Tissue Factor Pathway Inhibitor Attenuates Procoagulant Activity and Upregulation of Tissue Factor at the Site of Balloon-Induced Arterial Injury in Pigs

James St. Pierre, Lu-Ying Yang, Kamala Tamirisa, David Scherrer, Pamela De Ciechi, Paul Eisenberg, Eser Tolunay, Dana Abendschein

Abstract—Intravenous infusion of recombinant tissue factor pathway inhibitor (rTFPI) for 24 hours decreases neointimal thickening and luminal stenosis 1 month after balloon-induced injury to the carotid arteries in minipigs. This study was designed to determine whether the effect of rTFPI is accounted for by early decreases in procoagulant activity and thrombosis on the injured vessel wall. Vascular injury was induced by balloon hyperinflations in both carotid arteries of anesthetized pigs given no anticoagulant as a control (n=16), an intravenous infusion for 24 hours of rTFPI (0.5 mg/kg bolus and 25 μg·kg⁻¹·min⁻¹, n=14), or an intravenous infusion of unfractionated heparin (100 U·kg⁻¹·h⁻¹, n=19). Accumulation of radiolabeled autologous platelets was markedly decreased over 24 hours on injured arteries from animals given rTFPI (0.6×10⁶/cm²) compared with controls (2.5×10⁶/cm², P=0.0004). Deposition of radiolabeled fibrin was also decreased in rTFPI-treated animals (269±266 μg/cm²) compared with controls (2389±1673 μg/cm², P=0.04). Similar effects were observed with heparin. However, factor Xa activity, assayed after 24 hours by incubation of the injured arterial segments with the chromogenic substrate S-2222, was decreased more markedly on arteries from rTFPI-treated animals (0.14±0.13 OD) than those from heparin-treated animals (0.29±0.18 OD) compared with controls (0.47±0.24 OD, P=0.0007). In addition, arteries from rTFPI-treated animals showed a 4-fold lower induction of tissue factor protein compared with controls (P=0.0002). Attenuation of procoagulant activity and tissue factor–mediated thrombin generation in response to injury may account for the promising results with rTFPI in the porcine angioplasty model. (Arterioscler Thromb Vase Biol. 1999;19:2263-2268.)

Key Words: restenosis ■ tissue factor pathway inhibitor ■ tissue factor ■ thrombin

Restenosis after percutaneous transluminal coronary angioplasty occurs in ≈1/3 of treated patients¹⁻² and thus remains a major stigma of interventional cardiology. The mechanisms of restenosis are complex, but elastic recoil, proliferation of extracellular matrix and smooth muscle cells, and thrombosis have all been implicated.³⁻⁵ Thrombin has been an attractive target for pharmacological intervention to attenuate restenosis because it activates platelets, converts fibrinogen to fibrin-forming thrombus, and also serves as a potent mitogen for vascular smooth muscle cells.³ However, although brief administration of high doses of direct inhibitors of thrombin, such as recombinant hirudin, at the time of balloon-induced arterial injury have been shown to reduce neointimal thickening and stenosis in experimental animals,⁶⁻⁸ lower dosages employed in patients to avoid bleeding complications have decreased the rate of early clinical events but not the incidence of restenosis.⁹

The fact that antithrombin agents have not attenuated restenosis after angioplasty may be a consequence of persistent activation and association of activated factor X (factor Xa) with the injured vessel wall, which can continue to convert prothrombin to thrombin. In addition, activation of factor X by the complex of factor VIIa and tissue factor may be potentiated because tissue factor is upregulated in the wall of balloon-injured vessels¹⁰ and is increased in atherosclerotic plaques.¹¹⁻¹⁴

Recently, we have reported a marked reduction in neointimal thickening and stenosis 1 month after balloon-induced injury to the carotid artery in minipigs given a 24-hour, but not a 3-hour, constant intravenous infusion of recombinant tissue factor pathway inhibitor (rTFPI), the full-length, recombinant mimic of the physiological inhibitor of the complex of tissue factor and factor VIIa.¹⁵ However, whether the benefit of rTFPI resulted from inhibition of acute thrombus formation, vascular procoagulant activity, or both has not been defined. The goals of this study were to assess the effects of rTFPI on the accumulation of thrombus and the activity of factor Xa associated with balloon-injured carotid arteries in pigs, as well as the effect of rTFPI on the generation of tissue factor in response to balloon-induced arterial injury.

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**Protocol**

<table>
<thead>
<tr>
<th>Group 1 (n=20)</th>
<th>Group 2 (n=20)</th>
<th>Group 3 (n=20)</th>
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<tbody>
<tr>
<td>rTFPI: 0.5 mg/kg bolus and 25 μg/kg/min infusion</td>
<td>Heparin: 100 U/kg infusion</td>
<td>Control: no additional anticoagulant</td>
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<tr>
<td>-30 min: 200 U/kg heparin bolus</td>
<td>15 min: Balloon injury, carotids</td>
<td>23 h: Excise injured segments</td>
</tr>
<tr>
<td>60 min: Balloon injury, carotids</td>
<td>5 h: rTFPI bolus (n=6)</td>
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<tr>
<td>23 h: rTFPI bolus (n=6)</td>
<td>24 h: Excise injured segments</td>
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**Figure 1. Summary of experiment protocol.**

**Methods**

**Animal Preparations**

Procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Studies Committee at Washington University. Male domestic pigs (Oak Hill Genetics, Ewing, IL) weighing 17 to 22 kg were fed a nonatherogenic diet. Sciences, 1996) and were approved by the Animal Studies Committee.

**Experiment Protocol**

The pigs were randomly assigned to 1 of 3 treatment groups (Figure 1): rTFPI administered as a bolus (0.5 mg/kg) and subsequent infusion (25 μg · kg⁻¹ · min⁻¹) for 24 hours; additional heparin infused (100 U · kg⁻¹ · h⁻¹) for 24 hours; or no additional anticoagulation as a control. rTFPI was the full-length, 34-kDa human protein derived from Escherichia coli. The dosage of rTFPI was one we have shown recently to be effective for attenuating stenosis in minipigs with minimal perturbation of systemic hemostasis.

Fifteen minutes after the onset of infusions of antithrombotic agents, a balloon catheter (ProFlex 5, 8 mm ×2.0 cm; Mallinckrodt Inc) was advanced over a 0.014-in. guide wire into the left carotid artery, which is level with the 50- and third cervical vertebrae. The balloon was inflated 5 times to a distending pressure of 8 atm for 30 seconds with 60 seconds between inflations, which we and others have shown induces the multiple fractures of the internal elastic lamina that are required to induce a robust neointimal response. In some of the animals, the balloon catheter was then advanced into the right carotid artery and the injury procedure repeated. After carotid arterial injury, the femoral sheath was removed, and a bolus of unfractionated heparin (200 U/kg IV) was administered to prevent clot formation in the catheters.

In many of the animals, either immediately before carotid injury or 23 hours after injury, radiolabeled autologous platelets (8 × 10⁴) were injected into each animal at the time of platelet infusions. The assay of factor Xa and total procoagulant activity. The assayed segments were then placed in Molecular Biology Fixative (Streck Laboratories) and counted in a gamma counter to determine the number of radiolabeled platelets bound to the surface. Three to 4 weeks later, the segments were recounted for radiolabeled fibrinogen.

**Radiolabeling of Platelets and Fibrinogen**

Platelets in 30 mL of citrated (3.8%) autologous venous blood were freshly isolated and labeled with ¹¹¹In-oxine by methods modified from those of Heaton et al. ²¹ In brief, the blood was added to a tube containing 1 μmol/L (final concentration) of prostaglandin E₁, and centrifuged at 960g for 15 minutes. The supernatant containing the platelets was withdrawn, and the platelets were pelleted by centrifugation at 2000g. The platelets were resuspended and washed twice with 2.7 mmol/L phosphate buffer, counted in a hemocytometer, and incubated for 20 minutes at room temperature with 1 μCi of ¹¹¹In-oxine (Nycomed-Amersham). The radiolabeled platelets were washed twice with buffer and resuspended in buffer, and an aliquot (10 μL) was counted for radioactivity. Typically, 2 × 10⁴ platelets were labeled (7 ± 4.0 × 10⁴ disintegrations per minute per platelet, n=30), and >95% of the radioactivity was confirmed as being associated with the platelets after pelleting and counting the supernatant. The entire platelet suspension was reinjected into each animal.

Fibrinogen purified from porcine plasma was purchased (Sigma), and 2 mg was labeled with 1 μCi of Na¹¹¹I (ICN) by the chloramine T method with the use of Iodobeads (Pierce) as described previously.¹⁰ The ¹²⁵I-fibrinogen was separated from free Na¹¹¹I by gel filtration through a G-25 column. Radioactivity bound to protein (3 ± 1.0 × 10⁴ dpm/μg, n=30) was routinely >90% precipitable in 10% trichloroacetic acid. A 250-μg aliquot of radiolabeled fibrinogen was injected into each animal at the time of platelet infusions.

Vessel segments were counted initially for ¹¹¹In-labeled platelet radioactivity in a well gamma counter with a window setting of 130 to 900 keV. After allowing at least 3 weeks for the ¹¹¹In to decay (6 half-lives of 2.8 days), the vessels were recounted for ¹²⁵I-labeled fibrinogen as a control with a window setting of 20 to 80 keV. Accumulations of radiolabeled platelets and fibrinogen were determined from the decay-corrected deposited activity (dpm) divided by the product of activity per platelet or per microgram of fibrinogen injected and the luminal surface area (centimeters squared) as described previously.²¹

**Assay of Factor Xa and Total Procoagulant Activity on Carotid Segments**

Arterial segments were cannulated on each end with pieces of silicone tubing (0.24-mm internal diameter, 0.49-mm external diameter; Technical Products, Inc) adjusted so that ~1.5 cm of the luminal surface of the vessel was exposed between the ends of the tubing. The segment was immersed in a tray containing PBS at 35°C, and the lumen was equilibrated with 10 mL of PBS perfused at 1 mL/min.

To specifically characterize preformed factor Xa associated with the vessel wall, segments were incubated with S-2222 substrate (450 μmol/L final concentration of N-Benzoyl-L-Ile-Glu-Gly-Arg-p-nitroaniline; Chromogenics) in 0.2 mol/L Tris-HCl at pH 6.4 containing 2 mmol/L CaCl₂ (final concentration). After 30 minutes, the solution was withdrawn and proteolysis of S-2222 was measured using the change in absorbance at 405 nm in an automated microplate reader at 37°C (ThermoMax, Molecular Devices). Levels of factor Xa were defined by comparison with a standard curve generated with purified factor Xa.

To characterize the total procoagulant activity of the luminal surface attributable to bound factor Xa and thrombin as well as the potential for de novo elaboration of thrombin, segments were flushed with PBS and then perfused with recalcified (25 mmol/L CaCl₂, final concentration), citrated, human pooled plasma (American Red Cross, St. Louis, Mo) at 1 mL/min for 10 minutes as described previously.²¹ Samples of the perfusing plasma and effluent plasma after 10 minutes were assayed for fibrinopeptide A (FPA) with use of a
previously validated radioimmunoassay as an index of the rate of thrombin-induced fibrin formation. FPA values in the 10-minute effluent sample were subtracted from those in the perfusate to identify the increase attributable to thrombin elaborated on the vessel.

**Purification and Characterization of Antibodies to Porcine Tissue Factor**

Monoclonal antibodies were generated to the extracellular domain of recombinant porcine tissue factor (derived from E. coli), residues 1 to 208 (molecular weight ~22.8 kDa), which is 76% identical and 81% similar to human tissue factor. One of the antibodies, P199.8C7.A1, an IgG2a with a κ light chain, was purified from ascites by application to a Hi-Trap protein G column (Pharmacia Biotech) equilibrated and washed with 20 mmol/L sodium phosphate at pH 7.0. The antibody was eluted with 0.1 mol/L glycine–HCl at pH 2.7 and neutralized with 1 mol/L Tris buffer at pH 9.0. Antibody sensitivity and specificity were determined with use of ELISA and Western blotting assays according to standard methods. The lowest dilution of P199.8C7.A1 still capable of recognizing recombinant porcine tissue factor was 0.25 μg/mL.

Polyclonal antibodies were generated in rabbits by using the same recombinant porcine tissue factor and purified by using the Hi-Trap protein G column as described above. The purified polyclonal antibody was shown to recognize recombinant porcine tissue factor in a Western blot assay.

**Western Blot Analysis of Tissue Factor Protein**

The frozen vessels were ground in a mortar under LN₂, and the protein was extracted by sonication in ice-cold, Tris-buffered saline (TBS, 8 mL/g of tissue), pH 8.0, containing 0.1% SDS and protease inhibitors (30 μg/mL aprotinin, 10 μg/mL pepstatin, and 10 μg/mL PMSF; Sigma). The samples were incubated at 37°C for 30 minutes and centrifuged at 12 000g for 20 minutes. The supernatant was removed and recenterfuged at 39 000g for 30 minutes, and the pellet was resuspended in TBS containing 100 μg/mL EDTA with use of a Polytron. After incubation for 30 minutes at 37°C and another centrifugation at 12 000g for 10 minutes, the protein concentration in the supernatant was determined (Micro bichinonic acid protein assay reagent, Pierce). Extracted protein samples were reduced with 100 mmol/L DTT and denatured at 95°C for 10 minutes. The samples were separated by SDS–polyacrylamide gel electrophoresis (4% to 15% Ready Gels; Bio-Rad) at 200 V for 30 minutes, and the protein was transferred to nitrocellulose (Hybond ECL, Amersham) in Towbin’s Tris-glycine buffer containing 20% methanol at 100 V for 20 minutes. The blot was blocked and peroxidase activity was blocked by incubation for 30 minutes with H₂O₂ in methanol. Nonspecific binding of antibody was blocked by incubation for 45 minutes in PBS containing 1% BSA, 0.3% Triton X-100, 10% normal goat serum, and 1% normal pig serum. The sections were incubated overnight at 4°C with the rabbit anti-pig tissue factor antibody diluted 1:100 in the blocking solution. After the slides were rinsed with PBS, the sections were incubated for 45 minutes at room temperature with biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) diluted 1:200 with blocking buffer. The sections were then incubated with streptavidin-peroxidase for 10 minutes, stained for 2 minutes with 3-amin-9-ethylcarbazole (Histostain Plus, Zymed Laboratories), and counterstained with hematoxylin (Richard Allen Scientific).

**Statistical Analysis**

Data are reported as mean±SD. Platelet and fibrin deposition, factor Xa and total procoagulant activities, and fold changes in vascular tissue factor levels between treatment groups were compared by ANOVA with use of the Bonferroni/Dunn test or unpaired Student’s t test for specific contrasts. Significance was defined as P≤0.05.

**Results**

Sixty pigs were randomized to the 3 treatment groups. Nine did not complete the protocol either because they died after the balloon injury procedure (n=3) or because the infusion of anticoagulant was interrupted (n=6). Data from 2 other animals were not analyzed because the balloon-injured artery failed to exhibit multiple fractures of the internal elastic lamina. Thus, the final groups comprised 14 rTFPI-treated, 19 heparin-treated, and 16 control animals.

**Accumulation of Thrombus**

Deposition of radiolabeled platelets injected either immediately before the injury (24 hours before recovery of the vessels) or 23 hours after the injury procedure (1 hour before recovery of the vessels) was decreased markedly by constant infusions of either rTFPI or heparin compared with controls (Figure 2). The mean and SD in the 23- to 24-hour control group was high because of unexplained high values in 2 of the animals (31.1 and 16.6×10⁶/cm²). There were no differences in deposition of platelets between rTFPI- and heparin-treated animals.

Radiolabeled fibrinogen deposition at the site of injury did not differ between groups over the 23- to 24-hour interval but was decreased for both rTFPI- and heparin-treated animals compared with controls over the 0- to 24-hour interval (Figure 3). The mean and SD in the 0- to 24-hour control group was high because of a high value in 1 of the animals (847 ng/cm²). There were no differences in accumulation of fibrinogen between rTFPI- and heparin-treated animals.
Factor Xa and Total Procoagulant Activities

Factor Xa associated with the luminal surface of the arteries 24 hours after balloon-induced injury was decreased by infusion of either rTFPI or heparin, but the decrease with rTFPI was more marked compared with controls (Figure 4). In addition, thrombin-mediated procoagulant activity, reflected by FPA generation in recalcified human plasma perfused for 10 minutes over the luminal surface of the injured vessels, tended to be lower for rTFPI-treated animals (853±376 ng/mL, n=11) compared with control animals (1351±854 ng/mL, n=8, P=0.2). However, procoagulant activity on vessels from rTFPI-treated animals was significantly lower than that on vessels from heparin-treated animals (1952±1229 ng/mL, n=12, P=0.007).

Tissue Factor Protein

Two bands were observed by Western blot analysis of vessel extracts: a major band at 43 kDa, as predicted for tissue factor and observed in extracts of porcine brain, and a minor band at 38 kDa (Figure 5). Both were eliminated by preincubation of the samples with the antibody to tissue factor, indicating that the minor band was also derived from tissue factor. Recombinant porcine tissue factor used as a control separated at 23 kDa because it lacked the intracellular domain. Probing the blots with a monoclonal antibody against human rTFPI (provided by James Wun, PhD, at Monsanto) revealed a single band at 32 kDa, indicating that the tissue factor was not complexed with rTFPI (data not shown).

Ballooning overstitch injury resulted in a nearly 4-fold induction of vascular tissue factor protein (3.3±0.4 ng/µg of total protein, n=4) compared with noninjured arterial segments (0.8±0.1 ng/µg, n=4, P=0.00005; Figure 5). Heparin administration partially attenuated the induction of tissue factor (2.3±0.2 ng/µg, n=4, P=0.04, compared with control injured arteries), but rTFPI treatment essentially abolished the increase in tissue factor associated with vessel injury (0.9±0.1 ng/µg, n=4, P=0.0002, compared with control injured arteries).

Histological Localization of Tissue Factor

In balloon-injured arterial segments, tissue factor was detected transmurally, both associated with thrombus along dissection planes through the media and on the surface of smooth muscle cells (Figure 6). Staining was also prominent on the luminal surface and beneath the internal elastic lamina near sites of laminar disruption (Figure 6C). In contrast, tissue factor staining in noninjured carotid arterial segments was localized to the adventitia, especially around vessels of the vasa vasorum and the subintima beneath the internal elastic lamina (data not shown).

Discussion

Our results show that administration of rTFPI over the first 24 hours after balloon overstitch injury to the carotid arteries in pigs attenuates accumulation of thrombus at the injury site, an effect not quantitatively different from that achieved by constant infusion of unfractionated heparin (Figures 2 and 3). However, the potential for the injured vessel wall to generate thrombin, indicated by both the level of factor Xa activity on the luminal surface as well as the amount of tissue factor elaborated in response to injury, was decreased more markedly by rTFPI than by heparin (Figures 4 and 5).

Decreased factor Xa activity, likely in the form of prothrombinase (factors Xa/Va), associated with the site of balloon injury in rTFPI-treated animals probably resulted from inhibition of the complex of tissue factor and factor VIIa. This complex forms a quaternary inhibitory complex with rTFPI, factor Xa, and calcium that is incapable of generating additional factor Xa.25 Formation of the quaternary complex may be essential for the efficacy of rTFPI because Lindahl et al24 reported that the combination of rTFPI, factor Xa, and factor VIIa, but not either rTFPI alone or rTFPI and factor VIIa, reduced thrombus deposition on the subendothelial matrix under moderate shear rates in vitro. rTFPI may have also
animals showed no increase in tissue factor protein, averaging overstretch injury of rat aortas. However, rTFPI-treated finding is similar to the response reported after balloon surface of medial smooth muscle cells (Figure 6). This lamina, in association with mural thrombus, and on the detected transmurally beneath the traumatized internal elastic uninjured arteries (Figure 5). The increased tissue factor was expected. Injured carotid arteries from control animals exhibi-
mately reduce neointimal thickening 1 month after balloon overstretch injury in the carotid arteries of minipigs. Antico-
agulation with unfractionated heparin has also failed to attenuate restenosis after angioplasty, which appears to inhibit thrombin generation both by decreasing the number of platelets in thrombi that support assembly of prothrombinase and by interfering with platelet activation.

Despite variability in the accumulation of radiolabeled platelets and fibrinogen among control animals, it is apparent that both rTFPI and heparin reduced the net accumulation of thrombus over time (Figures 2 and 3). Interestingly, however, the same dosages of heparin administered in our previous studies did not significantly reduce neointimal thickening 1 month after balloon overstretch injury in the carotid arteries of minipigs. Antico-
agulation with unfractionated heparin has also failed to attenuate restenosis after angioplasty in patients. This suggests that acute reductions of thrombus accumulation per se are insufficient to achieve the benefit observed by inhibition of thrombin generation as with rTFPI.

**Study Limitations**

Our results showing less procoagulant activity on carotid arteries treated with rTFPI for 24 hours must be interpreted with caution in terms of the relevance for coronary angioplasty in humans. The injury induced by overstretch of the elastic peripheral arteries is more modest than the injury induced by angioplasty of the muscular coronary arteries, so vascular responses may differ. In addition, considering that tissue factor accumulates in atherosclerotic plaques, mural generation of thrombin in human atherosclerotic coronary arteries subjected to angioplasty may continue for a longer interval than predicted from animals with normal vessels. Another limitation of our experiments is that assays of factor Xa and procoagulant activity on excised vessels may not measure the same activity in vivo because washing procedures could have removed some of the anticoagulants associated with the bound factors.

In summary, we have shown that factor Xa activity on the luminal surface and the mural generation of tissue factor are both decreased markedly by administration of rTFPI during the first 24 hours after balloon-induced arterial injury. This results in both decreased thrombus accumulation on the luminal surface and, perhaps more important, a diminished capacity to generate intramural thrombin, which may provide
prolonged stimulation of the injured vessel wall, leading to neointimal thickening and luminal stenosis in this animal preparation. Accordingly, the promising results observed with rTFPI in the porcine carotid angioplasty preparation likely result from decreased local thrombin generation, an approach that may be proven clinically effective once conditions for thrombin generation after angioplasty of atherosclerotic coronary arteries are better understood.

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