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Role of the Endothelium in the Vascular Effects of the Thrombin Receptor (Protease-Activated Receptor Type 1) in Humans

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Objectives
The purpose of this study was to determine the role of the endothelium in the vascular actions of protease-activated receptor type 1 (PAR-1) activation in vivo in man.

Background
Thrombin is central to the pathophysiology of atherothrombosis. Its cellular actions are mediated via PAR-1. Protease-activated receptor type 1 activation causes arterial vasodilation, venoconstriction, platelet activation, and tissue-type plasminogen activator release in man.

Methods
Dorsal hand vein diameter was measured in 6 healthy volunteers before and after endothelial denudation. Forearm arterial blood flow, plasma fibrinolytic factors, and platelet activation were measured in 24 healthy volunteers during venous occlusion plethysmography. The effects of inhibition of prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor on PAR-1 responses were assessed during coadministration of aspirin, the “NO clamp” (L-NG-monomethyl arginine and sodium nitroprusside), and tetraethylammonium ion, respectively.

Results
Endothelial denudation did not affect PAR-1–evoked venoconstriction (SFLLRN; 0.05 to 15 nmol/min). Although aspirin had no effect, SFLLRN-induced vasodilation (5 to 50 nmol/min) was attenuated by the NO clamp (p < 0.0001) and tetraethylammonium ion (p < 0.05) and abolished by their combination (p < 0.01). The NO clamp augmented SFLLRN-induced tissue-type plasminogen activator and plasminogen activator inhibitor type 1 antigen (p < 0.0001) release, but tetraethylammonium ion and aspirin had no effect. SFLLRN-induced platelet activation was unaffected by NO or prostacyclin inhibition.

Conclusions
Acting via PAR-1, thrombin causes contrasting effects in the human vasculature and has a major interaction with the endothelium. This highlights the critical importance of endothelial function during acute arterial injury and intravascular thrombosis, as occurs in cardiovascular events including myocardial infarction and stroke. (J Am Coll Cardiol 2008;51:1749–56) © 2008 by the American College of Cardiology Foundation

Thrombin plays a central role in the coagulation cascade and thrombosis (1). It is one of the most powerful physiological agonists in the cardiovascular system, and its actions are fundamental to the processes of atherosclerosis and its thrombotic consequences.

In addition to the enzymatic generation of fibrin, thrombin stimulates a range of cell types including platelets, endothelial cells, and vascular smooth muscle cells. An extensive search for thrombin receptors ultimately culminated in the identification of a group of G-protein coupled receptors termed protease-activated receptors (PARs).

These receptors are characterized by a unique mechanism of activation whereby the receptor undergoes proteolytic cleavage, unmasking a short peptide sequence that remains tethered and auto-activates the receptor (2,3). To date, 4 different types of PARs have been identified: PAR-1, -3, and -4 are all activated by thrombin; PAR-2 is mainly activated by trypsin but transactivation of PAR-2 by cleaved PAR-1 has been recognized (4,5).
Protease-activated receptor type 1 receptors are the principal thrombin receptors in man and extensive studies in small animals and cell cultures suggest that they have a diverse and important role in various organs. Their activation stimulates a network of G-protein coupled signaling pathways that involve phospholipase C, protein kinase C, calcium release, mitogen-activated protein kinases, and potassium channels. However, there is significant species heterogeneity with pre-clinical studies of limited relevance to man. Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and be important in the clinical development of new therapeutic strategies.

To understand the physiological actions of thrombin in the human vasculature is challenging because direct thrombin instillation has the potential to cause acute thrombosis in situ and hence vascular occlusion. The use of a PAR-1 receptor agonist, however, permits the direct assessment of cellular responses to thrombin without the enzymatic activation of the coagulation cascade and fibrin formation. Using the short peptide mimetic SFLLRN, we have recently described the in vivo effects of PAR-1 activation in platelets, endothelium and vascular smooth muscle in man. For the first time, we were able to show that PAR-1 activation has unique and contrasting effects in the human vasculature including arterial dilation, venous constriction, platelet activation, and tissue-type plasminogen activator (t-PA) release. Given the central role of thrombin in the pathophysiology of cardiovascular disease, it is important to establish the mechanisms of these PAR-1–mediated effects and, in particular, the role of the endothelium. We therefore set out to explore the role of the endothelium in the vascular actions of PAR-1 activation in vivo in man.

Methods

Subjects. A total of 30 healthy nonsmokers (mean age 22 years; range 19 to 37 years) were recruited into the study. The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Participants were screened and excluded for clinically significant conditions including hypertension, hyperlipidemia, diabetes mellitus, asthma, and coagulopathy. No participant had suffered a recent infective or inflamma-

tory condition or had taken any medications in the 7 days prior to the study.

Vascular assessments. All studies were carried out in a quiet, temperature-controlled room (22°C to 24°C). Participants were semirecumbent (venous studies) or supine (arterial studies) and had abstained from alcohol for 24 h and from food and caffeine-containing drinks for at least 4 h prior to the study.

Venous studies. A 23-G needle was sited in a dorsal hand vein and total infusion rate kept constant at 0.25 ml/min in all studies. The hand was supported above the level of the heart and an upper arm cuff inflated to 40 mm Hg to obstruct venous return. The internal diameter of the dorsal hand vein was measured by the Aellig technique in 6 healthy volunteers. In brief, a magnetized lightweight rod rests on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion needle. The rod passes through the core of a linear variable differential transformer supported above the hand by a small tripod. Changes in diameter of the vein caused vertical displacement of the rod, leading to a linear change in the voltage generated by the transformer. This enabled calculation of absolute changes in vein size.

PROTOCOL 1. VENOUS EFFECTS OF PAR-1 ACTIVATION. First, we established the presence of functional endothelium. As dorsal hand veins do not have resting tone, norepinephrine (1 to 128 ng/min) was used to induce 70% reduction in vein diameter. Once stable venoconstriction was obtained, ace\-tycholine (1 nmol/min; Novartis Pharmaceuticals UK Ltd, Frimley, United Kingdom) was coinfused with norepinephrine for 8 min to demonstrate endothelium-dependent venodilation and an intact, functional endothelium. Following a 20-min saline infusion, the PAR-1 activating peptide, SFLLRN-NH$_2$ (0.05 to 15 nmol/min; Clinalfa AG, Laufelfingen, Switzerland), was administered intravenously before a final 20-min saline washout infusion.

PROTOCOL 2. VENOUS EFFECTS OF PAR-1 ACTIVATION FOLLOWING ENDOTHELIAL DENUDATION. At the end of protocol 1, the endothelium of the venous segment was denuded as previously described. In brief, a second 23-G butterfly needle was sited 3 to 4 cm downstream from the tip of the infusion-needle, and this segment of the vein was isolated by the use of occlusion wedges. Distilled water was infused through the venous segment at a rate of 5 ml/min for 15 min, thereby causing endothelial denudation that persists for at least 2 days. Aspirin (300 mg orally) was given 30 min prior to start of the first study and on each of the 2 subsequent days to prevent venous thrombosis. On the second day following denudation, subjects reattended and protocol 1 was repeated.

Arterial studies. All subjects underwent cannulation of the brachial artery with a 27-G standard wire steel needle under controlled conditions. The intra-arterial infusion rate was kept constant at 1 ml/min throughout all studies. Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography using mercury-in-
Silastic strain gauges as described previously (12,13). Supine heart rate and blood pressure were monitored at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer. Tirofiban (1.25 μg/min) was coinfused during the studies to inhibit potential PAR-1 activation-induced platelet aggregation in vivo (9). This dose of tirofiban does not affect platelet-monocyte binding, forearm blood flow, or baseline concentration of t-PA (9).

PROTOCOL 3: ROLE OF NITRIC OXIDE AND PROSTACYCLIN IN PAR-1–INDUCED VASODILATION. Forearm blood flow was measured by venous occlusion plethysmography in response to brachial artery infusion of SFLLRN (PAR-1 agonist; 5 to 50 nmol/min) with tirofiban (1.25 μg/min) in 8 healthy volunteers on 4 visits using a randomized controlled crossover study employing a 2-by-2 factorial design: with and without aspirin (600 mg orally; to inhibit prostacyclin [PGI₂] synthesis) and the “nitric oxide (NO) clamp.” Assuming total forearm blood flow of 25 ml/min, this will achieve end-organ concentrations of 0.2 to 2 μmol/l SFLLRN.

The NO clamp was used to determine the contribution of NO in PAR-1–mediated vascular effects. Following baseline intra-arterial tirofiban infusion, the NO synthase inhibitor, L-NG-monomethyl arginine citrate (L-NMMA; 8 μmol/min), was coinfused. To compensate for L-NMMA-induced basal vasoconstriction, forearm blood flow was returned to baseline using a titrated dose of exogenous NO in the form of intrabrachial sodium nitroprusside (SNP; 90 to 900 ng/min). This dose of SNP was coinfused with L-NMMA and continued throughout the study. This arrangement allows a constant “clamped” delivery of exogenous NO while endogenous NO synthase activity is inhibited.

PROTOCOL 4: ROLE OF CALCIUM-ACTIVATED POTASSIUM CHANNELS/ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR IN PAR-1–INDUCED VASODILATION. Forearm blood flow was measured in 8 other healthy volunteers in whom intrabrachial SFLLRN (5 to 50 nmol/min), bradykinin (30 to 300 pmol/min), and SNP (2 to 8 μg/min) were coinfused with either saline placebo or tetrodotoxin (TEA) (1 mg/min) on either of 2 visits using a randomized double-blind crossover design. Again, agonists were coinfused with intra-arterial tirofiban (1.25 μg/min), which was continued throughout the study. At the dose used, TEA is a nonselective potassium channel antagonist (14–16).

PROTOCOL 5: ROLE OF ENDOTHELlUM-DEPENDENT VASODILATORS IN PAR-1–INDUCED VASODILATION. In the final series of studies, TEA or saline placebo was coinfused with ascending doses of bradykinin and SFLLRN in 8 volunteers using a randomized double-blind crossover design. In this series, endothelium-derived hyperpolarizing factor (EDHF) activity was isolated by inhibiting NO and PGI₂ production on both visits. The NO clamp was employed as described previously (protocol 3), and cyclooxygenase activity was inhibited with a single 600-mg dose of oral aspirin 1 h before each study.

BLOOD SAMPLING. Seventeen-gauge venous cannulae were inserted into left and right antecubital fossae. Blood samples were drawn simultaneously from each arm at baseline. Blood samples were also drawn before SFLLRN or bradykinin infusion and after each dose of SFLLRN or bradykinin. Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, Umeå, Sweden; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd, Oxford, United Kingdom; for plasminogen activator inhibitor type 1 [PAI-1], beta thromboglobulin, and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2,000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at −80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria), PAI-1 antigen and activity (Elitest–PAI-1 Antigen and Zymutest PAI-1 Activity, HYPHEN BioMed, Neuville-sur-Oise, France), beta thromboglobulin (Asserachrom Btg, Diagnostica Stago, Asnières sur Seine, France), and vWF (Dako, Glostrup, Denmark) concentrations were determined by enzyme-linked immunosorbent assays. Full blood count was measured at baseline and at the end of the study.

PLATELET-MONOCYTE BINDING. In protocols 3 and 4, blood was collected from each arm for determination of platelet-monocyte binding at baseline and after the highest dose of SFLLRN. Samples of 5 ml of venous blood were collected and transferred into a tube containing the direct thrombin inhibitor, D-phenylalanyl-L-propyl-L-arginine chloromethylketone. Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labeled with fluorochromes for 20 min and platelet-monocyte aggregates measured as described previously (17).

Data analysis and statistics. Dorsal hand venous (18) and forearm plethysmographic (12) data were analyzed as described previously. Variables are reported as means ± SEM and analyzed using repeated measures analysis of variance (ANOVA) with post hoc Bonferroni corrections and 2-tailed Student t test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software, San Diego, California) and statistical significance taken at the 5% level. The authors had full access to the data and take responsibility for its integrity. All authors have read and agreed to the manuscript as written.

Results

Endothelium and PAR-1–induced venoconstriction. The role of the endothelium in PAR-1–induced vasomotor effects was assessed by comparing venous responses before and after local endothelial denudation. This was achieved through brief instillation of distilled water in an isolated dorsal hand vein segment. After pre-constriction with norepinephrine, the presence or absence of functional endothelium was confirmed by the coinfusion of acetylcholine (1 nmol/min). Acetylcholine caused venodilation in the presence of endothelium and venoconstriction in its absence.
Endothelium-derived vasodilators and PAR-1–induced release of fibrinolytic and coagulant factors. The SFLLRN increased net t-PA antigen and activity and PAI-1 antigen release but did not affect net PAI-1 activity (Fig. 4). This increase was augmented by the NO clamp (Fig. 4) but was not affected by aspirin or TEA (data not shown; p = NS; ANOVA). Bradykinin caused a dose-dependent increase in net t-PA antigen (p < 0.05; ANOVA) and activity (p < 0.0001; ANOVA) release but did not affect PAI-1 antigen and activity release (p = NS for both; ANOVA). Tetaethylammonium ion did not alter bradykinin-induced PAI-1 or t-PA release (p = NS for both; ANOVA). Unpaired analysis between the 2 subject groups (protocol 4 vs. protocol 5) suggests that the NO clamp did not alter bradykinin–induced t-PA or PAI-1 release (p = NS for all; ANOVA). Neither bradykinin nor SFLLRN affected vWF release (data not shown; p = NS; ANOVA).

Endothelium-derived vasodilators and PAR-1–induced platelet activation. SFLLRN increased platelet–monocyte binding but this was unaffected by inhibition of NO or PGI2 (Table 1). In contrast, SFLLRN increased beta-thromboglobulin (p < 0.001; ANOVA) that was augmented during the NO clamp (p < 0.01; ANOVA) (Fig. 5) but unaffected by aspirin (p = NS; ANOVA).

Discussion

Thrombin is one of the most powerful physiological agonists in the cardiovascular system, and its actions are fundamental to the processes of atherothrombosis. In a series of studies, we have here described the contrasting role of the endothelium in the PAR-1–mediated vascular actions of thrombin in vivo in man. Although not providing a major contribution to venoconstriction or PAI-1 release, the endothelium mediates PAR-1–induced arterial vasodilation and t-PA release. Our findings provide clear evidence of a major interaction between the vascular endothelium and thrombin in vivo in man. Furthermore, it highlights the critical importance of endothelial function at the time of acute arterial injury and intravascular thrombosis, such as during acute coronary syndromes.

Role of the endothelium in PAR-1–induced vasomotion. We have previously described the unexpected finding of PAR-1–induced venoconstriction in man (9). Although not caused by platelet aggregation (9), this effect could be mediated by either a direct action on vascular smooth muscle or via the release of endothelium–derived vasoconstrictors, such as endothelin or angiotensin II. To address this question, we assessed PAR-1 venoconstriction before and after endothelial denudation by instillation of distilled water. There was a modest trend toward enhanced venoconstriction after endothelial denudation, and we cannot exclude a small contribution from the endothelium that may also include the release of venodilatory mediators such as NO. However, PAR-1 continued to induce a marked dose-dependent venoconstriction even in the absence of the
endothelium, which suggests a dominant and direct effect of PAR-1 on the vascular smooth muscle cells.

In contrast to effects on the venous circulation, the PAR-1 agonist causes potent arterial vasodilation. This suggests a different effect on the arterial vasculature that is likely to be mediated by the endothelium. It would be difficult and ethically challenging to conduct comparable in vivo endothelial denudation studies in the arterial circulation of man. We chose, therefore, to use a pharmacological approach to the inhibition of the 3 main known mediators of endothelium-dependent vasodilation: PGI2, NO, and EDHF. Although PGI2 inhibition appeared to have no effect, inhibition of NO and potassium channels both attenuated the PAR-1–induced vasodilation. Consistent with some cross talk and compensatory up-regulation, combined inhibition of all vasodilator mechanisms appeared to produce greater inhibition, if not abolition, of the vasodilator actions of the PAR-1 agonist. This suggests that, unlike the venous circulation, PAR-1–mediated arterial actions are dominated by, and dependent on, the vascular endothelium.

PAR-1–induced release of endothelium-derived factors. In addition to vasomotion, PAR-1 has important effects on the release of endothelium-derived coagulant and fibrinolytic factors. In keeping with a wide range of other endothelial G-protein coupled receptor dilator agonists (19), we confirmed our earlier findings that the PAR-1 agonist causes endothelial t-PA release without affecting vWF. However, we also report here that SFLRN–induced t-PA release appeared to be augmented by the inhibition of endogenous NO production. Smith et al. (20) have reported similar findings when they examined bradykinin-evoked t-PA release in the presence and absence of L-NMMA. Because t-PA release is independent of NO and cyclooxygenase activity, it has been suggested that EDHF is responsible for its release (21). One could speculate that, by inhibiting NO activity, EDHF is up-regulated, and this accounts for the augmented t-PA release induced by SFLRN in our study and by bradykinin in Smith’s study. However, in contrast to TEA’s inhibitory effects on SFLRN–induced arterial vasodilation, it had no effect on SFLRN-evoked t-PA release. Similar findings have recently been reported by Muldowney et al. (22) who examined the role of EDHF in an in vitro model of thrombin-induced endothelial t-PA release. A variety of potassium channel antagonists, including TEA, had no effect on thrombin-induced t-PA release, but antagonists of specific epoxyeicosatrienoic acids appeared to inhibit thrombin-induced release of t-PA.

Another novel finding in our study was the increase in PAI-1 release, especially during NO synthase inhibition. To
date, there have been no reports of acute increases in plasma PAI-1 concentrations following the administration of endothelial agonists, especially using the forearm model (19). Even though the endothelium is an important source of PAI-1, we believe our findings are consistent with acute platelet release of PAI-1. There are several reasons to support our contention. First, although PAI-1 antigen concentrations increased, there was no corresponding rise in PAI-1 activity. Indeed, PAI-1 activity fell during marked release of t-PA. Plasminogen activator inhibitor type 1 is stored in platelet granules where its activity is very low (<5% of the activity seen in plasma) due to the absence of the stabilizing effect of vitronectin. In contrast, we would anticipate that endothelial-derived PAI-1 would remain active. Second, there was no concurrent rise in vWF confirming a selective effect on the endothelium with isolated t-PA release. Third, we also demonstrated concomitant platelet activation with marked increases in platelet-monocyte binding and release of beta-thromboglobulin; the latter is also stored in the α-granules of platelet. Finally, PAR-1–induced PAI-1 release was augmented during the NO clamp. Nitric oxide has important antiplatelet effects and, in the presence of its inhibition, increased platelet activation may have led to greater PAI-1 release.

**Clinical relevance.** Until recently, it has not been possible to undertake a safe clinical assessment of the vasomotor effects of thrombin due to its potent stimulatory effects upon the coagulation cascade. However, the synthetic activating peptide, SFLLRN, allows the examination of activation of the human PAR-1 thrombin receptor without activation of the coagulation cascade. This also permits the assessment of PAR-1 actions independent of the potential confounding effects that the activated coagulation pathway may have upon vascular responses.

We have demonstrated that many of the arterial effects of the PAR-1 agonist are dependent on, and mediated through, the endothelium and can therefore be used to assess endothelial function. To date, many endothelial G-protein-coupled receptor agonists have been used to assess endothelial function, such as acetylcholine and substance P. However, such agents are unlikely to have a major role in vascular physiology or pathophysiology and, as pharmacological tools, their relevance to the assessment of endothelial vasomotor function has limitations. As a more physiologically relevant tool, the PAR-1 agonist may be a more appropriate method of assessing endothelial function in the context of atherothrombosis. These novel insights into the vascular actions of the PAR-1 agonist will not only contribute to our understanding of human physiology and pathophysiology but also promise to inform the clinical development of novel antithrombotic PAR-1 receptor antagonists.

**Study limitations.** We chose to use SFLLRN as a PAR-1 agonist for several reasons. First, the vast majority of published work has employed SFLLRN as a PAR-1 activating peptide, and its actions have been widely characterized. Second, we have previous clinical experience of the in vivo actions of SFLLRN and this has facilitated the comparability of our current findings with our previous “first-into-man” clinical studies. Finally, SFLLRN is identical to the active cleaved sequence of the human PAR-1 receptor and represents a more physiologically relevant agonist of the receptor.

Although SFLLRN is selective for the PAR-1 receptor, it does have agonist activity at the PAR-2 receptor: 4-fold greater selectivity for the PAR-1 versus PAR-2 receptor (23). Therefore, there remains a possibility that a contribution of the observed actions of SFLLRN may represent PAR-2 antagonism. However, we do not believe this is likely for several reasons. First, we have
previously shown that SLIGKV, a highly selective PAR-2 activating peptide, causes only modest arterial vasodilation at high doses and, in contrast to PAR-1 activation, causes marked venodilation and does not cause arterial t-PA release in vivo (24). Moreover, the predicted end-organ concentration of the highest dose of SFLLRN used in our current and previous studies is 4-fold lower than the median effective dose for the PAR-2 receptor (23). However, we do accept that, in future studies, consideration should be given to the use of the more selective PAR-1 activating peptide, TFLLRN (23,25).

The role of NO in bradykinin-induced t-PA release remains controversial (19) and the present study has not definitively addressed this issue. Although NO donors do not induce t-PA release (26,27), inhibition of NO synthesis has been reported either to have no effect (21), or to increase (20), bradykinin-induced t-PA release. Our own unpaired analysis of data from different subject populations is in keeping with the findings of Brown et al. (21) and suggests that bradykinin-induced t-PA release is unaffected by either NO or prostaglandin inhibition. Further research into the pathways involved in bradykinin-induced t-PA release is needed to clarify the role of NO and other potential mediators.

Conclusions

Protease-activated receptor type 1 activation causes contrasting effects in the human vasculature. It causes endothelium-dependent arterial vasodilation and t-PA release as well as endothelium-independent venoconstriction and PAI-1 release. There appears to be a major interaction between the vascular endothelium and thrombin’s PAR-1–mediated effects in vivo in man. This highlights the critical importance of endothelial function particularly at the time of acute arterial injury and intravascular thrombosis, such as occurs during many acute cardiovascular events including myocardial infarction and stroke.

<table>
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<tr>
<th>Table 1</th>
<th>Percent Platelet-Monocyte Binding</th>
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<tr>
<td></td>
<td>Baseline</td>
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<tr>
<td></td>
<td>Infused Arm</td>
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<tr>
<td>Placebo</td>
<td>16.62 ± 4.38</td>
</tr>
<tr>
<td>Aspirin only</td>
<td>14.43 ± 1.95</td>
</tr>
<tr>
<td>NO clamp only</td>
<td>11.84 ± 1.52</td>
</tr>
<tr>
<td>Aspirin + NO clamp</td>
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*p < 0.001 versus baseline (analysis of variance). NO = nitric oxide.
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