Protease-Activated Receptor-1 Antagonist F 16618 Reduces Arterial Restenosis by Down-Regulation of Tumor Necrosis Factor α and Matrix Metalloproteinase 7 Expression, Migration, and Proliferation of Vascular Smooth Muscle Cells.

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ABSTRACT

Wound healing after angioplasty or stenting is associated with increased production of thrombin and the activation of protease-activated receptor 1 (PAR1). The aim of the present study was to examine the effects of a new selective PAR1 antagonist, 2-[5-oxo-5-(4-pyridin-2-ylpiperazin-1-yl)-penta-1,3-dienyl]-benzonitrile (F 16618), in restenosis and vascular smooth muscle cell (SMC) proliferation and migration using both in vivo and in vitro approaches. Daily oral administration of F 16618 inhibited the restenosis induced by balloon angioplasty on rat carotid artery in a dose-dependent manner. Furthermore, single intravenous administration of F 16618 during the angioplasty procedure was sufficient to protect the carotid artery against restenosis. In vitro, F 16618 inhibited the growth of human aortic SMCs in a concentration-dependent manner with maximal effects at 10 μM. At that concentration, F 16618 also prevented thrombin-mediated SMC migration. In vivo, oral and intravenous F 16618 treatments reduced by 30 and 50% the expression of the inflammatory cytokine tumor necrosis factor α (TNFα) 24 h after angioplasty. However, only acute intravenous administration prevented the induction of matrix metalloproteinase 7 expression. In contrast, F 16618 treatments had no effect on early SMC de-differentiation and transcription of monocyte chemoattractant protein-1 and interleukin-6 and late re-endothelialization of injured arteries. Furthermore, F 16618 compensated for the carotid endothelium loss by inhibiting PAR1-mediated contraction. Altogether, these data demonstrate that PAR1 antagonists such as F 16618 are a highly effective treatment of restenosis after vascular injury, by inhibition of TNFα, matrix metalloproteinase 7, and SMC migration and proliferation in addition to an antithrombotic effect.

Introduction

Wound healing after vascular injury, and in particular restenosis after balloon angioplasty and stenting, results from different processes such as inflammation, smooth muscle cell (SMC) de-differentiation leading to SMC migration and proliferation, and constrictive remodeling (Muto et al., 2007; Zargham, 2008). In fact, localized loss of endothelium and subendothelial structures allows the direct contact of
blood with SMCs. This leads to the generation of large amounts of the serine protease thrombin through activation of the coagulation cascade by the tissue factor pathway (Mar- 
mur et al., 1994). By binding the subendothelial extracellular 
matrix, thrombin remains functionally active, localized, and 
protected from inactivation by circulating inhibitors (Schrör 
et al., 2010). Thus thrombin cleaves circulating fibrinogen to 
fibrin, but also exhibits a wide range of functions by inter-
acting with the surface receptors of platelets, leukocytes, 
endothelial cells, and SMCs (Coughlin, 2000; Minami et al., 
2004; Steinberg, 2005; Hirano, 2007). Specifically, thrombin 
modulates endothelial permeability, vasomotor tone, leuko-
cyte trafficking, migration, and proliferation of vascular 
SMCs (Minami et al., 2004).

Thrombin cleaves the N-terminal segment of protease-ac-
tivated receptors (PARs). This unmask a new amino-termi-
nal tethered ligand that binds to the extracellular domain to 
directly activate these G protein-coupled receptors (Vu et al., 
1991; Déry et al., 1998; Coughlin, 2000; Hollenberg and Compton, 2002; Hirano, 2007). In normal arteries, PAR1 
expression is detected in platelets, leukocytes, and endothel-
ial cells (Macfarlane et al., 2001), but it is limited in SMCs. 
However, after vascular injury such as balloon angioplasty, 
PAR1 transcription is up-regulated in SMCs (Hirano, 2007). 
Up-regulation of PAR1 has been hypothesized as a key event 
in the development of vascular lesions and the hypercontrac-
tile response to thrombin (Fukunaga et al., 2006). This leads 
to neointimal formation and constrictive remodeling.

Thus, PAR1 antagonists may represent a powerful strat-
 egy for inhibiting the development of vascular lesions after 
arterial reconstruction procedures. Orally active PAR1 an-
tagonts were first developed as inhibitors of platelet aggre-
gation with a low impact on bleeding. SCH-530348 (ethyl 
N-[(3R,3aS,4aS,4R,7R,8aR,9aR)-4-[(E)-2-[5-(3-fluorophenyl)-
2-pyridyl]vinyl]-3-methyl-1-oxo-3a,4,4a,5,6,7,8,8a,9,9a-deca-
hydro-3H-benzo[f]isobenzofuran-7-yl(carbamate), which has po-
tent antithrombotic properties, is currently in phase 3 clinical 
trials for the treatment of acute coronary syndrome (Siller-
Matula et al., 2010). E5555 (aplatapax) moderately inhibits 
human platelet activity, has anti-inflammatory properties, and 
prevents arterial PAR1 up-regulation and hyper-responsive-
tness to thrombin (Kai et al., 2007; Siller-Matula et al., 2010).

We recently discovered a new PAR1 antagonist, 2-[5-oxo-5-(4-
pyrrolo[1,2-a]indole-4,7-dione carbamate), which has po-
tent antithrombotic action (Lee and Blaufox, 1985).

Materials and Methods

Drugs. F 16618 was synthesized in the Centre de Recherche 
Pierre Fabre (Castres, France) by the Division of Medicinal 
Chemistry as described previously (Perez et al., 2009). The PAR1 agonist 
SPLLRR (Ser-Phe-Leu-Leu-Arg-Gsp) was synthesized at the Labora-
tory of Amino Acids, Peptides, and Proteins, Faculté de Pharmacie, 
Montpellier, France. Mitomycin C (6-amino-1,1a,2,8a,8b-hexa-
hydro-8-(hydroxymethyl)-8a-methoxy-5-methyl azirino[2,3',3,4]
pyrrolo[1,2-a]indole-4,7-dione carbamate (ester) was purchased from 
Sigma-Aldrich (St. Louis, MO). Human thrombin was from Merck 
Biosciences (Darmstadt, Germany).

Carotid Artery Injury and F 16618 Treatment. Rats were 
placed and tested in an Association for the Assessment and Accred-
itation of Laboratory Animal Care-accredited facility in strict com-
pliance with all applicable regulations, and the protocol was carried 
out in compliance with French regulations and local ethical commit-
te guidelines for animal research. This conforms to the Guide for the 
Care and Use of Laboratory Animals published by the National 
Institutes of Health.

Balloon denudation of the left carotid artery endothelium was 
performed in male adult Sprague-Dawley rats (Charles River Labo-
ratories-Iffa Credo, L’Arbresle, France) weighing 250 to 270 g under 
isoflurane anesthesia. After exposure of the left carotid artery, a 2F 
Fogarty balloon catheter (Edwards Lifescience, Irvine, CA) was 
inserted into the external carotid branch of the aortic arch, inflated to 
produce slight resistance, and moved back and forth three times. 
F 16618 was administered in the rats by two different routes. F 
16618 or vehicle (1% methylcellulose) was administered orally once 
daily 3 days before and 14 days after balloon angioplasty. In another 
set of experiments, F 16618 or vehicle (40% polyethylene glycol/60% 
NaCl) was administered intravenously 5 min before angioplasty and 
during the chirurgical procedure. For each mode of treatment, 
the correspondence between the total administered doses and the 
plasma concentrations of F 16618 is given in Table 1. The control 

treatment group of sham-operated rats included 11 animals, and the vehicle group included 19 rats. The groups receiving oral or intravenous 
treatment included 9 to 13 and 8 to 10 rats, respectively.

After treatment for 24 h, the animals were sacrificed and segments 
of injured and unjured carotid arteries with endothelium at the edges of the lesion were collected for mRNA extraction and reverse transcription-PCR quantification (Supplemental Methods). After treatment for 14 days, animals were sacrificed, and carotid 
arteries were collected for morphometric and immunofluorescent 
analysis.

Carotid Artery Histomorphometric Analysis. Cross-sections of 
arteries were fixed with 4% paraformaldehyde and stained with 
hematoxylin/eosin solution. The internal and external medial areas 
were measured to determine media and neointima surface. A neoin-
tima/media ratio (N/M) was calculated using a video image analysis 
system (LEICA QWIN; Leica Imaging Systems, Cambridge, UK) 
and served as an index of restenosis measurement. The analysis was 
conducted by an investigator blinded to treatment.

Immunofluorescence. Segments of carotid artery were mounted 
embedding medium (Thermo Fisher Scientific, Cergy-Pontoise, 
France), frozen in isopentane precooled in liquid nitrogen, and stored 
at ~80°C. Immunostaining of PAR1 and von Willebrand factor 
(vWF) was performed on 7-µm-thick cross-sections. Tissue sections 
were permeabilized and saturated with 0.5% Triton X-100, 1% BSA, 
and 10% goat serum in phosphate-buffered saline (PBS) for 60 min. 
Slides were then incubated with PAR-1 (ATAP-1, 1:50; Santa Cruz 
Biotecnhology, Inc., Santa Cruz, CA) or vWF (1:50; Acris, Herford, 
Germany) antibodies or corresponding isotype (mouse IgG, 1:50; 
Vector Laboratories, Servion, Switzerland) with 1% BSA, 0.5% Tri-
TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oral</th>
<th>Intravenous</th>
</tr>
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<tbody>
<tr>
<td>Doses (mg/kg/day)</td>
<td>5</td>
<td>0.32</td>
</tr>
<tr>
<td>Plasma concentration (ng/ml)</td>
<td>4.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Plasma concentration (µM)</td>
<td>12.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Plasma concentration (ng/ml)</td>
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<td>6.3</td>
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<tr>
<td>Plasma concentration (µM)</td>
<td>18.2</td>
<td>38.3</td>
</tr>
<tr>
<td>Plasma concentration (μg/ml)</td>
<td>4.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Plasma concentration (μM)</td>
<td>12.2</td>
<td>19.4</td>
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For oral treatment, maximal plasma concentrations of F 16618 were detected 1 h 
after force-feeding. For intravenous treatment, the plasma concentrations were 
calculated from the mean rat weight of 260 g and the corresponding plasma volume 
(Lee and Blaufox, 1985).
ton X-100, and 3% goat serum in PBS overnight at room temperature. After washing in PBS, Alexa 594-conjugated goat anti-mouse IgG (1:400; Invitrogen, Cergy Pontoise, France) was added with 1% BSA and 3% goat serum in PBS for 1 h. In some experiments, 4,6-diamidino-2-phenylindole (1:500) was incubated with secondary antibody. After washing in PBS, tissues were mounted with Dako fluorescent mounting medium (Dako Denmark A/S, Glostrup, Denmark) and visualized with an Olympus (Tokyo, Japan) IX 50 microscope and a Roper Scientific (Trenton, NJ) camera. For PAR1 staining, fluorescence images were automatically collected and deconvoluted using a piezoelectric translator (PIFOC, Karlsruhe/Palmbach, Germany) and Metamorph software (Molecular Devices, Sunnyvale, CA). The immunolabeling of endothelium was quantified with NIH Image software program as the ratio of mean vWF fluorescence to internal vessel perimeter.

Cell Proliferation Assays. The human aortic SMCs were obtained from Lonza (Basel, Switzerland) and cultured following the manufacturer’s instructions. For serum-induced cell proliferation, SMCs were cultured with complete smooth muscle basal medium and 5% fetal calf serum and treated with 1 to 100 μM (0.34–34 mg/liter) F 16618 for 48 h. For PAR1 agonist-induced cell proliferation, cells were starved for 24 h with smooth muscle basal medium containing 0.1% serum and 0.1% supplement (insulin, epidermal growth factor, and basic fibroblast growth factor) and then incubated for 48 h with 1% DMSO (vehicle) or F 16618 (1–100 μM) with or without 10 μM SFLLR or 10 UI/ml thrombin (Sigma-Aldrich). For proliferation tests without PAR1 expression, cells at 60 to 80% confluence were transfected with scrambled or PAR1 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Silencing of PAR1 mRNA was checked by real-time PCR and immunofluorescence (Supplemental Methods and Supplemental Fig. 1). Cell proliferation was assessed using a WST-1-based proliferation assay (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Each experiment included 12 wells for each condition and was repeated at least three times. For these experiments, cells were used up to six passages maximum.

Cell Migration Assays. Cell migration was studied by performing wound healing assays. Human aortic SMCs (20,000 cells) were plated in low 35-mm dishes with culture inserts (Ibidi, Martinsried, Germany). In some experiments, confluent cells were incubated in the presence of 20 μM mitomycin C for 2 h to inhibit cell proliferation. Inserts were then removed with sterile forceps to create a wound field of approximately 500 μm x 500 μm. The left carotid arteries were removed, prepared without endothelium, and mounted in organ baths (Emka Technology, Paris, France) as described previously (Bocquet et al., 2009). Cumulative concentration-response curves were obtained with the PAR1 agonist peptide SFLLR (0.1–100 μM with half log dose increment). Thrombin-induced cotrad strip was assayed using a single dose (10 μg/ml) to avoid receptor desensitization. The PAR1 antagonist F 16618 or corresponding vehicle (1% DMSO) was added 30 min before the SFLLR concentration-response curve. The amplitude of the tension was measured irrespective of the time. A single concentration of antagonist was tested per tissue sample.

Statistical Analysis. For tension measurement experiments, results are expressed as percentage of mean (E_max) ± S.E.M., where E_max was obtained with the higher dose of SFLLR (100 μM). Concentration-response curves were fitted using Origin 7.5 software (OriginLab Corp., Northampton, MA) to calculate EC50 and pA2 values. For in vitro and in vivo experiments, one-way analysis of variance was performed followed by a Dunnet’s test to compare each group. Differences were considered significant at p < 0.05.

Results

F 16618 Prevents Balloon Injury-Induced Restenosis of the Rat Carotid Artery. The effects of the PAR1 antagonist F 16618 were tested in a rat model of restenosis induced by balloon angioplasty of the rat carotid artery. As seen in Fig. 1A, left, PAR1 is expressed mainly in endothelial cells and SMCs of uninjured carotids. Fourteen days after balloon angioplasty, PAR1 expression in injured carotid appeared in both neointima and media (Fig. 1A, right). The carotid artery developed a neointima layer characteristic of restenosis compared with sham-operated rats (Fig. 1B). Daily oral administration of F 16618, 3 days before and 14 days after angioplasty, produced a dose-dependent reduction of restenosis with a maximal effect at 10 mg/kg (25 μM) (Fig. 1C). However, F 16618 has a bell-shaped response curve with less antirestenotic activity for higher doses (Fig. 1C). In another set of experiments, the efficacy of F 16618 was evaluated by a single intravenous injection during the surgical procedure. This protocol also inhibited restenosis, but only for the highest dose of 1.25 mg/kg (76 μM) (n = 8; P < 0.05) (Fig. 1D). These data suggest antiproliferative and/or antimigratory effects of F 16618 on SMCs.

F 16618 Inhibits Human Aortic Smooth Muscle Cell Proliferation. To determine whether F 16618 inhibits SMC proliferation, we used human aortic SMCs. As shown by Western blot analysis (Supplemental Methods), these SMCs expressed PAR1 (Fig. 2A). F 16618 inhibited serum-induced cell proliferation in a concentration-dependent manner with a maximal inhibitory effect of 64.5 ± 0.5% at 100 μM (Fig. 2B). To ascertain the direct effect of F 16618 on PAR1, human aortic SMCs were treated with siRNA directed against PAR1. Transfection of SMCs with PAR1 siRNA significantly lowered the basal cell proliferation and prevented the antiproliferative effect of F 16618 (Fig. 2B). This shows that PAR1 participates in part in serum-mediated SMC proliferation. We then investigated F 16618 efficiency on SMC proliferation stimulated by specific PAR1 agonists. As seen in Fig. 2, C and D, F 16618 inhibits the proliferative action of both SFLLR and thrombin in a concentration-dependent manner, with a maximal inhibitory effect at 10 μM. At this concentration, F 16618 totally prevented PAR1 agonist-activated proliferation. In contrast, when human aortic SMCs were stimulated by angiotensin II, F 16618 did not have any effect (data not shown). In addition, transfection of SMCs with PAR1-siRNA abolished the proliferative effect of thrombin and the inhibitory action of F 16618 (Fig. 2C, right). These data demonstrate that F 16618 inhibits thrombin/PAR1-mediated SMC proliferation.

F 16618 Inhibits Human Aortic Smooth Muscle Cell Migration. To examine whether F 16618 was also able to inhibit cell migration, human aortic SMCs were stimulated by heparin binding-epidermal growth factor (HB-EGF) and monococyte chemoattractant protein-1 (MCP-1), both of which contribute to the migratory effects of thrombin (Brandes et al., 2001; Kalmes et al., 2001). At 10 μM, the PAR1 antagonist had no significant effect on basal migration in control
medium, but it suppressed the promigratory action of MCP-1 and HB-EGF (Fig. 3A). Similar results were observed in the presence of 20 μM mitomycin (not shown). The PAR1 antagonist also suppressed the promigratory effect of thrombin, which increased human aortic SMC migration by 27% (Fig. 3B).

**F 16618 Has No Effect on Expression of Smoothelin, Early Growth Response-1, and Platelet-Derived Growth Factor in Injured Arteries.** In vivo, de-differentiation of vascular SMCs evidenced by loss of their contractile phenotype stimulates their migration and proliferation (Zargham, 2008). To explore whether the F 16618 treatments prevented restenosis by inhibition of SMC de-differentiation, we quantified the mRNA expression of the contractile protein smoothelin 24 h after angioplasty. The injury decreased by 75% the expression of smoothelin mRNA (Fig. 4, top). Neither single intravenous administration of F 16618 nor oral treatment had an effect on smoothelin expression. This indicates that the PAR1 antagonist is unable to block the SMC de-differentiation.

Platelet-derived growth factor (PDGF) is a mediator of SMC de-differentiation, migration and proliferation (Zargham, 2008). Its expression is up-regulated by the transcription factor early growth response-1 (Egr-1), which is rapidly induced at the endothelial wound edge after balloon angioplasty (Khachigian, 2006). To better define the in vivo mechanism of action of F 16618, we investigated its effect on the expression of Egr-1 and PDGF 24 h after angioplasty. In injured carotid arteries, the global expression of Egr-1 and PDGF mRNAs decreased by 40% (Fig. 4, middle). Whatever the treatment, F 16618 had no effect on the expression of Egr-1 and PDGF. Together, the results suggest that the acute phase after injury is characterized by a change in SMC phenotype but it is not yet associated with proliferative events.

**F 16618 Reduces Tumor Necrosis Factor α and Matrix Metalloproteinase-7 Expression without Affecting mRNAs of MCP-1, Interleukin-6, Tissue Inhibitor of Metalloproteinase-1, and Tissue Inhibitor of Metalloproteinase-2.** The response to arterial injury is the sequence of inflammatory events and extracellular matrix remodeling, which stimulate SMC migration and proliferation (Inoue and Node, 2009). To characterize how a single intravenous administration prevents restenosis, we next examined the influence of F 16618 on expression of inflammatory cytokines and matrix metalloproteinase (MMP). Tumor necrosis factor α (TNFα) is a key regulator of inflammatory responses (Monraats et al., 2005). Twenty four hours after angioplasty, its mRNA was expressed 4-fold more in injured carotid arteries than in noninjured ones (Fig. 5A, top). Both oral and intravenous F 16618 treatment significantly reduced the injury-mediated expression of TNFα. Interleukin 6 (IL-6) controls monocyte activation, and MCP-1 is involved in monocyte recruitment into injured vascular walls (Welt and Rogers, 2002; Brasier, 2010). Balloon injury caused 9- and 13-fold increases in the expression of IL-6 and MCP-1 mRNAs, re-
either 1‰ DMSO (Vehicle) or 100 nM F 16618 were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM or PAR1-directed siRNA. Blot is representative of three experiments. B, proliferation of human aortic SMCs that are cultured in 5% serum for 48 h with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM (Vehicle) or F 16618 ranging from 1 to 10 µM (n = 5) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by thrombin (n = 3–5). C, serum-starved SMCs transfected or not with scrambled siRNA (−) or PAR1-directed siRNA were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by thrombin (n = 3–5). D, serum-starved SMCs were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by SFLLR (n = 5) for 48 h. Cell proliferation for 48 h was quantified by colorimetric assay with WST-1 reagent. Results are means ± S.E.M. of n experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle; #, P < 0.05, ###, P < 0.001 versus SFLLR or thrombin.

Effect of F 16618 on PAR1 Expression. A, PAR1 expression was assessed by Western blot analysis. Blot is representative of three experiments. B, proliferation of human aortic SMCs that are cultured in 5% serum for 48 h with 1‰ DMSO (Vehicle) or F 16618 (n = 3–6). Right, proliferation of human aortic SMCs that are transfected with scrambled siRNA (−) (n = 3) or PAR1-directed siRNA (n = 3) and cultured for 48 h in 5% serum with either 1‰ DMSO (Vehicle) or 100 µM F 16618. Left, serum-starved SMCs were treated with 1‰ DMSO (Vehicle, n = 5) or F 16618 ranging from 1 to 10 µM (n = 3). C, serum-starved SMCs transfected or not with scrambled siRNA (−) or PAR1-directed siRNA were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by thrombin (n = 3–5). D, serum-starved SMCs were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by SFLLR (n = 5) for 48 h. Cell proliferation for 48 h was quantified by colorimetric assay with WST-1 reagent. Results are means ± S.E.M. of n experiments. *, P < 0.05; **, P < 0.01; *** P < 0.001 versus vehicle; #, P < 0.05, ###, P < 0.001 versus SFLLR or thrombin.

Effect of F 16618 on Re-Endothelialization of Injured Carotid Surface. We next studied whether F 16618 interferes with the primary inflammatory response without preventing monocyte activation and recruitment, which are required for the healing process.

MMP7 is known to be present in media and stenotic regions of vessels from patients with supravalvular aortic stenosis and Williams Beuren syndrome, but it is not expressed in normal artery (Dridi et al., 2005). In rat, the MMP7 mRNA was also poorly expressed in noninjured carotid arteries, but its expression increased 4-fold 24 h after balloon angioplasty (Fig. 5B, top). It is noteworthy that only the acute intravenous administration of F 16618 prevented the injury-mediated expression of MMP7. The oral treatment had no effect. After angioplasty, the expression of tissue inhibitors of metalloproteinase (TIMP-1, TIMP-2) seemed to be oppositely controlled (Fig. 5B, middle). The mRNA expression of TIMP-1 was up-regulated, whereas that of TIMP-2 was repressed. Neither oral nor intravenous F 16618 treatment affected TIMP-1 and TIMP-2 expression. Altogether, these results show that a single administration of F 16618 during a surgical procedure prevents early activation of MMP7 without affecting the endogenous inhibitors TIMP-1 and TIMP-2.

Effect of F 16618 on Re-Endothelialization of Injured Carotid Surface. We next studied whether F 16618 was able to modulate re-endothelialization, which is an essential step in the normal wound-healing process of injured vessels. In all uninjured carotids, the labeling of the endothelial marker, von Willebrand factor (vWF), was visible on the entire endothelial layer (Fig. 6A, top). At magnification ×400, proximity of the internal elastin lamina from the endothelial monolayer lets appear a yellow labeling. Under our conditions of balloon angioplasty, the re-endothelialization remained incomplete 14 days after the carotid artery injury (Fig. 6A, bottom). Re-endothelialization was also partial in injured carotids of rats treated with oral or intravenous F 16618 (Fig. 6A, bottom). Analysis of the vWF-labeled vessel perimeter showed that the decrease in fluorescence was similar in vehicle- and F 16618-treated rats (Fig. 6B). This suggests that the PAR1 antagonist reduces the formation of neointima without

Fig. 2. Effects of F 16618 on PAR1-dependent growth of human aortic smooth muscle cells. A, PAR1 expression was assessed by Western blot analysis. Blot is representative of three experiments. B, proliferation of human aortic SMCs that are cultured in 5% serum for 48 h with 1‰ DMSO (Vehicle) or F 16618 (n = 3–6). Right, proliferation of human aortic SMCs that are transfected with scrambled siRNA (−) (n = 3) or PAR1-directed siRNA (n = 3) and cultured for 48 h in 5% serum with either 1‰ DMSO (Vehicle) or 100 µM F 16618. Left, serum-starved SMCs were treated with 1‰ DMSO (Vehicle, n = 5) or F 16618 ranging from 1 to 10 µM (n = 3). C, serum-starved SMCs transfected or not with scrambled siRNA (−) or PAR1-directed siRNA were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by thrombin (n = 3–5). D, serum-starved SMCs were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by SFLLR (n = 5) for 48 h. Cell proliferation for 48 h was quantified by colorimetric assay with WST-1 reagent. Results are means ± S.E.M. of n experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle; #, P < 0.05, ###, P < 0.001 versus SFLLR or thrombin.

Fig. 3. Migration of human aortic smooth muscle cells is prevented by F 16618. When SMC monolayers reached confluence, the insert was removed to create a wound field of approximately 500 µm width, and cells were stimulated in medium containing 10% serum. Cell migration to the wound surface was monitored for 0 and 8 h. Ten fields of the wound area were photographed with a light microscope at 100× magnification. A, human aortic SMCs were treated with 1‰ DMSO (CTRL) or 10 µM F 16618 and stimulated or not with 25 ng/ml MCP-1 and 25 ng/ml HB-EGF. The migration distance of the wound edge was quantified by using the NIH Image software program. Histograms are mean ± S.E.M. of n experiments. *, P < 0.05; ***, P < 0.001 versus SFLLR or thrombin.

Fig. 4. Effects of F 16618 on PAR1-dependent growth of human aortic SMCs that are cultured in 5% serum for 48 h with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM (Vehicle) or F 16618 ranging from 1 to 10 µM (n = 3). C, serum-starved SMCs transfected or not with scrambled siRNA (−) or PAR1-directed siRNA were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by thrombin (n = 3–5). D, serum-starved SMCs were treated with 1‰ DMSO (Vehicle) or F 16618 ranging from 1 to 10 µM. Cells were then stimulated or not by SFLLR (n = 5) for 48 h. Cell proliferation for 48 h was quantified by colorimetric assay with WST-1 reagent. Results are means ± S.E.M. of n experiments. *, P < 0.05; **, P < 0.01; *** P < 0.001 versus vehicle; #, P < 0.05, ###, P < 0.001 versus SFLLR or thrombin.
significant effect on the re-endothelialization process in our model of vessel injury.

**F 16618 Abolishes the SFLLR- and Thrombin-Induced Rat Denuded Carotid Artery Contraction.** The loss of endothelium is associated with enhanced contractile response induced by both SMC exposure to the vessel lumen and up-regulation of PAR1 (Fukunaga et al., 2006; Kai et al., 2007). Thus, we investigated the effect of F 16618 on vasoconstriction of rat denuded carotid arteries. Thrombin produced a contraction of the carotid, which was prevented with F 16618 treatment in a concentration-dependent manner (Fig. 7A). Half-maximal inhibitory concentrations of F 16618 treatment in a concentration-dependent manner induced a contraction of the carotid, which was prevented with F 16618 administered either orally 3 days before and 14 days after angioplasty at the dose of 1.25 mg/kg. Twenty four hours after balloon angioplasty, noninjured and injured carotids were harvested, and total mRNA was extracted. The cDNA expression was evaluated by real-time PCR analysis. The expression level of targeted mRNAs was expressed as ratio to hypoxanthine-guanine phosphoribosyl transferase (HPRT). Data are mean ± S.E.M. of four to six rats/group. **, P < 0.01 and ###, P < 0.001 versus noninjured carotid.

2009; Létienne et al., 2010b). Herein, we show that F 16618 inhibits late neointimal formation in rat injured carotid artery through anti-inflammatory activities and exhibits anti-proliferative, anti-migratory, and antivasoconstrictor properties in vitro and ex vivo.

**Chronic oral and acute intravenous administration of F 16618 similarly inhibited neointimal formation.** This shows that the blockade of PAR1 by a single administration of F 16618 during balloon angioplasty is sufficient to prevent neointimal hyperplasia. Antirestenotic effect of an acute perivascular administration of PAR1 antagonist has been reported previously in the rat carotid model (Andrade-Gordon et al., 2001). Single administration of 25 mg/kg (αS)-N'-(1S)-3-amino-1-[(phenylmethyl)-amino]carbonyl] propyl]-N-[[[1-(2,6-dichlorophenyl)methyl]-3-(1-pyrrolidinylmethyl)-1H-indazol-6-yl]amino]carbonyl][amino]-3,4-difluorobenzene propanamide) (RWJ-58259) effectively reduced the neointimal formation in injured arteries. In our study, a single intravenous administration of 1.25 mg/kg F 16618 was adequate to reduce neointimal hyperplasia.
higher efficiency of F 16618 could result from direct contact with circulating cells and also from its antithrombotic effect, a property not shared by RWJ-58259 (Andrade-Gordon et al., 2001).

In our experimental restenosis model, thrombosis precedes neointima formation. We recently demonstrated that a single intravenous administration of selective PAR1 antagonists, including F 16618, exerts antithrombotic activity in an arterio-venous shunt model in rats (Létienne et al., 2010a,b). Previous experiments with PAR1 agonist peptides suggested that PAR1 is not functional in rat platelets (Kinlough-Rathbone et al., 1993). PAR1 antagonists may exert their antithrombotic effect by inhibiting activation of endothelial cells at the wound edge. Early induction of Egr-1 and PDGF has been detected at the endothelial wound edge after catheter scrape injury to rat aorta (Khachigian, 2006). Here, we observed a reduction of Egr-1 and PDGF expression in the acute phase after angioplasty. These reductions indeed paralleled those of the endothelial markers thrombomodulin and intercellular adhesion molecule-1 (Supplemental Fig. 2). Such decreases probably result from removal of endothelium.

Thus, it is hard to conclude with our global mRNA analysis whether activation of endothelial cells at the wound edge is inhibited by F 16618.

By denuding the vessel of its protective endothelium, balloon angioplasty creates a local environment that favors vasospasm and contributes to constrictive remodeling. Thrombin is a potent vasoconstrictor that participates in initiation
of vasospasm at the site of vascular injury. In addition, up-regulation of PAR1 expression contributes to the enhanced contractile response to thrombin in injured arteries (Fukunaga et al., 2006). In the present study, F 16618 shows no effect on re-endothelialization 14 days after injury but inhibits the contractile response to PAR1 agonists in endothelium-denuded carotid arteries. Similar results have been obtained with other arteries (Bocquet et al., 2009). In vivo, the acute treatment with F 16618 may compensate for the vasoconstriction induced by endothelial loss and artery enlargement, thereby preventing vasospasm-induced remodeling.

During the acute phase after de-endothelialization, neointimal formation is initiated by vasospasm and also by local inflammatory reaction (Zargham, 2008). Consequently, activated platelets and leukocytes release cytokines, growth factors, and MMPs, which initiate SMC proliferation and migration (Welt and Rogers, 2002; Muto et al., 2007; Zargham, 2008; Inoue and Node, 2009). Increased plasma levels of MCP-1, MMPs, and TIMP-1 have been observed in human postangioplasty restenosis (Cipollone et al., 2001; Jones et al., 2009). In addition, a haplotype of the human gene coding for the inflammatory cytokine TNFα is associated with increased risk of restenosis (Monraats et al., 2005). In our experimental model, vascular injury induces marked expression of TNFα, IL-6, MCP-1, MMP7, and TIMP-1. Both acute and chronic F 16618 treatments reduce the expression of TNFα, which is produced by endothelial cells and leukocytes. In contrast, only acute delivery of F 16618 inhibits the expression of MMP7. This suggests requirement of high F 16618 concentration to penetrate into the vascular tissue to target macrophages and fibroblasts, which synthesize MMP7 (Woessner, 1995; Galis and Khatri, 2002). It should be noted that F 16618 partly affects the inflammatory response required for vascular healing because it has no effect on IL-6 and MCP-1 expression.

We also demonstrate that F 16618 suppresses in vitro migration and proliferation of human aortic SMCs at a concentration of 10 μM, which is below those efficient in vivo. Indeed, in the case of daily oral administration, maximal inhibitory effect was observed at a plasma concentration of 25 μM. Because at this concentration F 16618 does not prevent the loss of the contractile protein smoothelin, chronic administration of F 16618 could inhibit restenosis by reducing TNFα expression and migration/proliferation of de-differentiated SMC. For the intravenous administration during angioplasty, a concentration of 75 μM is needed for efficient inhibition of TNFα and MMP7 expression and neointima formation. In vivo, F 16618 interacts with SMC, but also with endothelial cells, fibroblasts, and circulating leukocytes. In humans, F 16618 will also interact directly with platelets. Therefore, the effective concentration is higher in vivo than in vitro.

Although our animal model of arterial injury provides evidence for a role of PAR1 signaling in the restenosis process, it cannot predict the efficacy of PAR1 antagonist in human pathology. In fact, immunosuppressors, anti-inflammatory drugs, antiplatelet agents, anticoagulants, calcium-channel blockers, and angiotensin-converting enzyme inhibitors reduced the late neointimal thickening in experimental models but have failed to show any benefit for the prevention of restenosis in humans (Welt and Rogers, 2002; Inoue and Node, 2009). By systemic administration, only drugs with pleiotropic actions show efficiency in humans (Douglas, 2007; Wessely, 2010). With its potent antioxidant and antiproliferative properties, the lipid-lowering agent probucol is effective in preventing restenosis in both rats and humans (Douglas, 2007). The phosphodiesterase type III blocker cilostazol, which is used as antiplatelet agent, exerts antiproliferative and lipid-lowering effect and also promotes SMC relaxation (Douglas, 2007). It is also effective in humans. Because the PAR1 antagonist F 16618 negatively modulates thrombosis, inflammation, cell proliferation and migration, and regulation of vascular tone, it could be suitable for preventing postangioplasty restenosis in humans.

However, the direct thrombin inhibitors heparin and bivalirudin, which also have pleiotropic actions, have demonstrated beneficial effects on restenosis in rabbit models but not in humans (Burchenal et al., 1998). This paradox may be explained by the fact that inhibition of thrombin alone is not sufficient to prevent restenosis in humans and drugs were administered for only 24 h after angioplasty in the clinical trial. Because F 16618 blocks PAR1 auto-activation, this compound inhibits signaling events induced not only by thrombin but also by coagulation factors VII and X and some MMPs (Camerer et al., 2000; Borensztajn et al., 2008). Moreover, F 16618 is an orally active drug, with fewer side effects than direct thrombin inhibitors (Létiéenne et al., 2010b). This allows the consideration of chronic treatment until complete healing. All of these characteristics suggest that chronic treatment with F 16618 could be effective in the prevention of human restenosis.

Whether systemic administration of F 16618 fails to prevent postangioplasty restenosis in humans, it remains an attractive strategy for locally inhibiting in stent restenosis. Indeed, the immunosuppressive, antiproliferative, and anti-inflammatory drug sirolimus prevented intimal hyperplasia in rat injured artery but has failed to show any benefit in humans when it was systemically administered (Inoue and Node, 2009). However, sirolimus and its derivates have shown beneficial effects when they were locally and chronically released by drug-eluting stents (Inoue and Node, 2009; Wessely, 2010). Stented arteries are subjected to sustained proinflammatory and prothrombotic status (Welt and Rogers, 2002). Hence, these processes may be prevented by the antithrombotic and anti-inflammatory properties of F 16618, in addition to its effect on neointimal hyperplasia. Moreover, at the high concentration of 75 μM, intravenous administration of F 16618 significantly delayed the time of thrombotic occlusion without affecting the bleeding time and without hemodynamic effect in rat (Létiéenne et al., 2010b). Indeed, the potent antithrombotic activity of F 16618 is potentiated when combined with aspirin or clopidogrel without further increasing the bleeding time. The broad therapeutic range of F 16618 may facilitate its use on coated stents in the context of standard of care in percutaneous coronary intervention and coronary artery bypass graft.

In conclusion, our results suggest that F 16618 prevents restenosis by limiting early inflammatory events, MMP release, SMC migration/proliferation, and vascular contraction. These properties could be of particular interest for acute treatment during percutaneous coronary intervention or chronic release by eluting stents.