Mast Cell Stabilization Reduces Hemorrhage Formation and Mortality After Administration of Thrombolytics in Experimental Ischemic Stroke

Daniel Strbian, MD; Marja-Liisa Karjalainen-Lindsberg, MD, PhD; Petri T. Kovanen, MD, PhD; Turgut Tatlisumak, MD; Perttu J. Lindsberg, MD, PhD

Background—Thrombolysis with tissue plasminogen activator (tPA) improves stroke outcome, but hemorrhagic complications and reperfusion injury occasionally impede favorable prognosis after vessel recanalization. Perivascularly located cerebral mast cells (MCs) release on degranulation potent vasoactive, proteolytic, and fibrinolytic substances. We previously found MCs to increase ischemic and hemorrhagic brain edema and neutrophil accumulation. This study examined the role of MCs in tPA-mediated hemorrhage formation (HF) and reperfusion injury.

Methods and Results—Exposure to tPA in vitro induced strong MC degranulation. In vivo experiments in a focal cerebral ischemia/reperfusion model in rats showed 70- to 100-fold increase in HF after postischemic tPA administration (P<0.001). Pharmacological MC stabilization with cromoglycate led to significant reduction in tPA-mediated HF at 3 (97%), 6 (76%), and 24 hours (96%) compared with controls (P<0.01, P<0.001, and P<0.01, respectively). Furthermore, genetically modified MC-deficient rats showed similarly robust reduction of tPA-mediated HF at 6 (92%) and 24 (89%) hours compared with wild-type littersmates (P<0.01 and P<0.001, respectively). MC stabilization and MC deficiency also significantly reduced other hallmarks of reperfusion injury, such as brain swelling and neutrophil infiltration. These effects of cromoglycate and MC deficiency translated into significantly better neurological outcome (P<0.01 and P<0.05, respectively) and lower mortality (P<0.05 and P<0.05, respectively) after 24 hours.

Conclusions—MCs appear to play an important role in HF and reperfusion injury after tPA administration. Pharmacological stabilization of MCs could offer a novel type of therapy to improve the safety of administration of thrombolytics.

Key Words: hemorrhage formation • mast cells • stroke • tissue plasminogen activator

Thrombolysis with tissue plasminogen activator (tPA) is the only approved pharmacological therapy for acute ischemic stroke aiming to provide early recanalization of an occluded cerebral artery. For every 1000 patients treated with tPA within 3 hours after symptom onset, >140 will avoid disability,1 and the therapeutic window may extend to 4.5 hours.2 A major obstacle is the risk for hemorrhage formation (HF). In consecutive patient cohorts of stroke thrombolysis, the risk of solid parenchymal hematomas, which commonly worsen the outcome, is 8% to 14%, and equal risk exists for hemorrhagic transformations.3–5 In placebo-controlled trials of thrombolysis, 6% of stroke patients developed substantial brain hemorrhages,2 and patients with acute myocardial infarction had a 1.1% risk of cerebral bleeding.6 Pulmonary embolism treated commonly with systemic tPA carries a risk of almost 3%.7

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The pathogenesis of postthrombolytic HF by tPA is not understood. Fibrinolytics activate clot-associated plasminogen, preferentially resulting in the formation of plasmin already bound to fibrin through lysine binding sites.8 The basal ganglia is a predilection site for HF, where spontaneous bleedings also frequently localize.

After successful thrombolysis, the reperfusion injury can elicit expansive brain swelling, which is responsible for most acute fatalities.9 Reperfusion injury originates at the blood–endothelium interface and includes acute blood–brain barrier (BBB) leakage, extravasation of plasma and erythrocytes, release of free radicals, and inflammatory mediators promoting adhesion of leukocytes and their emigration into the brain tissue.
Mast cells (MCs) are tissue-based stationary effector cells and form a first-line defense against various challenges. They have been found in a variety of locations in the nervous system in different species, including humans,10 and appear to be concentrated in relatively few locations, including the diencephalic parenchyma, particularly the thalamus, cerebral cortex, and the meninges, positioned in close proximity to potential cellular targets of mediator/cytokine action.11 Their metachromatic granules contain preformed vasoactive substances such as histamine, bradykinin, leukotrienes, serotonin, proteolytic enzymes such as chymase and tryptase, the anticoagulant heparin, chondroitin sulfate, and chemotactic factors (eg, neutrophil and eosinophil chemotactic factor). Degranulation and histamine release occur immediately after MC activation. Thereafter, de novo synthesis of vasoactive and chemotactic mediators (eg, prostaglandins, platelet-activating factor, and leukotrienes) and cytokines (eg, tumor necrosis factor-α and interleukins) ensues. Accordingly, MC-dependent hypersensitivity reactions have 2 distinct phases: the early phase, occurring in seconds to minutes and, several hours later, the secondary inflammatory phase, inducing the influx of neutrophils and eosinophils.

We recently found MC stabilization to decrease ischemic brain swelling and neutrophil infiltration12 and to reduce hematoma volume, brain swelling, and mortality after intracerebral hemorrhage.13 These findings, together with the characteristic perivascular location of MCs, the ability of their proteases to degrade the basement membrane proteins such as fibronectin,14–16 and our observation of colocalization of MCs and HF, led us to hypothesize that MCs might be involved in hazardous tPA-mediated cerebral bleedings after stroke. tPA led to robust MC degranulation in vitro. In vivo experiments showed that MC stabilization protected against tPA-mediated HF and brain swelling. This effect was associated with better neurological scores and reduced mortality. Similar results in genetically MC-deficient rats supported the crucial role of MCs.

Methods

Animals and Surgery

We used adult male Wistar rats (Harlan, Netherlands) and MC-deficient WsRe<sup>Ws</sup> rats (WsWs) (Japan SLC, Inc, Narita, Japan) and their wild-type (WT) litters (weight, 290 to 340 g). Rats were housed under diurnal lighting and allowed free access to food and water. The rats were anesthetized with intraperitoneal ketamine hydrochloride (50 mg/kg; Ketalar, Parke-Davis) and subcutaneous medetomidine hydrochloride (0.5 mg/kg; Domitor, Orion). A PE-50 polyethylene tube was placed into the left femoral artery to continuously monitor blood pressure (Olli 533, kone, Espoo, Finland) and to measure arterial pH, blood gases, and glucose (AVL OPTI, Rotkreuz, Basel, Switzerland). Another PE-50 tube was placed into the left femoral vein for drug and/or vehicle infusions. The rectal temperature was maintained at 37°C during the operation with a heating blanket and a thermoregulated heating lamp. The Animal Research Committee had approved the study protocol.

In Vitro Assay of MC Degranulation

Rat peritoneal MCs were obtained from Wistar rats as described.17 A standard amount of 2×10<sup>5</sup> MCs in 50 μL of phosphate-buffered saline was placed into Eppendorf tubes and preincubated for 10 minutes at 37°C. Then, freshly prepared tPA solution (Actilyse, Boehringer-Ingelheim) was added to give the final concentrations indicated (ranging from 0 to 50 μg/mL), and incubation was continued for 15 minutes to allow completion of MC degranulation. Immersing the tubes in ice-cold water stopped the reaction, and the cells were sedimented by centrifugation at 4°C. The histamine concentration in the supernatant was determined fluorometrically according to Bergendorff and Uvnäs with modifications.18 Histamine release by tPA was expressed as a percentage of the maximal histamine release induced by the standard MC secretagogue compound 48/80 (1 μg/mL) set as 100%.

Middle Cerebral Artery Occlusion

We used a nylon thread model as described.19 Reperfusion was accomplished by withdrawing the suture occluder after 90 minutes. Sham-operated animals underwent the same surgery except middle cerebral artery occlusion (MCAO).

Pharmacological Protocols and Animal Grouping

Several series with different timings of in vivo experiments were performed. With 6 hours of follow-up after the induction of MCAO, we first established a model to evaluate tPA-induced HF, inflammatory cell infiltration, and the potential effect of the timing of the tPA infusion (before or after reperfusion) (Table, experiment 1). Then we focused on the effect of pharmacological MC stabilization (sodium cromoglycate, a classic inhibitor of MC degranulation) on tPA-induced HF (Table, experiment 2). Because sodium cromoglycate crosses the BBB inadequately,20,21 it was administered intracerebroventricularly.

To assess the effect of posts ischemic interval and the treatment effect on HF and outcome, we performed experiments with pharmacological MC stabilization with 3- and 24-hour follow-up (Table, experiments 4 and 5). Because of a short biological half-life (90 minutes), we administered cromoglycate in the 24-hour groups as an intracerebroventricular bolus (750 μg/10 μL of saline) followed by continuous intracerebroventricular infusion of the same concentration using Alzet osmotic pumps (model 2001; 200 μL, 1 μL/h) and the Brain Infusion Kit 2 (both Durect Corporation, Cupertino, Calif) according to instructions. Finally, to confirm the role of MCs, we investigated the effect of tPA in genetically modified MC-deficient WsWs rats22,23 carrying a defective gene for c-kit (ligand for stem cell factor required for MC differentiation) and in their WT litters (Table, experiments 3 and 6). In comparison to WT rats, newborn Ws/Ws rats are anemic and MC deficient. Anemia, however, is ameliorated by the age of 10 weeks, whereas the MC deficiency increases, which has led to wide use of adult rats in specific investigations of MC function.22 In the present work, we used adult rats (aged 13 to 14 weeks); Table I in the online-only Data Supplement shows their hematological (Coulter Counter T-660, Coulter Electronics, London, UK) and hemostatic markers (Coagulometer KC-40, Lemgo, Germany) obtained by the mechanical clot method from rat plasma with 3.8% sodium citrate added.

Ten milligrams per kilogram of body weight is the standard dose of tPA thrombolysis in rats in studies focusing on both therapeutic efficacy and HF.24,25 and the infusion protocol was identical to that used for humans (Table). Experiments were blinded, and group assignments were randomized.

Tissue Processing

After termination with 120 mg IP of pentobarbital and cardiac perfusion, the brains were quickly removed and dissected into six 2-mm-thick coronal slices with the use of a standard brain-cutting matrix. Each third slice was cut into two 1-mm portions (rostro, caudal). The rostral part was embedded in Tissue-Tek (Sakura Finetek Inc, Tokyo, Japan), snap-frozen in liquid nitrogen, and kept at −80°C until 15-μm sections were cut for BBB leakage analysis12 (online-only Data Supplement). Five-micrometer sections were cut from the caudal site of all slices and processed for histochemical stainings. The remaining slices were incubated for 15 minutes in triphenyltetrazolium chloride (TTC) at 37°C and immersion-fixed in 10% formaldehyde.
Histopathological Examinations

Five-micrometer tissue sections were cut from the caudal part of the third slice and stained with hematoxylin-eosin and toluidine blue, a standard metachromatic histopathological technique to detect the heparin-containing granules present only in MCs (Figure ID in the online-only Data Supplement), and stained with chloroacetate esterase (Leder)\textsuperscript{26} to also detect polymorphonuclear neutrophils and heparin-containing granules present only in MCs (Figure ID in the online-only Data Supplement). Light microscopy was performed by an experienced hematopathologist (M.-L.K.-L.) with an Olympus BH-2 device, followed by photography with a Nikon Eclipse E600 microscope connected to a digital camera (Carl Zeiss) connected to the microscope. Finally, total areas containing extravasated erythrocytes and representing intracerebral hemorrhage were photographed with an AxioCam MRc digital camera (Nikon). The densities of intravascular and emigrated neutrophils were counted in systematically placed target areas of the temporoparietal lesion core, the parasagittal penumbra, and the deep thalamic and basal ganglia. Cerebrovascular areas were photographed with a Nikon Coolpix 995 digital camera (Nikon). The densities of intravascular and emigrated neutrophils were counted in systematically placed target areas of the temporoparietal lesion core, the parasagittal penumbra, and the deep thalamic and basal ganglia.

Intracerebral Hemorrhage Formation

Five-micrometer sections were cut from the caudal site of all 6 slices and stained with hematoxylin-eosin. We measured the areas of HF from these 6 hematoxylin-eosin–stained brain sections using an Axioplan 2 microscope (Carl Zeiss, Hallbergmoos, Germany). All areas with HF were photographed with an Axiocam MRc digital camera (Carl Zeiss) connected to the microscope. Finally, total areas containing extravasated erythrocytes and representing intracerebral hemorrhage were calculated from digital images with the use of image analysis software (National Institutes of Health, Bethesda, Md).

Lesion Sizes and Hemispheric Expansion

The 6 TTC-stained brain slices were photographed with a digital camera. Corrected lesion/infarction volumes were calculated as described.\textsuperscript{27} TTC reliably detects infarcted tissue 24 hours after MCAO, and data support its reliability at 6 hours,\textsuperscript{28,29} but extensive data supporting its use below this time point are not available. The percentage of hemispheric expansion was derived from the volumetric increase relative to the intact hemisphere (% of hemispheric expansion=[(right hemispheric volume/left hemispheric volume)−1]×100).

Neurological Evaluation

Neurological performance was scored at 24 hours, as follows: 0, normal; 1, contralateral paw paralysis; 2, 1 plus decreased resistance to lateral push; 3, 2 plus circulating behavior; 4, no spontaneous walking with depression of consciousness; and 5, death.\textsuperscript{27}

Statistical Analysis

Data are presented as mean±SE. Normally distributed parametric data sets in multiple groups were compared with 1-way ANOVA followed by the Holm-Sidak post hoc test. Nonnormally distributed data sets in multiple groups were compared with Kruskal-Wallis ANOVA followed by the Dunn post hoc test. Numerical data in comparisons of 2 groups showed normal distribution, and an unpaired t test was used. Neurological scores are reported as medians and individually for each animal. Comparison of mortality between groups was performed with the Fisher exact test. A 2-tailed value of P<0.05 was considered significant.

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

Physiological Variables
No significant differences were observed in physiological parameters (mean arterial blood pressure, temperature, pH, PaCO₂, Pao₂) among the study groups (data not shown).

Histopathological Findings
Ischemic neuronal changes in right hemispheres were confirmed by light microscopy of hematoxylin-eosin–stained tissue sections. tPA treatment was frequently associated with erythrocyte extravasation (Figure IA in the online-only Data Supplement), generally manifested as a perivascular hemorrhagic cuff (Figure IB in the online-only Data Supplement) arising from segmental structural deterioration of the vessel wall (Figure IC in the online-only Data Supplement), often accompanied by disseminated intraparenchymal hemorrhages or by a subarachnoid hemorrhage. In sections stained with toluidine blue detecting heparin present only in granulated MCs, metachromatic perivascular cells were found frequently siding small cerebral cortical and thalamic penetrating vessels (Figure ID in the online-only Data Supplement), often in widened Virchow-Robin spaces as described. Histopathological evaluation revealed enhanced polymorphonuclear neutrophil adhesion, extravasation, and migration on tPA treatment (Figure IF and IG in the online-only Data Supplement).

In Vitro Assay of MC Degranulation
A dose-dependent release of histamine indicated a degranulation of the MCs (Figure 1). The tPA-dependent histamine release reached up to 50% of the maximal degranulation-associated histamine release by the classic MC-secretagogue compound 48/80. Thus, even small concentrations of tPA, achievable also in vivo during therapeutic thrombolysis, strongly stimulated MCs to degranulate and release histamine in vitro.

Lesion Volumes
TTC-based corrected lesion volumes were not influenced by the treatment assignments in any of the experiments (Table).

Intracerebral Hemorrhage Formation
tPA induced HF at a similarly robust rate (70- to 100-fold) given either before or after reperfusion (Figure 2A). Pharmacological stabilization of MCs significantly reduced the HF caused by tPA at all time points (Figure 2B). Furthermore, MC deficiency in the WsWs rats was associated with a significant reduction of tPA-mediated HF (Figure 2C).

BBB Leakage
Fluorescence signal intensities, representing magnitude of extravasation, were significantly smaller after MC stabilization.
Hemispheric Expansion
Pharmacological stabilization of MCs led to significant reduction of brain swelling at all time points (Figure 3A). Moreover, WsWs rats had significantly less brain swelling than their WT littermates (Figure 3B).

Neutrophil Response
tPA administered either 5 minutes before or 90 minutes after reperfusion increased the postischemic neutrophil infiltration, but the earlier infusion had a more substantial effect (Figure 4A). Interestingly, a several-fold increase in neutrophil infiltration took place also in the noninfarcted hemisphere. In the WsWs rats, postischemic neutrophil counts were 40% of the WT littermates (Figure 4B).

Neurological Scores and Mortality
Evaluated at 24 hours, MC stabilization with cromoglycate led to significantly better neurological scores (median 2) compared with saline-treated (n=3) and tPA-treated (median 5) animals (P=0.002, Kruskal-Wallis ANOVA; Figure III in the online-only Data Supplement). WsWs rats had significantly less brain swelling than their WT littermates (Figure 3B).

Discussion
Hemorrhages and vascular reperfusion injury can devastate prognosis after stroke thrombolysis. Postthrombolytic hemorrhages occur in 8% to 11% of patients2–4 and in 1.1% of acute myocardial infarction patients and 3% of pulmonary emboli patients with no cerebral ischemia.6,7 We report a novel cellular mechanism underlying these catastrophic phenomena and identify a therapeutic pharmacological target. tPA degranulated MCs in vitro (Figure 1), a finding not reported for any fibrinolytic drug. tPA increased HF robustly in vivo after focal ischemia regardless of its timing (Figure 2A). Prevention of MC degranulation with sodium cromoglycate decreased tPA-induced HF at all time points (Figure 2B). The specificity of this finding was supported by reduced HF in MC-deficient rats (Figure 2C). Furthermore, both MC stabilization and MC deficiency led to reduced BBB leakage (Figure II in the online-only Data Supplement), less brain
swelling (Figure 3), and attenuation of neutrophil accumulation (Figure 4). Because these findings also translated into a better neurological outcome and reduced mortality, these observations are of potential relevance to clinical use of thrombolytic therapy (eg, stroke, acute myocardial infarction, and pulmonary embolism) for >10 million patients annually worldwide.

The mechanism of postthrombolytic HF is generally not understood, but it occurs with all fibrinolytic substances, such as tPA, streptokinase, prourokinase, and reteplase.31,32 They are potent serine proteases that, in addition to their effect of activating plasminogen to dissolve fibrin locally, invariably are potent serine proteases that, in addition to their effect of activating plasminogen to dissolve fibrin locally, invariably induce plasminemia.33 Plasmin is a multiactive substance with proinflammatory activity, degrades a range of extracellular matrix proteins, and activates matrix metalloproteinases (MMPs), which digest matrix proteins34 and cause brain tissue damage.35 Inhibition of MMPs was shown to reduce tPA-mediated mortality in experimental ischemia/reperfusion.36 Our data support the hypothesis that tPA aggravates the breakdown of BBB elements and increases extravasation of red blood cells, leading to HF (Figure 2A and Figure 1A in the online-only Data Supplement). This phenomenon depends to a large extent on MC degranulation (Figure 2, B and C), an effect also promoted by tPA itself (Figure 1). HF may be mediated by the strong anticoagulant heparin, which is produced in mammals only by MCs. MC-dependent BBB damage may be mediated by their numerous preformed substances influencing vascular permeability (eg, histamine, bradykinin). Furthermore, heparin, locally released from perivascularly located MCs, may prevent the formation of hemostatic plugs to patch up BBB to prevent red blood cell extravasation and thereby contribute to HF. Furthermore, our observation of tPA-induced direct MC degranulation in vitro deserves further human experiments and clarification of whether this is a class effect of fibrinolytics or specific to tPA.

The integrity of the BBB is largely determined by the basal lamina, the main 3 constituents of which are laminin, fibronectin, and collagen type IV. The integrin-labeled basal lamina loses its integrity very early after the onset of ischemia.37 Cerebral MCs release chymase,30,38,39 which cleaves fibronectin and activates procollagenases to liberate MMPs15,16 even in the presence of the tissue inhibitor of metalloproteinase.40 MCs also directly release gelatinases A (MMP-2) and B (MMP-9),41 which are known to degrade collagen type IV. Thus, MC-derived chymase and collagenase activity may participate in the early postischemic basal lamina degradation, which, in association with MC-derived heparin release, gives rise to gross hemorrhages. This was supported by reduced BBB leakage after MC stabilization and in MC-deficient rats (Figure II in the online-only Data Supplement).

Postthrombolytic reperfusion injury includes BBB damage, plasma extravasation, and swelling, causing intracranial pressure gradients even at 4 hours, and causes most fatal outcomes (eg, transtentorial herniation).9 Our data show a significant reduction of brain swelling by MC stabilization (Figure 3A), and the specificity of this effect was supported by findings in WsWs rats (Figure 3B). This agrees with our recent findings that MCs regulate ischemic brain swelling12 and intracerebral hemorrhage–related edema.13 tPA did not reduce the lesion size, as we focused on the untoward postreperfusion effects of tPA rather than therapeutic recanalization. This led us to use a nylon thread model with controlled reperfusion and not a blood clot model, which requires further examination of the efficacy of the present drug combination.

Another hallmark of reperfusion injury is infiltration of neutrophils, which accumulate within several hours after reperfusion42,43 (Figure 4); this also occurs in the human brain.44 It is believed to compromise microcirculation and BBB integrity and to release free radicals. We observed a consistent 2- to 3-fold increase in neutrophil infiltration in the ischemic hemispheres after tPA and up to a 6-fold increase in the intact hemisphere (Figure 4A), suggesting an independent proinflammatory effect of tPA. Such an effect of tPA that may relate to tPA toxicity was reported previously in a similar MCAO model and reversed by an N-methyl-D-aspartate antagonist.45 In our study, neutrophils were largely intravascular, but the count of neutrophils already emigrated into the brain parenchyma was also significantly increased by tPA (data not shown). This was also influenced by MCs because in the WsWs rats the tissue neutrophil counts were <40% of those of the WT littermates (Figure 4B); this finding was similar in emigrated and intravascular neutrophils (data not shown) (Figure IF and IG in the online-only Data Supplement). HF could be the trigger of the accumulation of neutrophils, but we recently also reported increased neutrophil infiltration without tPA-mediated HF, observing smaller posts ischemic neutrophil counts after MC stabilization and in MC-deficient rats.12 Therefore, we believe that MCs recruit neutrophils independently of the HF.

Tissue-based MCs release chemoattractants (eg, neutrophil and eosinophil chemotactic factor, platelet-activating factor, tumor necrosis factor-α) that may contribute to the early chemotactic gradient after which blood-borne neutrophils start to transmigrate. The proinflammatory effect of tPA might be mediated by plasmin, which is able to induce the synthesis of platelet-activating factor and activates the terminal complement cascade.46 Injected into the brain, it recruits neutrophils.35 Accordingly, stabilization of the nonmigrating MCs may provide an early opportunity to prevent infiltration of inflammatory cells and their deleterious effects.

The present study protocol mimics the time window for clinical thrombolysis of 3 (possibly 4.5) hours after stroke onset.2 We have shown that MC stabilization and MC deficiency significantly reduce tPA-mediated HF, hemispheric expansion, and neutrophil infiltration in rats. Our key findings also translated into significant neurological improvement and reduced mortality and were supported by similar results in the genetically MC-deficient rats. In conclusion, pharmacological stabilization of MCs should be studied further as a potential novel therapy to prevent the occasional devastating complications of administration of thrombolytics.

Sources of Funding
This study was supported by grants (to Dr Lindberg and Dr Tatlisumak) from the Finnish Academy, the University of Helsinki,
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References

This study investigated thrombolytics-related hemorrhage formation in an experimental stroke model in rats. We found that, in addition to the fibrinolytic effect leading to clot lysis, alteplase (recombinant tissue plasminogen activator) also possesses proinflammatory properties and activates and degranulates mast cells. On degranulation, mast cells release potent proteases (eg, chymase, tryptase, and metalloproteases) that can target the proteins in the vessel wall and the basal lamina. The increased vascular permeability can lead to brain edema and potentially to frank hemorrhage formation. The mast cell stabilizer cromoglycate, administered before alteplase, reduced these detrimental effects and led to improved neurological outcome and reduced mortality. Furthermore, genetically mast cell–deficient mice showed minimal brain edema and alteplase-related hemorrhage formation and improved neurological outcome and mortality compared with wild-type littermates. In addition to proteolytic enzymes, mast cells release vasodilators such as histamine and bradykinin, as well as heparin, which may locally compromise hemostasis and predispose to hemorrhage and edema formation with an unfavorable prognosis. This study revealed a novel proinflammatory mechanism that may provide a novel pharmacological target if confirmed in the clinical setting. Mast cell stabilization therefore deserves further study as an adjuvant to thrombolysis.
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Circulation. 2007;116:411-418; originally published online July 2, 2007; doi: 10.1161/CIRCULATIONAHA.106.655423
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/116/4/411

Data Supplement (unedited) at:
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