed using 0.67 mg/mL Evans blue (Sigma) diluted in growth medium containing BSA (40 mg/mL) (Sigma). Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density at 650 nm was measured in the lower chamber. Experiments were performed in duplicate or triplicate and repeated multiple times.

**Miles Assay of Vascular permeability.** The Miles assay is a mouse model for vascular barrier integrity. Wild-type C57BL/6 and PN-1-deficient mice backcrossed for 10 generations into the C57BL/6 background were as previously described and bred in-house. We performed a modified Miles assay to test the effect of PN-1-deficiency on the endothelial barrier protective activity of aPC as follows: 30 µL Evans blue dye (37 mg/mL in 0.9% sterile saline) was injected intravenously and 10 min after, 30 µL of murine VEGF (6.5 ng/µL) or 30 µL VEGF + 1µL aPC (1µg/µL) or 30 µL PBS (Control) was injected intradermally into the shaved dorsal skin of mice. Evans blue binds to plasma proteins and therefore extravasates along with them at sites of increased vascular permeability. Thirty minutes later, mice were euthanized, their dorsal skin removed, and lesions were excised using an 8-mm biopsy punch (Miltex). Evans Blue was extracted over 24 hours at 56°C in formamide (Sigma Aldrich) and its content was quantified by reading at 650 nm in a microplate reader against a standard
curve of Evans Blue dye. Experiments were performed in accordance with European legislation on the protection of animals.

**Reverse Transcription and Quantitative Real-time PCR.** Total RNA from cultured EAhy926 cells was extracted with Trizol (Life Technologies) and reverse-transcribed using the Superscript II Reverse transcriptase (Life Technologies) as described by the manufacturer. The resulting cDNA was used as a template for quantitative PCR analysis of PN-1 and GAPDH mRNA expression in a LightCycler system with SYBR Green detection (Roche Applied Science). PN-1 primers were: forward 5’-CCGCTGAAAGTTCTTGG-CA-3’ and reverse 5’-CAGCACCTGTAGGATTATGTCG-3’. The following run protocol for PN-1 was used: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 60°C, 10 sec; 72°C, 20 sec. The lightCycler run protocol for GAPDH or EPCR was as follows: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 65°C, 10 sec; 72°C, 20 sec. EPCR primers were: forward 5’-CCAACACCACGATCATTCA-3’ and reverse 5’-ATACCGAGTGTGTTTAGG-3’. GAPDH primers were: forward 5’-GGGCACCCTGGGCTAAACTGA-3’ and reverse 5’-TGCTCTTGCTGGGCTG-3’. The level of mRNA encoding PN-1 or EPCR were normalized relative to GAPDH mRNA level.

**Immunoblot analysis of EPCR in EAhy926 cells.** Cells transfected with Irrelevant siRNA or PN-1-siRNA, or cells treated with either Irrelevant IgG or the polyclonal blocking anti-PN-1 IgG, were incubated or not with a potent ADAM17 inhibitor, GM6001 (10 µmol/L) for 2 hours and were washed with cold PBS before lysis in buffer (10 mmol/L Tris/HCl, pH 8, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L sodium orthovanadate, 1 % Nonidet P-40), containing a complete cocktail of protease inhibitors (Sigma). Protein concentration was determined by the Bio-Rad protein assay, 20 µg of lysate were boiled and the proteins were separated by laemmli-10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk, probed with the appropriate antibodies: anti- EPCR (2 µg/mL), or anti-GAPDH (2 µg/mL) antibodies followed by horseradish peroxidase (HRP) conjugated secondary antibodies (Jackson Research) at a 1:50,000 dilution. Immunoreactivity was visualized by chemiluminescence. Anti-GAPDH antibody was used for normalization. Blots were quantified by using ImageJ software.

**Immunoblot analysis of EPCR in lung extracts from mice.** The lungs from wild-type C57BL/6 and PN-1-deficient mice were snap-frozen and homogenized in lysis buffer,
containing a cocktail of protease inhibitors. After centrifugation for 5 min. at 15000 x g at 4°C, 10 µg of lysate were subjected to 10 %-SDS-PAGE and immunoblotted with anti-EPCR as described above. Anti-β actin (2 µg/mL) monoclonal antibody was used for normalization. Blots were quantified by using ImageJ software.

**Lysate preparation and immunoblot analysis of pro-ADAM17 in EAhy926 cells.** Cells transfected with Irrelevant siRNA or PN-1-siRNA were incubated or not with the furin inhibitor, decanoyl-RVKR-cmk (30 µmol/L) and were washed with cold PBS before lysis in buffer as described above. 100 µg of lysate were subjected to 10%-SDS-PAGE and immunoblotted with the anti-ADAM17 (4 µg/mL). Anti-GAPDH antibody was used for normalization. Blots were quantified by using ImageJ software.

**In vitro furin activity.** Recombinant furin (0.65 µmol/L) was incubated with increasing concentrations of PN-1 (0 to 1.2 µmol/L) in 20 µl of (20 mmol/L phosphate-buffered saline, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% PEG 8000, pH 7.4) for 90 minutes at 37°C. Furin inhibition was checked by measuring residual furin activity using the fluorogenic substrate pERTKR-AMC (50 µmol/L) (Excitation 380nM- Emission 460 nM) with a microplate spectrofluorimeter reader, or checked by immunoblot analysis of the PN-1/furin covalent complexes with the anti-PN-1 and anti-furin monoclonal antibodies followed by HRP-conjugated secondary antibodies as described above.

**EPCR and PN-1 staining and immunofluorescence microscopy.** EAhy926 cells were seeded on gelatine-coated glass coverslips. Cells were fixed in 4 % paraformaldehyde (PFA) at room temperature for 20 minutes, permeabilized or not with 0.1% Triton-X100 and blocked with 5% goat serum (Dako) in PBS for 2 h. After washing in PBS, cells were incubated overnight at 4°C with the mouse anti-PN-1 monoclonal antibody (10 µg/mL) and the anti-furin polyclonal antibody (10 µg/mL). After washing in PBS, cells were incubated with Alexa 555-conjugated goat anti-mouse IgG (Life Technologies) and Alexa 488-conjugated goat anti-rabbit IgG (Life Technologies) for 2 h at room temperature, mounted and visualized with a confocal laser-scanning microscope (LSM-510-META, Zeiss, Mannheim, Germany) equipped with a x40 oil-immersion objective. Simultaneous two-channel recording was performed with a pinhole size of 1.00 Airy Units by using excitation wavelengths of 488 and 555 nm. The specificity of the labelling was proved by the absence of signal when the primary antibody was omitted or when using an irrelevant antibody.
**Immunoprecipitation and immunoblot analysis of furin and PN-1 in EAhy926 cells.**

Immunoprecipitations were carried out using the same amount of protein for each sample, as determined with the Bio-Rad protein assay. After preclearing the samples with protein A-Sepharose for 60 min at 4°C, cell lysates were incubated with the rabbit anti-human furin polyclonal antibody (10 µg/mL) overnight at 4°C followed by the addition of protein A/G-coated magnetic beads and incubation was continued for 2 h at 4°C. The magnetic separation process ensued and immunoprecipitates were solubilized with 2 % SDS, separated by SDS-8%-15% gradient gel and transferred to nitrocellulose membranes. Membranes were probed with the anti-PN-1 (5 µg/mL) and anti-furin (2 µg/mL) monoclonal antibodies followed by HRP-conjugated secondary antibodies. Densitometric quantification of the blots was performed by using ImageJ software.

**Statistical analysis.** Results are expressed as means ± SD from an appropriate number of experiments as indicated in the figure legends. The statistical analysis was done using the Student t-test, or the Mann-Whitney test for mice experiments. \( P < 0.05 \) was considered statistically significant.

**References**


