Endogenous Tissue Plasminogen Activator Enhances Fibrinolysis and Limits Thrombus Formation in a Clinical Model of Thrombosis

Andrew J. Lucking, Kyle R. Gibson, Elspeth E. Paterson, Dana Faratian, Christopher A. Ludlam, Nicholas A. Boon, Keith A.A. Fox, David E. Newby

Objective—Using a clinical model of deep arterial injury, we assessed the ability of exogenous and endogenous tissue plasminogen activator (t-PA) to limit acute in situ thrombus formation.

Approach and Results—Ex vivo thrombus formation was assessed in the Badimon chamber at low and high shear rates in 2 double-blind randomized cross-over studies of 20 healthy volunteers during extracorporeal administration of recombinant t-PA (0, 40, 200, and 1000 ng/mL) or during endogenous t-PA release stimulated by intra-arterial bradykinin infusion in the presence or absence of oral enalapril. Recombinant t-PA caused a dose-dependent reduction in thrombus area under low and high shear conditions (P<0.001 for all). Intra-arterial bradykinin increased plasma t-PA concentrations in the chamber effluent (P<0.01 for all versus saline) that was quadrupled in the presence of enalapril (P<0.0001 versus placebo). These increases were accompanied by an increase in plasma D-dimer concentration (P<0.005 for all versus saline) and, in the presence of enalapril, a reduction in thrombus area in the low shear (16±5; P=0.03) and a trend toward a reduction in the high shear chamber (13±7%; P=0.07).

Conclusions—Using a well-characterized clinical model of coronary arterial injury, we demonstrate that endogenous t-PA released from the vascular endothelium enhances fibrinolysis and limits in situ thrombus propagation. These data support a crucial role for the endogenous fibrinolytic system in vivo and suggest that continued exploration and manipulation of its therapeutic potential are warranted. (Arterioscler Thromb Vasc Biol. 2013;33:1105-1111.)

Key Words: endogenous fibrinolysis ■ endothelial function ■ endothelium ■ thrombosis ■ tissue plasminogen activator

The endogenous fibrinolytic system protects against intravascular fibrin formation and thrombosis, and seems to be particularly important within the coronary circulation.1 In the presence of a developing thrombus, tissue plasminogen activator (t-PA) is rapidly released from a dynamic storage pool within the vascular endothelium through its stimulation by various factors that include bradykinin, thrombin, and factor Xa.2 The released t-PA causes a 1000-fold increase in the enzymatic conversion of plasminogen to plasin.3,4 This ensures that rapid plasmin generation, fibrin degradation, and clot dissolution are tightly regulated and localized to sites of vascular injury. Thus, the rapidity and extent of acute t-PA release from the endothelium is a critical factor in determining the efficacy of local endogenous fibrinolysis and in preventing intravascular thrombus propagation.5,6 Although many workers have assessed basal plasma concentrations of t-PA and its principal inhibitor, plasminogen activator inhibitor type 1, these do not reflect the capacity of the endothelium to release t-PA acutely nor the dynamic ability to dissolve intravascular thrombus.

Robust methods to determine acute t-PA release within the human forearm and coronary circulations have been developed.5,7 In the forearm circulation, local t-PA release has been demonstrated in response to a variety of physiological and pharmacological stimuli, including thrombin receptor agonism,8 substance P,6 tumor necrosis factor α,10 and bradykinin.11 Indeed, the fibrinolytic capacity of the endothelium predicts future adverse cardiovascular events in patients with coronary disease and may provide additional insights into endothelial function.12 However, the primary end point in all studies to date has been the quantification of t-PA release and it is unknown whether this acute endogenous release of t-PA can truly influence or modify dynamic thrombus formation.

The demonstration of antithrombotic efficacy in man is challenging. Most techniques evaluate specific plasma or cellular components in isolation and are performed under static conditions in vitro. In contrast, in vivo thrombus initiation and growth occur in whole blood, under conditions of continuous flow and in the presence of vascular injury. Thus, the assessment of the efficacy of antithrombotic interventions may not be
reflected by static or unidimensional systems used by in vitro assays. An in vivo model for use in clinical studies presents significant safety issues and does not currently exist. The Badimon chamber is an ex vivo model of thrombosis with the ability to assess thrombus formation on a pathophysiologically relevant substrate under conditions of continuous flow. It has previously been used to assess novel antithrombotic interventions but has not been used to assess fibrinolysis.

By combining 2 well-characterized and established techniques, the aim of the present study was to assess the ability of exogenous and endogenous t-PA to limit thrombus formation in a model of deep coronary arterial injury.

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

Results
All study visits were completed for each subject without significant adverse events.

Extracorporeal Recombinant t-PA
Plasma t-PA antigen concentrations in the venous effluent of the chamber (6.0±0.6, 44±6.6, 26±13, and 1567±177 ng/mL) approximated to the anticipated plasma t-PA concentrations (5, 40, 200, and 1000 ng/mL respectively; Figure 1). There was a dose-dependent increase in plasma D-dimer concentrations (P<0.0001; Figure 1) accompanied by a corresponding dose-dependent reduction in total and fibrin-rich thrombus under both low and high shear conditions (P<0.001 for all; Figure 2). Thrombus in the low shear chamber had a greater proportion of fibrin compared with the high shear chamber (23±5% versus 8±2%; P<0.001; Figure 2).

Endogenous Fibrinolysis
Oral and intra-arterial drug administrations were well tolerated without significant adverse effects. Consistent with previous studies, transient patchy flushing and skin edema of the infused arm occurred with bradykinin infusion. There were no changes in heart rate, blood pressure, and noninfused forearm blood flow during or between study days (data not shown). As previously described, sodium nitroprusside and bradykinin produced dose-dependent forearm vasodilatation (P<0.001 for all; data not shown).

When compared with saline, sodium nitroprusside had no effect on fibrinolytic parameters in the presence of placebo or enalapril (data not shown). Intra-arterial bradykinin increased plasma t-PA antigen and activity in the chamber effluent during treatment with placebo and enalapril (P<0.01 for all versus saline; Figure 3). Bradykinin-induced t-PA antigen concentrations were increased 4-fold, and t-PA activity increased 7-fold in the presence of enalapril (P<0.0001 for both versus placebo; Figure 3) such that systemic spillover and elevation of plasma t-PA antigen and activity in the noninfused arm were observed (P=0.01 and P<0.001, respectively, versus saline; Figure 3). These changes in plasma t-PA antigen and activity were accompanied by an increase in plasma D-dimer concentrations in the chamber effluent during placebo and enalapril treatment (P=0.003 and P=0.001, respectively, versus saline; Figure 3), confirming that t-PA released from the forearm endothelium-enhanced fibrinolysis within the chamber. Plasminogen activator inhibitor type 1 antigen concentrations were unaffected by the presence of enalapril or during intra-arterial administration of bradykinin (Figure 3).

The changes in fibrinolytic markers were accompanied by a reduction in total thrombus area in the low shear chamber during bradykinin infusion (15±5%; P=0.026 versus saline) and a trend toward a reduction in the high shear chamber (13±7%; P=0.07 versus saline; Figure 4). There were no differences in thrombus area in either the low or the high shear chambers in the presence of placebo (Figure 4).

Discussion
Recent work has provided novel insights into the role of endogenous fibrinolysis. Using intra-arterial infusion in the human forearm and coronary circulations, we and others have demonstrated that a variety of agonists can stimulate t-PA release from the human vascular endothelium in vivo. In the current study, we have confirmed that acutely released endogenous endothelial t-PA is functionally active, enhances local fibrinolysis, and can limit in situ thrombus formation. This establishes the clinical significance of this method of assessing endothelial function as well as underscoring the importance of this aspect of vascular function.

The endothelium has several important complementary functions, including regulation of vasomotor tone, coagulation, fibrinolysis, and inflammation. Most clinical studies have focused on endothelium-dependent vasomotion. Although a useful surrogate marker for the role of the endothelium in atherothrombosis, the observation of impaired t-PA release with preservation of vasomotor function in response to endothelium-dependent vasodilators in cigarette smokers and patients with hypertension highlights both the complexity of vascular biology and suggests that fibrinolytic capacity may be a more sensitive marker of endothelial dysfunction in some circumstances.

Figure 1. The effect of recombinant tissue plasminogen activator (t-PA) administered into the extracorporeal circuit of the Badimon chamber on plasma fibrinolytic components measured in the chamber effluent (n=8). Data shown are mean±SE of the mean.
Despite a growing body of evidence, a key limitation of studies to date is that the effects of acute endogenous t-PA release on in situ thrombus formation have not been demonstrated. Is endogenous endothelial t-PA released under agonist stimulation functionally active and able to enhance fibrinolysis of in situ thrombus? Clearly, it would be unethical to induce stimulation functionally active and able to enhance fibrinolysis and there is currently no model for assessing thrombus formation in vivo in the human circulation. In the present study, we attempted to resolve these problems by combining 2 well-established techniques: the Badimon Chamber, an ex vivo clinical model of thrombus formation, and the forearm technique, using intraarterial infusion of bradykinin to generate enhanced concentrations of endogenous t-PA from the vascular endothelium. This methodology provides an elegant means of stimulating local t-PA release from the endothelium before channeling the venous effluent of the forearm, containing freshly released endogenous t-PA, through the Badimon chamber allowing an assessment of ex vivo thrombus formation to be made. We initially validated our model using the extracorporeal administration of exogenous recombinant t-PA before assessing the effects of endogenous t-PA. The doses of t-PA used in the initial studies were selected based on data from previous forearm studies and measurements of t-PA concentration made during studies of systemic thrombolysis for myocardial infarction. We estimated that 40 ng/mL was the maximum t-PA antigen concentration achievable in healthy volunteers during bradykinin infusion in the presence of angiotensin-converting enzyme (ACE) inhibition. Concentrations of 200 and 1000 ng/mL were selected to represent the lower and upper ends, respectively, of plasma t-PA antigen concentrations achieved during systemic therapeutic fibrinolysis. On the basis of this rationale, we were able to demonstrate that across a clinically relevant dose range, thrombus formation was reduced in a dose-dependent manner. Consistent with its mechanism of action, exogenous t-PA was associated with reductions in fibrin-rich clot and an elevation in D-dimer concentrations in the venous effluent.

Having validated our model, we then wanted to explore whether freshly released endogenous t-PA would have similar effects to exogenous t-PA. We used both systemic ACE inhibition and intraarterial bradykinin administration to maximize endogenous t-PA release. Using this approach, we were able to increase plasma t-PA concentrations to those seen with the lowest dose of exogenous t-PA. Consistent with this, we observed comparable reductions in thrombus area and increases in D-dimer formation. These data, therefore, confirm the importance of the endogenous fibrinolytic system and the use of the forearm model as a relevant model to assess acute endogenous fibrinolytic capacity. Furthermore, we believe that this methodology provides a robust, versatile, and relevant means to assess novel agents with the potential to modulate the endogenous fibrinolytic system and thus may benefit patients with a broad range of disease processes.

We elected to use bradykinin as the agonist to stimulate t-PA release for 2 principal reasons. First, it is pathophysiologically relevant. Although an inflammatory mediator, it is also released during the contact phase of coagulation and is a potent endothelial cell activator, stimulating release of t-PA from the endothelium through a B2 receptor–mediated mechanism. Thus, after activation of the intrinsic coagulation pathway, the liberation of bradykinin may represent an important negative feedback loop in which bradykinin-induced t-PA release inhibits thrombus formation within the vascular lumen when localized endothelial denudation occurs. Second, the use of bradykinin affords a safe and efficacious means of maximizing t-PA release. Ordinarily, bradykinin has a very short plasma half-life of ≈15 s with >95% of its
metabolism occurring through ACE. In the presence of ACE inhibition, bradykinin-induced vasodilatation and t-PA release are selectively and markedly potentiated, and this may account for some of the anti-ischemic actions of ACE inhibition in vascular disease. Accordingly, in the presence of placebo, infusion of bradykinin resulted in more modest increases in plasma t-PA antigen and activity in the infused arm, sufficient to result in an increase in plasma D-dimer concentration confirming enhanced fibrinolysis but not sufficient enough to detect a significant change in thrombus formation in the Badimon chamber. However, in the presence of ACE inhibition, there was a roughly 4-fold increase in t-PA antigen and 7-fold increase in t-PA activity sufficient to result in systemic spillover and a marked elevation of plasma t-PA antigen and activity in the noninfused arm. These marked increases in local and systemic plasma t-PA antigen

Figure 3. The effect of intra-arterial bradykinin infusion on plasma fibrinolytic components measured in the noninfused arm (clear bars) and the chamber effluent (hashed bars; n=12). Data shown are mean±SE of the mean. PAI-1 indicates plasminogen activator inhibitor type 1; and t-PA, tissue plasminogen activator.
and activity are consistent with our previous studies and resulted in an increase in plasma D-dimer concentrations and an accompanying reduction in thrombus formation. In contrast to a previous study in patients with moderate-to-severe heart failure, basal plasma plasminogen activator inhibitor type 1 antigen concentrations were not affected by ACE inhibition, suggesting that in this healthy volunteer cohort, enhanced endogenous fibrinolysis was the result of an increase in t-PA activity rather than a reduction in plasminogen activator inhibitor type 1 antigen concentration.

Although the magnitude of reduction in thrombus area with endogenous t-PA release was relatively modest and seen only in the presence of ACE inhibition, this occurred despite the marked dilutional effect that occurs because of the substantial increase in blood flow associated with concomitant bradykinin-induced vasodilatation. At the site of unstable plaques within the coronary circulation, it is likely that local concentrations will be much higher and, with impending vessel occlusion, dilutional effects will be minimized. Furthermore, we studied healthy volunteers with intact vasomotor responses and no vascular dysfunction. In our previous studies, ACE inhibition had a disproportionately large effect on bradykinin-induced t-PA release in patients with established heart disease. Indeed, in these patients, we were able to demonstrate up to 4.5 µg of endogenous t-PA release from the forearm and achieve 99 IU/mL of t-PA activity that approached the activity seen with systemic therapeutic fibrinolysis (100–1000 IU/mL). This may, in part, be because of impaired vasodilatation causing the accumulation of greater plasma concentrations of released t-PA. Taken together, these considerations suggest that local t-PA concentrations generated in the coronary circulation in vivo, particularly in patients treated with ACE inhibition, may be higher than those we achieved here. It does, however, remain possible that bradykinin and enalapril are affecting aspects of platelet function, coagulation, or the fibrinolytic system that we have not measured here.

**Study Limitations**

We acknowledge the modest sample size and exclusive use of healthy volunteers. It would be desirable to increase subject numbers and make similar assessments in patients with vascular disease but the invasive nature and complexity of combining 2 exacting techniques present many challenges particularly when attempting to apply this methodology to larger cohorts and patient groups.

Measurement of coronary t-PA release is of greatest relevance to coronary pathophysiology but can only be performed in selected subjects undergoing invasive coronary angiography. However, consistent findings between the forearm and coronary circulations support the notion that the use of the forearm model is a reasonable surrogate, particularly in the context of this proof-of-concept study.

For the reasons discussed earlier, we believe that our rationale for choosing bradykinin as the agonist to stimulate t-PA release is sound, although we acknowledge that the local concentration generated at the surface of an unstable atherosclerotic plaque and the exact role played by bradykinin in potentiating the endogenous fibrinolytic system in vivo remain incompletely understood.
Conclusion
Using a well-characterized clinical model of arterial injury, we have demonstrated that endogenous t-PA released acutely from the human vascular endothelium enhances fibrinolysis and limits in situ thrombus formation. These data confirm the functional significance of t-PA released during agonist stimulation, support a crucial role for the endogenous fibrinolytic system and suggest that further studies to explore its therapeutic value are warranted.

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Disclosures
None.

References
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Materials and Methods

Subjects

Twenty healthy non-smokers aged between 18 and 26 years were enrolled into the study that was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and the written informed consent of all volunteers. Exclusion criteria were bleeding diathesis, the use of regular medication or any clinically significant illness. Except for study medication, none of the subjects received vasoactive medication during the study and all abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least six hours before each study. Studies were carried out with subjects lying supine in a quiet, temperature-controlled room maintained between 22°C and 25°C.

Ex Vivo Thrombosis Studies

Thrombus formation was measured using the Badimon Chamber. In brief, a pump was used to draw blood from an antecubital vein, via a length of polyethylene tubing, through a series of three cylindrical perfusion chambers maintained at 37°C in a water bath. Carefully prepared strips of porcine aorta (Pel-Freez Biologicals, USA), from which the intima and a thin layer of media had been removed, acted as the thrombogenic substrate. The rheological conditions in the first chamber simulate those of patent coronary arteries (low shear rate, approximately 212 s⁻¹), while those in the second and third chambers simulate those of mildly stenosed coronary arteries (high shear rate, approximately 1690 s⁻¹). Each study lasted for five minutes during which blood flow was maintained at a constant rate of 10 mL/min using a peristaltic pump (Masterflex model 7013, Cole-Palmer Instruments, USA), positioned distal to the chambers. Following perfusion of blood, the chambers were flushed with 0.9%
saline for one minute under the same rheological conditions. All studies were performed using the same perfusion chambers. Immediately after each study, the porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde for 72 hours at 4°C prior to being prepared for histological analysis.

**Histological Analysis**

As thrombus forms along the entire length of the exposed porcine aortic strip, the cross-sectional area gives a reliable reflection of total thrombus.\(^3\) Following fixation, the proximal and distal 1-mm sections of the exposed substrate were discarded and the remainder cut into four pieces. These pieces were paraffin-wax embedded and 5 \(\mu\)m sections were prepared from each in order that quantification was performed on sections taken from all parts of the exposed tissue strip.

Sections were stained with Masson’s trichrome stain to detect total thrombus or with an anti-fibrin II \(\beta\) chain mouse monoclonal antibody (clone T2G1; Accurate Chemical & Scientific Corporation, USA) to detect fibrin. For immunohistochemical staining, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide and serum free protein block (Dako, Glostrup, Denmark). Sections were then incubated with the primary antibody at a final concentration of 10 \(\mu\)g/mL for 1 hour at room temperature. Detection was performed using EnVision™ (Dako, Denmark) and treatment with 3,3’-diaminobenzidine substrate chromogen (Dako, Denmark). Finally, sections were counterstained with haematoxylin. No staining was present on specimens not incubated with the primary antibody or on areas of the specimens not exposed to blood flow.
A purpose-designed semi-automated scanning microscope and image analysis system (Ariol 3.1, Applied Imaging, USA) was used to quantify thrombus area and composition. Digital images of each section were acquired at ×20 magnification. High-resolution classifiers, based on colour and shape parameters, were established to detect total thrombus and fibrin staining. Results from at least six sections were averaged to determine thrombus area for each chamber as described previously.⁴,⁶

**Forearm Blood Flow**

To confirm intrarterial infusion of the vasoactive agents, blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silicone elastomer strain gauges as previously described.⁷ Heart rate and blood pressure were monitored in the non-infused arm throughout each study with a non-invasive, semi-automated oscillometric sphygmomanometer (Boso Medicus, Germany).

**Blood Sampling and Fibrinolytic Assays**

Blood samples were taken immediately distal to the perfusion chambers (and from the non-infused arm where appropriate) during each study and collected into citrate for PAI-1 assay and acidified buffered citrate (Stabilyte™, Biopool International, USA) for t–PA and D-dimer assays. Samples were kept on ice before being centrifuged at 2000 g for 30 min at 4 °C. Platelet-free plasma was decanted and stored at -80 °C prior to assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit, Technoclone, Austria), PAI-1 antigen (Zymutest, Hyphen Biomed, France) and D-dimer (Asserachrom® D-dimer, Roche, Switzerland) concentrations were determined using enzyme-linked immunosorbent assays.
Study Design

Exogenous t-PA Administration

To assess the effect of recombinant t-PA (rt-PA) administered into the extracorporeal circuit, eight subjects attended on two separate occasions, two weeks apart in a double-blind randomised crossover design. On each visit, two *ex vivo* thrombosis studies were performed 45 min apart during extracorporeal administration of rt-PA (Boehringer Ingelheim, Germany; infused to achieve anticipated final concentrations of 40, 200 and 1000 ng/mL) or saline control. Study drugs were added to the extracorporeal circuit using a calibrated syringe driver (Alaris Arsena GH, Cardinal Health, USA) and allowed to mix prior to entering the perfusion chambers as previously described.8 Samples for fibrinolytic components were taken from the chamber effluent and thrombus formation assessed by measuring fibrin and total thrombus area.

Endogenous t-PA Release

We assessed the ability of endogenous t-PA released from the vascular endothelium to limit acute thrombus formation in a separate cohort of 12 subjects who attended on two separate occasions, two weeks apart, having received matched placebo or the angiotensin-converting enzyme (ACE) inhibitor, enalapril, 10 mg once daily for seven days prior to attendance in a double-blind randomized crossover design. On each study day, the final dose of placebo or enalapril was taken at 08.00 hr. Four hours later, the subjects rested recumbent, and strain gauges and cuffs were applied to the forearms. The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper’s Needle Works Ltd., Birmingham, UK) under local anaesthesia (1% lidocaine). The total rate of intra-arterial infusions was maintained
constant at 1 mL/min. Forearm blood flow was measured every 3 min during infusion of vasodilators and every 5 min during infusion of saline. Intrabrachial infusions of bradykinin (Clinalfa AG; endothelium-dependent vasodilator releasing t-PA) at 1,000 pmol/min\textsuperscript{9,10} and sodium nitroprusside (David Bull Laboratories, UK; endothelium independent vasodilator not releasing t-PA) at 8 µg/min\textsuperscript{11,12} were given for 12 min in a randomized order. Saline was infused for 30 min between vasodilator infusions. Samples for fibrinolytic components were taken from the chamber effluent and the non-infused arm. Total thrombus area was assessed at baseline (i.e. during intrabrachial infusion of saline) and during infusion of bradykinin and sodium nitroprusside.

**Data Analysis and Statistics**

The sample size was selected based on previous studies suggesting that we required a sample size of n=8-12 to have an 80% power to detect differences of 6-10% in thrombus area at p<0.05\textsuperscript{2,13-15}

Plethysmographic data were extracted from the Chart data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by a template spreadsheet (Excel v12.3; Microsoft Corp., USA) as previously described\textsuperscript{11,12,16}

Continuous variables are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism (GraphPad Software, USA) using paired Student’s t-tests and analysis of variance (ANOVA) as appropriate. Statistical significance was taken at P<0.05.
References


