Direct Vascular Effects of Protease-Activated Receptor Type 1 Agonism In Vivo in Humans

Ingibjörg J. Gudmundsdóttir, MD; Ian L. Megson, PhD; Jillian S. Kell, BSc; Christopher A. Ludlam, MD; Keith A.A. Fox, MD; David J. Webb, MD; David E. Newby, MD, PhD

Background—Protease-activated receptor type 1 (PAR-1) has been proposed as the principal thrombin receptor in humans, although its actions in vivo have not been defined. The aim of the present study was to determine the direct vascular actions of PAR-1 agonism in humans.

Methods and Results—Dorsal hand vein diameter was measured by the Aellig technique in 14 healthy volunteers during local intravenous SFLLRN (PAR-1 agonist; 0.05 to 15 nmol/min) and SLIGKV (PAR-2 agonist; 1.6 to 160 nmol/min) infusions. The venous effects of SFLLRN were further assessed in the presence or absence of norepinephrine or the glycoprotein IIb/IIIa antagonist tirofiban. Forearm blood flow was measured by venous occlusion plethysmography in 16 volunteers during infusion of SFLLRN (1 to 50 nmol/min), SLIGKV (160 to 800 nmol/min), and the endothelium-dependent vasodilator bradykinin (100 to 1000 pmol/min). Platelet-monocyte binding (a sensitive measure of platelet activation) and plasma tissue plasminogen activator (tPA), plasminogen-activator inhibitor 1, and von Willebrand factor concentrations were measured at intervals throughout the study. SFLLRN caused dose-dependent venoconstriction ($P<0.001$) that was unaffected by norepinephrine or tirofiban co-infusion. In forearm resistance vessels, SFLLRN increased forearm blood flow ($P<0.001$), tPA release ($P<0.001$), and platelet-monocyte binding ($P<0.0001$) without affecting plasma plasminogen-activator inhibitor 1 or von Willebrand factor concentrations. SLIGKV caused venous ($P<0.001$) and arterial ($P<0.01$) dilatation without tPA release.

Conclusions—We have demonstrated that PAR-1 agonism causes platelet activation, venous constriction, arterial dilatation, and tPA release in vivo in humans. These unique and contrasting effects provide important insights into the physiological and pathophysiological role of thrombin in the human venous and arterial circulations. (Circulation. 2006; 114:1625-1632.)

Key Words: platelets ■ blood flow ■ veins ■ arteries ■ receptor, PAR-1 ■ receptor, PAR-2

Thrombin is a powerful physiological stimulant in the cardiovascular system. Apart from its central enzymatic role in the coagulation cascade, it directly activates platelets, leukocytes, and vascular smooth muscle and endothelial cells. Thrombin is therefore a vital link between thrombosis, cellular activation, and inflammation, key pathogenic factors in atherothrombotic disorders.

Clinical Perspective p 1632

Given the apparent direct cellular effects of thrombin, cloning methods were used to conduct an extensive search for its receptor. This led to the identification of G-protein-coupled protease-activated receptors (PARs) characterized by a unique mechanism of activation whereby proteolytic cleavage unmasks a short peptide sequence that remains tethered and activates the receptor. Four different types of PARs have been identified. PAR-1, -3, and -4 are all activated by thrombin. The type 1 receptor has been proposed as the principal thrombin receptor in humans. In contrast, the PAR-2 receptor is activated by trypsin, by tryptase, and to a lesser extent by coagulation factors upstream of thrombin. It appears to be of importance in inflammatory conditions that induce endothelial PAR-2 expression and vasodilatation.

PAR-1 receptor agonism has been extensively studied in vitro and is associated with platelet activation and aggregation, vasodilatation, and angiogenesis. Although studies in small animals suggest that PAR-1 and PAR-2 agonism induces vasodilatation, there is significant species heterogeneity, and rodent models are of limited relevance to humans. Exploring the role of PAR-1 receptors in the human vasculature would deepen our understanding of the physiological role of thrombin and would be of interest in the development of new therapeutic strategies such as PAR-1 receptor antagonists and direct thrombin inhibitors.
Therefore, the aims of the present study were to determine the direct in vivo vascular effects of PAR-1 agonism in the human vasculature. Specifically, we wished to assess the direct role of PAR-1 activation on platelet activation and aggregation, venous and arterial tone, and the release of endothelium-derived factors in vivo in humans.

Methods

Subjects
Fifty-two healthy male and female nonsmokers (age, 20 to 38 years) were recruited into the study (Table 1). Participants had not been taking any regular medications, over-the-counter medications, herbal supplements, or vitamins. They did not have clinically significant coexisting conditions, including hypertension, hyperlipidemia, diabetes mellitus, asthma, and coagulopathy, and had not suffered a recent infective or inflammatory condition. 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.

Platelet Studies

Protocol 1: Platelet Aggregometry

Blood was sampled with a 19-gauge needle from the antecubital fossa into a 50-mL syringe, transferred into tubes containing 3.8% citrate, and centrifuged at 130 g for 20 minutes at room temperature to obtain platelet-rich plasma. Blood was centrifuged at 1200 g for 10 minutes to obtain platelet-poor plasma for reference samples. Platelet aggregation was measured at 37°C using standard optical platelet aggregometry.18,19

Preliminary in vitro platelet studies were carried out to construct concentration-response curves to define the concentration of the PAR-1 agonist SFLLRN-NH2 that caused platelet aggregation and activation. SFLLRN-NH2 (Auspep, Parkville Victoria, Australia) was compared with thromboxane A2 agonist U46619 (Sigma, Gillingham, UK) in both platelet-rich plasma and washed platelets.18 To assess the specificity of the PAR-1–activating peptide, both SFLLRN-NH2 and U46619–induced platelet aggregation was assessed in the presence and absence of the PAR-1 antagonist RWJ-58259.13 (S3-N-[(1S)-2-amino-3-[(phenylmethyl)-aminocarbonyl]propyl]-alpha-[11-((2,6-dichlorophenyl)methyl)-3-(1-pyrrolidinyl)methyl]-1H-indazol-6-yl]amino]carbonyl]amino]-3,4-difluorobenzene propionamide; American Peptide Co, Sunnyvale, Calif) in blood collected into 2 D-phenylalanyl-L-propyl-L-arginine chloromethylketone tubes containing either tirofiban (50 ng/mL) or SFLLRN-NH2 (0.06 to 6 µg/mL). Five minutes after sampling, blood was incubated with appropriate monoclonal antibodies labeled with fluorochromes for 20 minutes, and platelet-monocyte aggregates were measured as described previously.20

TABLE 1. Baseline Characteristics of Study Volunteers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, %</td>
<td>79 (41/52)</td>
</tr>
<tr>
<td>Age, y</td>
<td>24±0.5</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23±0.4</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>67±2</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>132±2</td>
</tr>
<tr>
<td>Diastolic</td>
<td>73±1</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>42±0.01</td>
</tr>
<tr>
<td>White cell count, ×10⁹/L</td>
<td>4.98±0.37</td>
</tr>
<tr>
<td>Platelets, ×10⁹/L</td>
<td>213±11</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *Data from vascular studies only.

Vascular Studies
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 hours and from food and caffeine-containing drinks for at least 4 hours before the study.

Venous Studies
A 23-gauge needle was sited in a dorsal hand vein, and the total infusion rate was 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.21 In brief, a magnetized lightweight rod rested on the summit of the infused vein 1 cm downstream from the tip of the infusion needle. The rod passed through a core of a linear variable differential transformer, supported above the hand by a small tripod. Changes in vein diameter caused vertical displacement of the rod, leading to a linear change in the voltage generated by the linear variable differential transformer. This enabled calculation of absolute changes in vein size.

Protocol 3: Effect on Venous Tone
Because dorsal hand veins do not have resting tone, norepinephrine (1 to 128 ng/min) was used to induce and maintain a 50% to 70% reduction in vein diameter throughout the study to allow detection of either venodilatation or venoconstriction. This was followed by co-infusion of the PAR-1–activating peptide SFLLRN-NH2 (0.05 to 5 nmol/min) or the PAR-2–activating peptide SLIGKV-NH2 (1.6 to 160 nmol/min; Clinalfa).

Arterial Studies
All subjects underwent brachial artery cannulation with a 27-standard-wire-gauge steel needle under controlled conditions. 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.22,23 Stpine heart rate and blood pressure were monitored at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer. Tirofiban (1.25 µg/min) was co-infused during PAR-1 activation to inhibit in vivo potential platelet aggre-
gation. The doses of tirofiban and SFLLRN-NH₂ were chosen to achieve end-organ concentrations equivalent to those with efficacy in in vitro studies (protocol 1) and assumed a brachial artery blood flow of 25 mL/min.

**Blood Sampling**

Venous cannulas (17 gauge) were inserted bilaterally into the antecubital fossae. Blood samples were drawn simultaneously from each arm during infusion of saline, tirofiban, and each dose of the PAR-activating peptides and bradykinin. They were collected into acidified buffered citrate (Stabilyte, Trinity Biotech Plc, Co Wicklow, Ireland; for tissue plasminogen activator [tPA] assays) and into citrate (BD Vacutainer, BD UK Ltd, Oxford, UK; for plasminogen activator inhibitor type 1 [PAI-1] and von Willebrand factor [vWF] assays). Samples were kept on ice before centrifugation at 2000 \( g \) for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at \(-80°C\) before assay. Plasma tPA antigen and activity (tPA Combi Actibind Elisa Kit, Technoclone, Vienna, Austria) and PAI-1 antigen and activity (ElitestPAI-1 Antigen and Zymutest PAI-1 Activity, Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by ELISAs. Full blood count was measured at baseline and at the end of the study. Blood was also collected from each arm to determine platelet-monocyte binding (see protocol 2) at baseline, at the highest dose of SFLLRN-NH₂, during saline washout, and at the highest dose of bradykinin.

**Protocol 5: PAR-1 Activation**

After a 20-minute baseline saline infusion, tirofiban (1.25 µg/min) was infused throughout the study. Thirty minutes after the tirofiban infusion was started, the PAR-1–activating peptide SFLLRN-NH₂ was co-infused at 5, 15, and 50 nmol/min for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute saline infusion before bradykinin was infused at 100, 300, and 1000 pmol/min for 8 minutes at each dose.

**Protocol 6: PAR-2 Activation**

After a 20-minute baseline saline infusion, the PAR-2–activating peptide SLIGKV-NH₂ was infused at 160, 360, and 800 nmol/min for 8 minutes at each dose separated by 6 minutes of saline washout infusions. This was followed by a 30-minute saline infusion before bradykinin was infused at 100, 300, and 1000 pmol/min for 8 minutes at each dose.

**Data and Statistical Analyses**

Dorsal hand venous26 and forearm plethysmographic23 data were analyzed as described previously. Variables are reported as mean±SEM and analyzed using repeated-measures 1- or 2-way ANOVA with post-hoc Bonferroni corrections and 2-tailed Student t test as appropriate. Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc, San Diego, Calif). Statistical significance was taken at \( P<0.05\).

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

In Vitro Effects of PAR Agonism on Platelets

Both SFLLRN-NH₂ and U46619 caused dose-dependent platelet aggregation (median effective concentration \( [EC_{50}] = 1.26 \) and 0.78 µmol/L, respectively; Figure 1A and 1B; \( n=8 \)). The PAR-1 antagonist RWJ-58259 selectively and fully inhibited SFLLRN-NH₂–induced aggregation (Figure 1C; \( n=8; P<0.001 \), 1-way ANOVA) but not U46619-induced (Figure 1D; \( n=8; P=NS \)) platelet aggregation. In contrast, the direct thrombin inhibitor lepirudin had no effect on SFLLRN-NH₂–induced platelet aggregation (\( n=6; P=NS \); data not shown).

The clinical-grade SFLLRN-NH₂ also caused a dose-dependent increase in platelet aggregation (\( [EC_{50}] = 0.66 \) µmol/L) that was inhibited by tirofiban (Figure 2A; \( n=6; P<0.001 \), 2-way ANOVA). In contrast, PAR-1 activation caused a dose-dependent increase in whole-blood platelet-monocyte binding (\( [EC_{50}] = 0.23 \) µmol/L) that was not affected by tirofiban (Figure 2B; \( n=6; P=NS \), 2-way ANOVA). As anticipated, PAR-2
activation with SLIGKV-NH₂ induced neither platelet aggregation nor platelet-monocyte binding (n=3; data not shown).

**In Vivo Effects of PAR Agonism on Dorsal Hand Veins**

After venoconstriction (50% to 70%) was induced and maintained with norepinephrine, SFLLRN-NH₂ and SLIKGV-NH₂ caused dose-dependent venoconstriction (n=8; P<0.001, 1-way ANOVA) and venodilatation (n=6; P<0.001, 1-way ANOVA), respectively. In the absence of norepinephrine, SFLLRN-NH₂ caused a dose-dependent venoconstriction (P<0.001, 1-way ANOVA) that was able to induce complete constriction of the venous segment. This was not mediated by platelet aggregation because co-infusion of tirofiban had no effect on SFLLRN-NH₂–induced venoconstriction (Figure 3). SFLLRN-NH₂ was well tolerated by all subjects with no adverse effects. Vein patency was maintained at all times with no clinically apparent in situ thrombus formation.

**In Vivo Effects of PAR Agonism on Forearm Resistance Vessel Tone**

There was no change in heart rate, blood pressure, or noninfused forearm blood flow throughout either study. Intra-arterial tirofiban had no effect on resting forearm blood flow (P=NS). Both PAR-1 activation with SFLLRN-NH₂ and PAR-2 activation with SLIGKV-NH₂ caused dose-
dependent vasodilatation (Figure 4A and 4C; n=8; P<0.001 and P<0.01, respectively, 1-way ANOVA) with a rapid onset and offset of action. As anticipated, bradykinin increased forearm blood flow (Figure 4B; P<0.001, 1-way ANOVA) and was unaffected by tirofiban co-infusion (Figure 4D; P=NS versus no tirofiban).

**Figure 4.** Forearm vasodilatation induced by the PAR-1–activating peptide SFLLRN-NH2 (A; □; n=8; P<0.001, 1-way ANOVA), the PAR-2–activating peptide SLIGKV-NH2 (C; ○; n=8; P<0.01, 1-way ANOVA), and bradykinin (B, ●; D, ○; n=8; P<0.001, 1-way ANOVA). Values are mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

**In Vivo Effects of PAR Agonism on Endothelium-Derived Factors**

Plasma tPA antigen and activity concentrations increased in a dose-dependent manner during SFLLRN-NH2 and bradykinin but not SLIGKV-NH2 infusion (Figure 5; P<0.001 for all). Plasma PAI-1 antigen and activity and vWF concentrations were unaffected by all infusions (P=NS for all; Table 2). Although there was an apparent rise in plasma PAI-1 antigen concentration at 50 nmol/min SFLLRN-NH2, it did not achieve statistical significance (P=0.08, paired t test versus baseline) and was not associated with increases in PAI-1 activity or vWF. There were no differences in peripheral blood hematocrit (0.416±0.006 versus 0.426±0.008) or platelet counts (213±16×10⁹ versus 216±15×10⁹/L) at baseline and the end of the study. SFLLRN-NH2 increased in
vivo platelet-monocyte binding (from 16±5% to peak of 74±5%; Figure 6; P<0.0001, 1-way ANOVA) that declined toward baseline with time.

The PAR-2–activating peptide SLIGKV-NH₂ had no effect of plasma tPA concentrations (P=NS for all; data not shown).

**Discussion**

In a series of clinical studies, we have, for the first time, described the direct vascular effects of PAR-1 agonism and established the induction of in vivo platelet activation, venoconstriction, vasodilatation, and tPA release, as well as confirming the vasodilatory effects of PAR-2 agonism. These unique and contrasting effects of PAR-1 activation provide important insights into the physiological and pathophysiological roles of thrombin in the venous and arterial circulations of humans.

**Platelet Effects of PAR-Activating Peptides**

Inhibition of platelet activation has been of major benefit in the prevention and treatment of cardiovascular diseases. Thrombin is one of the most potent physiological stimulants of platelet activation that appears to be mediated largely through the PAR-1 receptor, although major contributions are also provided by PAR-4 and glycoprotein Ib receptors. To date, strategies to reduce the action of thrombin have focused on direct inhibition of its enzymatic activity. Recently, PAR-1 antagonists have been developed as potential antiplatelet agents that do not affect the coagulation cascade. It is therefore important to define the in vitro and in vivo effects of PAR-1 agonism in humans. As anticipated, we have demonstrated that PAR-1 but not PAR-2 agonism caused concentration-dependent platelet activation and aggregation. Moreover, in the presence of complete inhibition of platelet aggregation with the glycoprotein IIb/IIIa receptor antagonist tirofiban, PAR-1 agonism continued to induce platelet activation as measured by platelet-monocyte binding both in vitro and in vivo. This is consistent with our previous findings that although glycoprotein IIb/IIIa receptor antagonism inhibits platelet aggregation, it does not affect platelet activation or platelet-monocyte binding.

**Vascular Effects of PAR-Activating Peptides**

PAR-1 appears to be the principal receptor responsible for the vascular actions of thrombin and includes the regulation of vasomotion, platelet aggregation, inflammation, and angiogenesis. However, investigation of the in vivo vascular effects of thrombin presents challenges because of the direct activation of the coagulation cascade and platelets that may result in intravascular thrombosis. Here, we have chosen to use PAR-activating peptides that cause direct cellular stimulation without enzymatic activation of the coagulation cascade.

The direct vascular effects of PAR-1 receptor agonism have not been previously reported in humans. Our study protocol was vigilant to minimize any potential prothrombotic effects associated with PAR-1–induced platelet aggregation. We defined the in vitro concentrations of glycoprotein IIb/IIIa receptor antagonism that would permit the direct in vivo intravascular infusion of PAR-1–activating peptide without inducing platelet aggregation and potential thrombosis. This appears to have been successfully achieved, given the absence of significant adverse clinical side effects or thrombosis.

**Figure 6.** Platelet-monocyte binding during the infusion of the PAR-1 agonist SFLLRN-NH₂ in the infused (open bars) and non-infused (gray bars) arms. Values are mean±SEM.
Venous Effects of PAR-Activating Peptides

Thrombin and the PAR-1 agonist SFLLRN-NH$_2$ have previously been shown to cause endothelium- and nitric oxide–dependent venous and arterial dilatation in canine and porcine ring segments.$^{28,29}$ Although confirming previous observations of PAR-2–mediated venodilatation,$^{30}$ we have rather unexpectedly described a dose-dependent venoconstriction with PAR-1 activation. This may be mediated by platelet activation, release of endothelium-derived vasoconstrictors, or a direct effect on venous smooth muscle cells. Neither norepinephrine nor tirofiban co-infusion appeared to affect this potent venoconstrictor effect. In combination with the apparent absence of in situ thrombosis, this suggests that PAR-1–induced venoconstriction was not mediated by platelet aggregation, although we cannot exclude an effect of platelet activation.

Arterial Effects of PAR-Activating Peptides

In contrast to the venous effects, both PAR-1 and PAR-2 activation caused dose-dependent vasodilatation of forearm resistance vessels. We have not assessed the mechanism of this vasodilatation, but animal and clinical models suggest that this is likely to be endothelium and nitric oxide dependent.$^{11,30}$ However, data from animal studies have limited relevance in humans because of the wide species variability in PAR-1 receptor expression and function. The mechanism of PAR-1–induced vasodilatation needs to be addressed in future clinical studies.

PAR receptor expression varies between endothelial cell cultures originating from different human blood vessels,$^{31}$ which may partly explain the contrasting responses between PAR-1 and PAR-2 in the arterial and venous circulations. Our findings that PAR-2 activation causes venous and arterial dilatation are consistent with previous findings$^{30}$ and support the vascular role of PAR-2 receptors in inflammatory conditions.

Effects of PAR-Activating Peptides on Endothelium-Derived Factors

Intra-arterial PAR-1 but not PAR-2 agonism caused an acute dose-dependent increase in local endothelial tPA release. In contrast to in vitro human endothelial culture studies,$^{32}$ this occurred in the absence of the release of other endothelium-derived factors such as PAI-1 and vWF. However, there was an apparent rise in plasma PAI-1 antigen ($P=0.08$) with high-dose PAR-1 agonism, but given the absence of an effect on PAI-1 activity and vWF, this may reflect the associated platelet activation and release of platelet-derived PAI-1. PAI-1 is stored in platelet α-granules, where its activity is $\approx 5\%$ of plasma because of the absence of the stabilizing effect of vitronectin. Thus, PAR-1 agonism appears to have a selective profibrinolytic effect on the arterial endothelium.

Physiological Significance of Vascular PAR-1 Activation

We have, for the first time, described the unexpected and contrasting vascular effects of PAR-1 agonism in vivo in humans. How do we interpret these effects? In an intact normal vessel, homeostatic mechanisms attempt to maintain vessel patency and minimize intravascular thrombus forma-

tion. Dorsal hand veins do not have resting tone, and the induction of venodilatation will not affect venous blood flow. Therefore, in the presence of developing venous thrombosis, venodilatation would not be beneficial, whereas venoconstriction will potentially limit thrombus propagation and embolization. In contrast, it would be anticipated that increasing blood flow and endogenous fibrinolysis would limit arterial thrombosis by ensuring rapid clearance and dissolution of a developing thrombus. We therefore propose that the vascular effects of PAR-1 agonism in vivo in humans can be understood in terms of limiting intravascular thrombosis and maintaining vessel patency. We speculate that these physiological effects may be disturbed in patients with cardiovascular disease or prothrombotic disorders.

Conclusions

PAR-1 agonism causes platelet activation, venoconstriction, vasodilatation, and tPA release in vivo in humans. This has important implications in our understanding of the physiological vascular effects of thrombin and the pathogenesis of thromboembolic and atherothrombotic disorders.

Acknowledgments

We thank Pamela Dawson, Fiona Strachan, and all the staff in the Wellcome Trust Clinical Research Facilities (Edinburgh) for their help with these studies.

Sources of Funding

This work was supported by a British Heart Foundation Junior Research Fellowship grant (FS 05/028), the British Medical Association Lansell and Lawson grant, and a small project grant from the National Health Service Research and Development Fund.

Disclosures

None.

References


In addition to its well-known central enzymatic role within the coagulation cascade, thrombin is a powerful direct cellular activator of platelets, leukocytes, and vascular smooth muscle and endothelial cells. It is therefore a vital link between thrombosis and inflammation, key pathogenic factors in atherothrombotic disease. It has recently been discovered that thrombin causes direct cellular activation through a group of G-protein–coupled protease-activated receptors (PARs). Although the PAR-1 receptor is believed to mediate most of the receptor-mediated effects of thrombin, the cellular actions of PAR-1 activation have not been described in vivo in humans. In the present study, we establish for the first time the in vivo vascular effects of PAR-1 activation and demonstrate that it causes venous constriction, arterial dilatation, platelet aggregation, and argatroban, on prothrombin time and INR values.


Direct Vascular Effects of Protease-Activated Receptor Type 1 Agonism In Vivo in Humans
Ingibjörg J. Gudmundsdóttir, Ian L. Megson, Jillian S. Kell, Christopher A. Ludlam, Keith A.A. Fox, David J. Webb and David E. Newby

Circulation. 2006;114:1625-1632; originally published online October 2, 2006;
doi: 10.1161/CIRCULATIONAHA.106.638478
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/15/1625

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/