The Contribution of Activated Factor XIII to Fibrinolytic Resistance in Experimental Pulmonary Embolism

Guy L. Reed, MD; Aiilyan K. Houng, BS

Background—The resistance of thrombi to fibrinolysis induced by plasminogen activators remains a major impediment to the successful treatment of thrombotic diseases. This study examines the contribution of activated factor XIII (factor XIIIa) to fibrinolytic resistance in experimental pulmonary embolism.

Methods and Results—The fibrinolytic effects of specific inhibitors of factor XIIIa–mediated fibrin-fibrin cross-linking and α2-antiplasmin–fibrin cross-linking were measured in anesthetized ferrets with pulmonary emboli. Five experimental groups were treated with heparin (100 U/kg) and/or tissue plasminogen activator (TPA, 1 mg/kg) and the percent (mean±SD) lysis of emboli was determined: (1) control, normal factor XIIIa activity (14.1±4.8% lysis); (2) inhibited factor XIIIa activity (42.7±7.4%); (3) normal factor XIIIa activity+TPA (32.3±7.7%); (4) inhibited factor XIIIa activity+TPA (76.0±11.9%); and (5) inhibited α2-antiplasmin–fibrin cross-linking+TPA (54.7±3.9%). Inhibition of factor XIIIa activity increased endogenous lysis markedly (group 1 versus 2; P<0.0001), to a level comparable to that achieved with TPA (group 2 versus 3; P<0.05). Among groups receiving TPA, selective inhibition of factor XIII-mediated α2-antiplasmin–fibrin cross-linking enhanced lysis (group 3 versus 5; P<0.0005). Complete inhibition of factor XIIIa also amplified lysis (group 3 versus 4; P<0.0001) and had greater effects than inhibition of α2-antiplasmin cross-linking alone (group 4 versus 5; P<0.0005). No significant fibrinogen degradation occurred in any group.

Conclusions—Factor XIIIa–mediated fibrin-fibrin and α2-antiplasmin–fibrin cross-linking both caused experimental pulmonary emboli to resist endogenous and TPA-induced fibrinolysis. This suggests that factor XIIIa may play a critical role in regulating fibrinolysis in human thrombosis. (Circulation. 1999;99:299-304.)

Key Words: embolism ■ fibrinolysis ■ plasminogen activators ■ thrombus

The success of plasminogen activators and adjunctive agents in the therapy of thrombotic disease remains limited by the intrinsic resistance of thrombi to fibrinolysis. This fibrinolytic resistance is evident in patients with acute thrombotic coronary occlusion: treatment with plasminogen activators results in full coronary reperfusion in only 33% to 55% of patients at 90 minutes.1-3 The resistance of thrombi to lysis by plasminogen activators may be even more marked in patients with venous thromboembolism. In deep venous thrombosis treated with tissue plasminogen activator (TPA), nearly two thirds of patients have minimal or no significant lysis evident on repeat venography at 24 hours.4,5 In patients with pulmonary embolism, TPA restores blood flow within 24 hours to only about a third of occluded lung segments, as judged by serial perfusion scanning.6,7 In grim contrast to the therapeutic successes seen in patients treated for acute myocardial infarction with TPA, those treated for pulmonary embolism with TPA show no proven reduction in mortality.6,7 Indeed, some experts suggest that the mortality of pulmonary embolism, despite more modern therapies, has remained unchanged for the past 30 years.8,9 Because improved thrombolysis could translate to reduced mortality and morbidity, there is a pressing need for a better understanding of the causes of fibrinolytic resistance.

In patients with venous thromboembolism, the standard therapy is to inhibit new thrombus formation with heparin.10 Although heparin accelerates inhibition of thrombin and factor Xa by antithrombin, it does not accelerate endogenous fibrinolysis, in vitro or in patients.5-7 Plasminogen activators are the only agents currently available for accelerating the degradation of thrombi (ie, causing clots to undergo lysis faster than they would dissolve by endogenous fibrinolysis). Plasminogen activators convert plasminogen to plasmin, the fibrinolytic enzyme that degrades the thrombus. The physical properties of thrombi (age, mass, retraction, and composition) and local blood flow are thought to influence net fibrinolysis by plasmin.11 Also, fibrinolytic resistance is likely to be mediated by specific molecular factors such as α2-antiplasmin,12-14 plasminogen activator inhibitor-1 (PAI-1),15,16 thrombin-activated fibrinolysis inhibitor,17 and activated factor XIII (XIIIa). In vitro studies show that these factors regulate fibrinolysis by inhibiting plasmin or endogenous...
plasminogen activators or by modifying the susceptibility of fibrin to proteolysis by plasmin. Still, little is known about the relative importance of these factors in regulating endogenous or pharmacological fibrinolysis in vivo.

In particular, the contribution of coagulation factor XIIIa to fibrinolytic resistance in vivo is poorly understood. Factor XIII is a zymogen composed of 2 catalytic A subunits and 2 noncatalytic B subunits. Factor XIII is activated to a functional transglutaminase (factor XIIIa) when thrombin cleaves an activation peptide from the amino terminus of the A subunits. Factor XIIIa catalyzes the formation of covalent bonds between glutamine and lysine residues in the γ and α chains of adjacent fibrin molecules, which markedly increase the mechanical durability of the fibrin polymer. Factor XIIIa also rapidly cross-links α2-antiplasmin, the fast-acting plasmin inhibitor, to fibrin. In vivo, human thromboemboli show evidence of extensive cross-linking by factor XIIIa, and highly cross-linked thrombi are more resistant to lysis in vitro. Conversely, genetic factor XIII deficiency in humans is associated with rebleeding after trauma, suggesting that thrombi in these patients may have an enhanced susceptibility to fibrinolysis. The relative contribution of fibrin-fibrin cross-linking or of α2-antiplasmin–fibrin cross-linking to fibrinolytic resistance in vitro is still debated. In this study, we examined the contribution of factor XIIIa–mediated fibrin-fibrin cross-linking and α2-antiplasmin–fibrin cross-linking to the fibrinolytic resistance of experimental pulmonary emboli.

Methods

Materials were obtained from the following suppliers: aprotinin, Sigma; calcium chloride, Mallinckrodt; purified factor XIII and fibrinogen, American Diagnostica; goat anti-mouse antibody, Cappel and 2-antiplasmin–fibrin cross-linking to α2-antiplasmin–fibrin cross-linking in vitro. To determine the dose of factor XIII inhibitor or α2-antiplasmin peptide necessary to inhibit factor XIII–mediated cross-linking, we mixed various concentrations of anti–factor XIII antibody 9C11 (10 μg/mL; 0 to 10 μg with fresh-frozen plasma (45 μL), bovine thrombin (100 μU/mL; 3 μL), and calcium chloride (0.4 mol/L; 2.5 μL). The mixture was allowed to clot for 90 minutes at 37°C; the clots were then compressed and washed 3 times in 500 μL of saline to remove unbound protein. The clots were solubilized in 95 μL of 9 mol/L urea for 5 μL of β-mercaptoethanol at 37°C for 30 minutes. They were then mixed in 100 μL of SDS sample buffer with 20 μL of bromophenol blue–glycerol solution and incubated at 85°C for 5 minutes. Proteins were electrophoresed on 6% SDS-polyacrylamide gels and electrophoetized to polyvinylidene membranes for immunoblotting with a monoclonal antibody (4A5) to the γ chain of fibrin and a polyclonal antibody to the carboxy terminus of α2-antiplasmin, as we have described.

To inhibit the cross-linking of α2-antiplasmin to fibrin, we synthesized a peptide (NH2-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Leu-Lys) spanning the cross-linking site on the amino terminus of α2-antiplasmin, as we have described. The purity of the peptide was analyzed by high-performance liquid chromatography, and its composition was verified by amino acid analysis on a Waters Picotag system. The α2-antiplasmin peptide was then solubilized in 20 mmol/L Tris-HCl, and the pH was adjusted to 7.0. Various concentrations of peptide (7.5 μL, 0 to 5 mmol/L final) were mixed with 20 μL of plasma, 1.25 μL of calcium chloride (0.4 mmol/L), and 1.25 μL of thrombin (100 U/mL) and clotted for 90 minutes at 37°C as described above. The clots were solubilized and analyzed by immunoblotting as described above.

Pulmonary Embolism Experiments

The protocol for studying pulmonary embolism in ferrets has been described. In brief, male ferrets (~1 kg) were anesthetized with ketamine and acepromazine. After the animals were fully anesthetized, the jugular vein and carotid artery were exposed by an anterior midline incision and cannulated with 20-gauge catheters. Pooled, citrated human plasma was mixed with 125I-fibrinogen (≈1 000 000 cpm/mL). Individual clots were formed by mixing 125I-fibrinogen–labeled plasma (45 μL) with 2.5 μL of bovine thrombin (100 U/mL) and 2.5 μL of calcium chloride (0.4 mol/L). In some experiments, antibody 9C11 (3.1 μL; 10 μg) was added to each mixture to inhibit factor XIII activity or α2-antiplasmin peptide (3 μL; 1.5 mmol/L final concentration) was added to attenuate α2-antiplasmin cross-linking. After incubation at 37°C for 90 minutes, the clots were compressed and washed 3 times with saline to remove unbound protein. The radioactivity of the clots was measured in a gamma counter immediately before injection. Blood samples were drawn at baseline and at the end of the experiment. Sodium iodide (10 mg) was injected to block thyroid uptake. Three clots were emobilized into the lungs by injection through the internal jugular vein. Successful embolization was evinced by the accumulation of radioactivity in the thorax.

All animals received weight-adjusted heparin at 100 U/kg (bolus), a dose sufficient to keep the activated partial thromboplastin time (aPTT) at >150 seconds throughout the procedure. TPA was given as a continuous infusion over 2 hours (1 mg/kg in 5 mL of normal saline). Animals were observed for a total of 4 hours after pulmonary embolization and then killed by lethal injection of anesthesia or CO₂ inhalation. The thorax was dissected, and all intrathoracic structures were removed for gamma counting to detect residual thrombi. The percentage of clot lysis was determined for each ferret by dividing the total residual radioactivity in the thorax by that in the initial thrombi. A total of 28 animals were studied; 3 were excluded: 1 because of anesthesia-related death, 1 because of improper TPA infusion, and 1 because of failed embolism.

Anti-Factor XIII Monoclonal Antibody Production and Purification

The generation of monoclonal antibody 9C11 against the catalytic A subunit of human factor XIII has been described. The hybridoma producing 9C11 was cloned by limiting dilution and expanded into ascites in pristane-primed BALB/c mice. Antibody was purified from filtered ascites by precipitation with 40% ammonium sulfate. After resuspension and dialysis into 10 mmol/L KH₂PO₄, pH 7.2, proteins were absorbed on a DEAE–Affigel Blue Sepharose column and monoclonal antibody 9C11 was eluted with a linear gradient spanning 0 to 100 mmol/L NaCl, as we have described. Eluted protein was collected in fractions and analyzed by SDS-PAGE on 10% gels.
This experimental protocol was approved by the Harvard Medical Area Standing Committee on Animals. The Harvard Medical School animal management program is accredited by the American Association of Laboratory Animal Care, and the procedures were conducted in accordance with National Institutes of Health standards, as set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH] 85–23, revised 1985), the Public Health Service Policy on the Humane Care and Use of Laboratory Animals by Awardee Institutions, and the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

Fibrinogen Assays
Blood samples were collected on K₃EDTA (0.15% solution final) with aprotinin (50 U kallikrein per milliliter). Platelet-poor plasma was obtained by centrifugation of whole blood and assayed for fibrinogen by the sodium sulfite method.

Statistical Tests
The data were analyzed by a 1-way ANOVA followed by the Bonferroni-Dunn procedure for testing multiple comparisons.

Results

Inhibition of Factor XIII Activity During Clotting
The cross-linking by factor XIII of the γ chains of fibrin is one of the fastest catalytic processes mediated by the enzyme. Indeed, in the presence of fibrin, the activation of factor XIII by thrombin is accelerated. In previous studies, we derived a monoclonal antibody (9C11) that fully inhibits all factor XIII–mediated cross-linking in primate plasma. To determine the amount of 9C11 required for inhibiting factor XIII activity in the present studies, we examined its dose-related effects on fibrin–fibrin γ chain and α2-antiplasmin–fibrin cross-linking during clotting. In comparison with clots formed in the absence of inhibitor or in the presence of the nonspecific alkylation agent iodoacetamide, clots formed with 9C11 at doses of 5 and 10 μg/clot showed no significant fibrin–fibrin γ chain (Figure 1, top panel) or α2-antiplasmin–fibrin cross-linking (Figure 1, bottom panel). On the basis of these studies, we selected a 9C11 dose of 10 μg for our experiments.

Selective Inhibition of α2-Antiplasmin Cross-Linking to Fibrin
We used a peptide inhibitor to examine the effect on fibrinolytic resistance of factor XIIIa–mediated cross-linking of α2-antiplasmin to fibrin. This peptide represents the amino terminal cross-linking site of α2-antiplasmin and competitively inhibits the cross-linking of α2-antiplasmin to purified fibrin. Figure 2 (top) shows that increasing concentrations of the peptide quenched α2-antiplasmin–fibrin cross-linking, as reflected by a decrease in the high-molecular-weight α2-antiplasmin immunoreactivity (indicated by the arrow) and an increase in the lower-molecular-weight un–cross-linked α2-antiplasmin, which migrates as a broad (heavily glycosylated) protein at ~70 kDa. Increasing concentrations of the peptide had no apparent effect on the formation of fibrin γ–γ cross-links, as assessed by immunoblotting (Figure 2, bottom). On the basis of these studies, we selected a peptide concentration of 1.5 mmol/L for our studies.

Role of Factor XIII Activity in Endogenous Lysis
To determine the importance of factor XIII in endogenous fibrinolysis (ie, lysis caused by the ferret’s own fibrinolytic system), we compared the rates of dissolution of pulmonary emboli in animals treated with and without factor XIII inhibitor (Figure 3, left). All animals received heparin at a weight-adjusted bolus of 100 U/kg; this dose was sufficient to keep the aPTT at >150 seconds throughout the experiment. Lysis of pulmonary emboli in the control group was 14.1±4.8% (mean±SD), whereas in the group treated with factor XIII inhibitor was 3 times as much (42.7±7.4%: P<0.0001). This experiment indicated that inhibition of
Role of Factor XIIIa Activity in Pharmacological Lysis

We next examined the effect of factor XIII activity on the fibrinolytic resistance of pulmonary emboli in ferrets treated with TPA (1 mg/kg) over 2 hours, a regimen similar to that used to treat pulmonary embolism in humans. As above, all animals were treated with heparin, the standard therapy for human pulmonary embolism. One experimental group received pulmonary emboli with normal factor XIII activity, another pulmonary emboli in which both factor XIIIa–mediated fibrin-fibrin and α2-antiplasmin cross-linking had been quenched by 9C11, and a third pulmonary emboli in which the cross-linking of α2-antiplasmin to fibrin had been selectively inhibited by the α2-antiplasmin peptide.

In the group with normal factor XIII activity, TPA caused more lysis (Figure 3, TPA, 32.5±7.7%) than was seen in the group that received no plasminogen activator (control, 14.1±4.8%; P<0.005). Also, there was a nonsignificant trend (evaluated by the Dunn-Bonferroni correction) to more fibrinolysis in the group that received the factor XIII inhibitor but no TPA (Figure 3, F13-I) in comparison with the group with normal factor XIII activity that received TPA (42.7±7.4% versus 32.3±7.7%; P<0.05). Overall, factor XIIIa activity was an important determinant of fibrinolysis in animals treated with TPA because the factor XIII inhibitor group (Figure 3, TPA+F13) showed significantly more lysis than the group with normal factor XIII activity (76.0±11.9% versus 32.3±7.7%; P<0.0001). In particular, factor XIII–mediated cross-linking of α2-antiplasmin to fibrin made a specific contribution to fibrinolytic resistance because selective inhibition of this cross-linking also significantly accelerated lysis by TPA (Figure 3, TPA+α2AP) in comparison with lysis in animals with normal factor XIIIa activity (54.7±3.9% versus 32.3±7.7%; P<0.0005). Still, selective inhibition of α2-antiplasmin–fibrin cross-linking was less effective at amplifying lysis than was inhibition of all factor XIIIa–mediated cross-linking (54.7±3.9% versus 76.0±11.9%; P<0.0005), suggesting that fibrin-fibrin cross-linking also contributed to fibrinolytic resistance.

Effects on Fibrinogen Levels

We then determined whether the inhibition of total factor XIIIa activity or the selective inhibition of α2-antiplasmin–fibrin cross-linking enhanced the systemic degradation of the clotting factor fibrinogen during fibrinolysis. Fibrinogen levels were measured for all animals before and after the experiment. Figure 4 compares the residual fibrinogen levels at the end of the study for each group, expressed as a percentage of the initial fibrinogen value. There was no significant decrease in fibrinogen levels (below 100%) for any of the experimental groups. This indicated that nonspecific degradation of fibrinogen did not occur when TPA was administered alone, in combination with inhibition of factor XIII, or in combination with inhibition of α2-antiplasmin cross-linking.

Discussion

The intrinsic resistance of thrombi to endogenous and pharmacological fibrinolysis remains an important cause of morbidity and mortality in patients with thrombotic disease. In the experiments described here, we examined the contribution...
of factor XIIIa-mediated fibrin-fibrin cross-linking and α2-antiplasmin–fibrin cross-linking to the fibrinolytic resistance of pulmonary emboli. These studies required potent and specific inhibitors of the 2 factor XIIIa-mediated processes.

We used a monoclonal antibody (9C11), which is capable of quenching all factor XIIIa–mediated cross-linking,29 and verified that during clotting it completely inhibited both fibrin γ-chain cross-linking and α2-antiplasmin–fibrin cross-linking. To selectively inhibit only factor XIIIa–mediated α2-antiplasmin–fibrin cross-linking, we used a peptide corresponding to the amino terminus of human α2-antiplasmin, which contains the cross-linking site.32 We also verified that this peptide attenuated α2-antiplasmin–fibrin cross-linking without significantly perturbing factor XIIIa–mediated fibrin γ-chain cross-linking.

We analyzed the effect of these 2 inhibitors on the fibrinolytic activity of formed pulmonary emboli in an established model of pulmonary embolism in ferrets.29 To simulate the standard therapy for humans with pulmonary embolism, and to inhibit the accretion of new thrombus on these emboli, all animals were given doses of heparin sufficient to significantly prolong the aPTT (>150 seconds) throughout the experiment.29 Inhibition of factor XIIIa activity tripled the rate of endogenous fibrinolysis (42.7 ± 7.4% versus 14.1 ± 9.8%). This was a profound effect, because the amount of endogenous lysis in clots with inhibited factor XIIIa activity was as much as, or perhaps slightly more than, the amount induced by TPA in clots with normal factor XIIIa activity (32.2 ± 7.7%).

A parallel enhancement was seen in the lysis of pulmonary emboli by TPA (1 mg/kg): inhibition of both factor XIIIa–fibrin and α2-antiplasmin–fibrin cross-linking substantially increased lysis (76.0 ± 11.9%) over that seen with the same dose of TPA alone (32.3 ± 7.7%). In addition, selective inhibition of α2-antiplasmin–fibrin cross-linking amplified TPA-induced lysis (54.7 ± 3.9%) in comparison with that induced by TPA alone (32.3 ± 7.7%). This effect underscores the inhibitory role played by the cross-linking of α2-antiplasmin to fibrin during initiation of fibrinolysis. That even higher fibrinolysis was achieved with TPA in factor XIIIa–mediated fibrin–fibrin cross-linking and α2-antiplasmin–fibrin cross-linking was both inhibited fully (76.0 ± 11.9%) suggests that both cross-linking processes are important inhibitors of fibrinolysis induced by TPA. If full heparinization did not completely prevent the absorption and activation of ferret factor XIII onto these clots after embolization, some degree of cross-linking may have occurred in the thrombi of all experimental groups. The effect of this cross-linking would be to blunt the increased fibrinolysis attributed to the factor XIII inhibitors. This would not change the conclusion that factor XIIIa cross-linking is a major cause of fibrinolytic resistance, but it would imply that its role may be even larger than was observed in these experiments.

By providing new in vivo evidence about the role of factor XIIIa in the regulation of fibrinolysis, these studies should help resolve past controversy. Although in vitro studies indicate that clots in which factor XIII is deficient (or inhibited) undergo lysis at accelerated rates,27,28 the magnitude and molecular causes of this effect have been debated. In vitro studies suggest that fibrin-cross-linked α2-antiplasmin is an important determinant of the susceptibility of plasma clots to fibrinolysis.21,25,27 In fact, Jansen et al27 concluded that while factor XIIIa–mediated cross-linking inhibited fibrinolysis, its major inhibitory effect was mediated through the cross-linking of α2-antiplasmin to fibrin. In contrast, Francis and Marder,26 in studies of supraphysiologic doses of factor XIII, concluded that fibrin–fibrin cross-linking in and of itself contributes to fibrinolytic resistance. Their assertions were given credence by the finding that coronary fibrinolysis in dogs was accelerated by a weak, nonspecific inhibitor that prevented only the slowest factor XIIIa–mediated process, fibrin α-chain cross-linking.37 The use of 2 potent, selective inhibitors of factor XIIIa–mediated cross-linking in the present study permitted us to establish that fibrin–fibrin cross-linking and fibrin–α2-antiplasmin cross-linking both contribute to fibrinolytic resistance.

In addition to factor XIIIa, it is likely that other molecular factors in the thrombus (eg, PAI-1 and α2-antiplasmin) cause the fibrinolytic resistance seen in thrombotic diseases such as pulmonary embolism. Both PAI-1 and α2-antiplasmin are detected readily in human thrombi.38 Several studies in vivo have shown that PAI-1 contributes to the resistance of thrombi to endogenous fibrinolysis.39–41 Whether PAI-1 contributes to the resistance of thrombi to pharmacological fibrinolysis with full-dose plasminogen activators remains to be settled; high doses of TPA appear to overwhelm the inhibitory capacity of PAI-1, and agents like streptokinase are impervious to it.44 However, there is clear evidence from the present study, and from other studies in which inhibitory antibodies were tested, that α2-antiplasmin causes resistance to endogenous as well as pharmacological fibrinolysis in venous thromboemboli in vivo.29,45

In humans, pulmonary emboli appear to develop from the fragmentation of propagating thrombi in the deep venous system. Anticoagulants that interfere with the activity of thrombin prevent thrombus propagation by inhibiting the new deposition of fibrin.10 Still, despite effective anticoagulation, the inherent fibrinolytic resistance of formed thrombi prevents optimal treatment of patients with thrombotic disease. In these studies of heparinized animals, we found that factor XIIIa–mediated cross-linking played a critical role in limiting the endogenous and pharmacological fibrinolysis of formed experimental pulmonary emboli; this argues that factor XIII inhibitors eventually may be of potential use in the treatment of thrombotic disease. By virtue of its inhibitory effects on endogenous fibrinolysis, it is also likely that factor XIIIa facilitates the growth of new or forming thrombi, a hypothesis that merits investigation in suitable models of developing venous thrombosis.

Acknowledgments

This work was supported in part by National Institutes of Health grant HL-57314 (to G.L.R.). We gratefully acknowledge the editorial assistance of Thomas McVarish. This report is dedicated to the memory of Edgar Haber, MD.

References

1. Lincoff AM, Topol EJ, Califf RM, Sigenon KN, Lee KL, Ohman EM, Rosenschein U, Ellis SG. Significance of a coronary artery with thrombolysis in myocardial infarction grade 2 flow “patency” (outcome


The Contribution of Activated Factor XIII to Fibrinolytic Resistance in Experimental Pulmonary Embolism
Guy L. Reed and Aiilyan K. Houng

Circulation. 1999;99:299-304
doi: 10.1161/01.CIR.99.2.299

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 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/99/2/299

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/