Local Heat Shock Priming Promotes Recanalization of Thromboembolized Microvasculature by Upregulation of Plasminogen Activators

Martin Rücker, Thilo Schäfer, Claudia Scheuer, Yves Harder, Brigitte Vollmar, Michael D. Menger

Objective—Thromboembolization and subsequent microvascular perfusion failure is implicated in the pathology of a variety of diseases, including transient ischemic attack (TIA), stroke, and myocardial infarction, and also for the complications after interventional and microsurgical procedures in coronary heart disease and peripheral arterial occlusive disease. In vitro heat shock priming has been suggested to induce plasminogen activators, which are the major upregulators of the fibrinolytic system. Herein, we determined whether local heat shock priming endogenously upregulates plasminogen activators also in vivo, and whether this promotes recanalization of thromboembolized microvasculature.

Methods and Results—To induce thromboembolization, a suspension of preformed microthrombi (maximum diameter: 40 μm) was injected via the femoral artery into the left hindlimbs of anesthetized rats. Local heat shock priming (42.5°C, 30 minutes) was performed 24 hours before embolization and resulted in a significant increase of endothelium-derived plasminogen activator expression. The study of the microcirculation by intravital microscopy revealed in all tissues analyzed (muscle, periosteum, subcutis, and skin) that heat shock priming significantly (P<0.05) accelerates recanalization of the thromboembolized microvasculature when compared with nonprimed and sham-primed controls. Importantly, the addition of plasminogen activator inhibitor-1 to the microthrombi suspension completely blunted the heat shock-induced acceleration of microvascular recanalization.

Conclusions—Heat shock induces endogenous hyperfibrinolysis by upregulation of plasminogen activators that promote recanalization of thromboembolized microvasculature. (Arterioscler Thromb Vasc Biol. 2006;26:1632-1639.)

Key Words: heat shock ■ intravital microscopy ■ microcirculation ■ plasminogen activator inhibitor-1 ■ thromboembolization ■ urokinase plasminogen activator

In myocardial ischemia and stroke, thromboembolization of the microvasculature essentially contributes to infarction and necrosis. In addition, vascular interventional procedures, such as PTA and PTCA, but also coronary bypass surgery and peripheral reconstructive microsurgery, may be complicated by downstream microvascular thromboembolization with the consequence of nutritive capillary perfusion failure. Recanalization of thromboembolized microvasculature can be achieved by urokinase therapy; however, this bears some risk for bleeding and aggravation of organ dysfunction, particularly if a surgical procedure is involved. In contrast, endogenous induction of hyperfibrinolysis would represent a more elegant approach; however, little information is available as to whether this may be capable of successfully promoting recanalization of thromboembolized microvasculature.

Preconditioning by heat shock requires the exposure of the tissue to a supraphysiological but sublethal temperature, which results in a transient change of cellular biosynthesis with an accelerated induction of only a few distinct proteins, including heat shock proteins.

The serine proteases urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) initiate the endogenously mediated lysis of platelet emboli. Both enzymes derive from endothelial cells and convert plasminogen to the fibrinolytic protease plasmin. Various types of stimuli are known to directly modulate plasminogen activator synthesis and release from endothelial cells. In vitro, stress conditioning by heat shock has been shown to induce plasminogen activators in human umbilical vein endothelial cells. With the use of a rat hindlimb microcirculation model, we herein demonstrate for the first time to our
knowledge that local heat shock priming induces upregulation of plasminogen activators also in vivo, and that this promotes endogenous hyperfibrinolysis, which results in accelerated recanalization of thromboembolized microvasculature.

Methods

Animals

Experiments were performed in 72 Sprague-Dawley rats with a body weight of 280 to 350 grams. The study complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and was approved by the local animal care committee.

Surgical Procedure

Under pentobarbital anesthesia (50 mg/kg intraperitoneal; Abbott, Chicago, Ill), the animals underwent tracheotomies and were placed on a heating pad to guarantee 37°C body temperature. Polyethylene catheters were inserted into the right carotid artery and left jugular vein. The catheters allowed monitoring of blood pressure, continuous infusion of saline (1 mL/100 g per hour), withdrawal of blood, and injection of fluorescent dyes for intravital microscopy. The left hindlimb preparation for microcirculatory analysis was performed according to the technique described previously in detail. The preparation exposed tibial peristeum, gracilis and semitendinous muscles, subcutis and skin, and was supplied by the femoral vessels. All branching vessels were ligated up to the superficial epigastric artery, in which a catheter was inserted with the tip directed toward the femoral artery.

Microvascular Thromboembolization

Arterial platelet-rich thrombi were prefomed in vitro in a moving high-pressure closed compartment system. For each experiment a 1-mL insulin syringe was filled with thrombin (60 U/mL; T6634; Sigma-Aldrich, Taufkirchen, Germany). In a second syringe, blood was drawn from the carotid artery. After 20 seconds, the 2 syringes were interconnected and the suspension containing blood and thrombin (4:1) was moved during 3 minutes ~70 times from one syringe to the other. In additional experiments, a rat-specific plasminogen activator inhibitor (PAI-1) (30 pg/mL; #102; American Diagnostica, Pfungstadt, Germany), the principal physiological inhibitor of both tPA and uPA, was added to the microthrombi suspension. The syringes were left standing for 30 minutes until embolization. To exclude thrombi >40 μm, the thrombus suspension was filtered through a nylon strainer (2340; Falcon; Becton Dickinson, Heidelberg, Germany). For thromboembolization of the microvasculature in vivo, 30 μL of the filtered thrombi suspension was gently injected into the femoral artery.

Western Blot Analysis of uPA, tPA, and PAI-1

Muscle and skin tissue was harvested after 24 hours and homogenized in lysis buffer (10 mmol/L Tris pH 7.5, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5% Triton-X-100, 0.02% Na2, 0.2 mmol/L phenyl-methyl-sulfonyl fluoride), incubated for 30 minutes on ice, and centrifugated for another 30 minutes at 16 000 g for 4 °C. Equal amounts of protein per lane (90 μg) were separated discontiuously on 10% sodium dodecyl sulfate polyacrylamide gels under denaturating conditions and transferred to a polyvinylidifluoride membrane (BioRad, Munich, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 hours with a chicken–anti-rabbit–uPA/ mouse–anti-rabbit–tPA/ rabbit–anti-rabbit–PAI-1 antibody (1:50; American Diagnostica), a goat–anti-rat–uPA antibody (1:50; Santa Cruz Biotechnology) or a rabbit–anti-rabbit–PAI-1 antibody (1:50; American Diagnostica) followed by the secondary horseradish peroxidase-conjugated bovine–anti-chicken (1:3500; Santa Cruz Biotechnology), rabbit–anti-goat (1:2000; R&D, Wiesbaden, Germany), and donkey–anti-rabbit (1:2500; Amersham Biosciences, Freiburg, Germany) IgG antibodies, respectively. Protein expression was visualized using luminol-enhanced chemiluminencescence and exposure of membranes to blue light-sensitive autoradiography film. Signals were densitometrically assessed and normalized to β-actin signals to correct unequal loading.

Experimental Groups

In a first group of animals (n=8), stress conditioning by local heat shock priming was performed 24 hours before thromboembolization. Animals undergoing a sham procedure of local heat shock priming was performed 24 hours before thromboembolization, and the hindlimb was exposed in a waterbath to 30°C for 30 minutes. In these animals, the muscle temperature was kept at ~36°C to 37°C. Muscle temperature was monitored with a needle thermo-probe (LICOX-System; GMS, Kiel-Mielendorf, Germany).

Immunohistochemistry

After elimination of endogenous peroxidase activity and nonspecific protein binding, specimens were incubated overnight at 4°C with either anti-uPA-antibody (1:200; #1191; American Diagnostica), anti-tPA-antibody (1:200; kindly provided by J.J. Emeis; Leiden, Netherlands) or anti-PAI-1-antibody (1:200; #1062; American Diagnostica), which react specifically with rat uPA, rat (PA and rat PAI-1, respectively. As secondary antibody, either a biotinylated donkey–anti-chicken–IgG (Dianova, Hamburg, Germany) or goat–anti-rabbit–IgG antibody (DAKO-Cytomation, Hamburg, Germany) was used. Thereafter, streptavidin-horseradish peroxidase complex was added for 30 minutes at 20°C, followed by 5 minutes of treatment with 3,3’-diaminobenzidine. Slides were counterstained with hematoxylin. For negative controls, slides were treated similarly but without the primary antibody. All control stainings were negative.

The staining intensity was evaluated blindly according to Page et al; A “0 to 4” score was applied, depending on color intensity and extent, ie, 0 indicates negative, comparable to control section; 1, very weakly positive; 2, weakly positive; 3, positive; and 4, strongly positive.

Intravital Fluorescence Microscopy

The tissues were positioned on a micromanipulator-adjusted stage and covered with a glass slide to prevent drying and exposure to ambient air. The microcirculation was analyzed with a blue filter (450 to 490 nm/520 nm) after intravenous injection of fluorescent dyes for intravital microscopy and exposure of membranes to blue light-sensitive autoradiography film. Signals were densitometrically assessed and normalized to β-actin signals to correct unequal loading.

Video Analysis

Microscopic images were recorded by a charge-coupled device video camera (FK-6990, COHU, Prospective Measurements, San Diego, Cali) and transferred to a video system. Microcirculatory parameters were quantified off-line using a computer-assisted image analysis system (Capimage, Zeintl, Heidelberg, Germany). After thromboembolization, microvascular recanalization was analyzed by studying the re-onset of capillary perfusion. In addition, a 4-hour follow-up assessment included the determination of: (1) the fraction of perfused capillaries (%), defined as percentage of the length of red blood cell (RBC) perfused capillaries relative to the total length of all capillaries per unit area; (2) capillary V_{ABC} (mm/s); and (3) capillary diameters (μm). Volumetric capillary blood flow (CBF) (pL/s) was calculated for each microvessel as CBF = π*(D/2)^2 * V_{ABC}.

Stress Conditioning

For stress conditioning, left hindlimbs of anesthetized rats were heated in a waterbath 24 hours before thromboembolization. During local heating, muscle temperature was increased to 42.5°C and was kept constant for 30 minutes. Animals undergoing a sham heating procedure were also anesthetized 24 hours before thromboembolization, and the hindlimb was exposed in a waterbath to 30°C for 30 minutes. In these animals, the muscle temperature was kept at ~36°C to 37°C. Muscle temperature was monitored with a needle thermo-probe (LICOX-System; GMS, Kiel-Mielendorf, Germany).
(n=8), and a further group of animals was designed to study the effect of heat shock preconditioning alone without thromboembolization (n=8). To study the role of plasminogen activators, further heat shock-primed (n=8) and nonheat shock-primed animals (n=8) underwent thromboembolization with PAI-1-supplemented microthrombi suspensions. The microcirculation of muscle, periosteum, subcutis and skin was analyzed before and at 30 minutes, 60 minutes, 120 minutes, 180 minutes, and 240 minutes after thromboembolization. Tissue samples for immunohistochemistry were obtained at thromboembolization (additional animals) and 4 hours recanalization.

**Statistical Analysis**
Results are expressed as means±SEM. Differences between groups were assessed by 1-way ANOVA, differences within each group were analyzed by 1-way repeated measures ANOVA. To isolate overall differences, appropriate Student-Newman-Keuls or Dunn post-hoc tests were performed. Differences were considered significant at P<0.05.

**Results**

**Systemic Circulatory Parameters**
In all animals, mean arterial blood pressure (>100 mm Hg) and heart rate (340 to 400 minutes⁻¹) were in normal range without significant differences between the groups studied.

**Expression of uPA, tPA, and PAI-1**
In nonheat shock-primed controls, immunohistochemistry revealed almost lack of expression of uPA and an only slight expression of tPA, which was found predominantly localized in arteriolar endothelial cells (Figure 1). After heat shock, expression of uPA and tPA was significantly enhanced (Table), predominantly in endothelial and smooth muscle cells (Figure 1). PAI-1 increased slightly on heat shock-priming but was less pronounced compared with uPA and tPA (Table; Figure 1). In all experiments, uPA, tPA, and PAI-1 expression was not affected by adding PAI-1 to the microthrombi suspension.

Quantitative Western blot analysis confirmed a significant upregulation of uPA and tPA at 24 hours after heat shock preconditioning, whereas PAI-1 expression was only slightly increased in muscle and not affected in skin compared with nonheat shocked controls (Figure 2).

**Microvascular Thromboembolization**
Directly after injection, intravital microscopy revealed that the microthrombi were arrested in the downstream microcirculation (Figure 3). This resulted in a complete shutdown of microvascular perfusion in all tissues analyzed.

**Microvascular Recanalization**
During the first 30 minutes after thromboembolization, nonheat shock-primed and sham heat shock-primed controls showed almost complete lack of recanalization. Only 1 of 8 and 1 of 4 preparations revealed signs of recanalization (Figure 3). During the second 30 minutes after thromboembolization, all preparations developed recanalization (Figure 3), but with different quality of microvascular reperfusion. After 30 minutes only 10% of the initially perfused capillaries were found reperfused (Figure 4). Recanalization improved during the next 2 hours, as indicated by an increase of perfused capillaries to ≈50% of baseline, but without further recovery during the 4-hour observation period (Figure 4).

This was associated with a markedly lowered volumetric CBF (P<0.05; Figure 5). There were no differences in recanalization between muscle, periosteum, subcutis and skin, and no significant differences between nonheat shock-primed and sham heat shock-primed animals (Figure 4).

Heat shock priming effectively accelerated initial recanalization of the obstructed microvasculature. Within the first 15 minutes after thromboembolization, all 8 tissue preparations showed recanalization (Figure 3). At 30 minutes, already 60% of the initially perfused capillaries were found reperfused in either of the tissues analyzed (P<0.05; Figure 4). Recanalization over the subsequent 3.5-hours further improved capillary perfusion to ≈80% of baseline, which was significantly higher than that of nonheat shock-primed and sham heat shock-primed controls (P<0.05; Figure 4).
Importantly, heat shock-primed tissues showed already at baseline a significantly higher volumetric CBF ($P<0.05$) than nonconditioned tissues (Figure 5). Further, heat shock produced reactive hyperemia during initial recanalization and preserved CBF over the entire post-thromboembolization period.

Addition of PAI-1 to the thrombi resulted in prolonged failure of recanalization ($P<0.05$). Until 2 hours after thromboembolization, zero and, at 4 hours only 2, of 8 preparations showed recanalization (Figure 3). This was associated with a significantly ($P<0.05$) lowered fraction of perfused capillaries (Figure 4) and a marked ($P<0.05$) compromise of CBF (Figure 5) compared with nontreated controls. Heat shock priming accelerated the onset of recanalization and improved the microcirculation (Figures 4 and 5; $P<0.05$), although the addition of PAI-1 was associated with a reduction of capillary density and CBF when compared with heat shock-conditioned but non–PAI-1–treated controls (Figures 4 and 5; $P<0.05$).

**Microvascular Response to Heat Shock Priming**

In animals without any treatment, analysis of capillary perfusion over the 4-hour observation period showed ≈100% of the capillaries perfused without significant changes of CBF (Figures 4 and 5). Heat shock priming without thromboembolization did not affect capillary density (Figure 4) and confirmed the increased CBF compared with nonheat-shocked controls (Figure 5).

**Discussion**

The major novel findings of the present study are that heat shock priming: (1) upregulates plasminogen activators in vivo; (2) accelerates recanalization of thromboembolized microvasculature; and (3) counteracts the function of PAI-1. Thus, local upregulation of heat shock proteins induces endogenous hyperfibrinolysis.

Thromboembolization significantly contributes to the shutdown of microvascular perfusion, infarction, and necrosis.1,3 The microemboli may clot primarily arteriolar and capillary segments of the microvasculature, and thus produce initially a negative angiogram.19 Over time, obstruction of the vasculature may aggravate, producing relevant infarction, or may dissolve because of spontaneous recanalization.20,21

The study of the mechanisms of thromboembolization requires an adequate experimental model, which should consider the development of both infarction and spontaneous recanalization. O’Shaughnessy et al22 introduced a thromboembolization model, demonstrating that platelet emboli, originating from the site of arterial vessel repair, pass downstream, and block the microcirculation.22 This confirms the particular risk of interventional and microsurgical procedures for thromboembolism. Herein, we have not chosen this model, because the development of thromboemboli is heterogeneous, and the cremaster muscle allows only a 2-hour study period, which is too short to analyze spontaneous recanalization. We have used the rat hindlimb model, because this allows repetitive intravital microscopy of the microcirculation of muscle, periosteum, subcutis, and skin for a period of 6 hours.12 Further, we have injected in vitro produced microthrombi, mimicking platelet-rich arterial thrombi, which adequately standardizes the experiments.15

The process of platelet-rich arterial thrombus formation can be partitioned into platelet adhesion, coagulation factor activation and thrombus propagation.23 Accordingly, in the model used thrombi were generated by addition of thrombin (coagulation factor activation). Because a low pressure in vitro system can produce large “white” thrombi, however, with a “red” tail,24 we have chosen a moving high-pressure closed compartment system,13 which generates arterial “white” thrombi, rich in platelets and fibrin, intermingled with only a few erythrocytes and leukocytes.13 The moving
High-pressure closed compartment system mimics conditions of pressure and turbulences as known in arterial thrombus formation in vivo. The platelet-rich thrombi were filtered through a 40-μm mesh to exclude large thrombi. Only small thrombi of 40 μm were used for the experiment to guarantee that embolization takes place within terminal arterioles. This microembolization mimics the clinical situation of ischemic stroke and flap tissue failure.

Because terminal arterioles are not regularly visible by intravital microscopy, recanalization was not assessed by direct visualization of the lysis of arrested microthrombi, but indirectly by evaluation of the re-onset of capillary perfusion within the downstream microcirculation. This can reliably be performed, because downstream capillary perfusion failure, which additionally indicates tissue viability, has been shown to correlate with embolization-induced arteriolar vessel obstruction.

Previous studies have demonstrated that heat shock increases capillary perfusion in flap tissue. Thus, the increased baseline CBF may have contributed to the accelerated recanalization. However, although baseline skin CBF did not differ significantly between heat shock preconditioning versus non-preconditioned controls, heat shock significantly accelerated recanalization, similarly as observed in the other tissues, in which baseline CBF was different between the 2 groups. Thus, the heat shock-mediated increase in basal CBF may not be considered a primary determinant for recanalization.

Beside acceleration of recanalization and improvement of capillary perfusion, heat shock increased CBF above baseline levels initially during recanalization. This reactive hyper-

**Figure 2.** Western blot analysis of PAI-1, uPA, and tPA in muscle (A,B) and skin (C) at 24 hours after heat shock preconditioning (hs) (n=4) and in nonheat shock-primed controls (con) (n=4). Representative examples from muscle (A) and quantitative analysis from muscle (B) and skin (C). Note the significant increase of uPA and tPA after heat shock priming, whereas PAI-1 was only slightly increased. Mean±SEM; *P<0.05 vs nonheat shock-primed controls.

**Figure 3.** Intravital microscopy of thrombolysis in a transverse arteriole. The thrombus (arrows) is visualized by negative contrast using FITC–dextran plasma staining (A) and directly by rhodamine-staining of platelets (B). C and D, Identical arteriole after onset of recanalization. Magnification ×80. E and F, Rate and time course of recanalization of microthrombi (E, n=8) and microthrombi containing PAI-1 (F, n=8). Note the acceleration of recanalization by heat shock preconditioning (closed circles) compared with nonpreconditioned (open circles) and sham-preconditioned (open diamonds) controls.
emia, which classically is produced only after short ischemia periods, may be caused by the rapid lysis-associated reduction of ischemia time by heat shock priming.

Because anesthesia and immersion of the hindlimb may also represent a stress conditioning, we have exposed additional animals to a sham procedure, which was not associated with tissue heating. Of interest, these animals did not show acceleration of recanalization and improvement of capillary perfusion. This supports that the protective effect on the microcirculation is caused by heat induction rather than anesthesia or hindlimb manipulation-associated stress.

By converting plasminogen to plasmin, plasminogen activators are the main upregulators of the fibrinolytic system.\(^8\) The serine proteases uPA\(^26\) and tPA\(^27\) are synthesized by endothelial cells. In vivo, tPA is the principal plasminogen activator, whereas uPA serves as an amplifier of the tPA-mediated fibrinolysis after its activation by tPA-mediated generation of plasmin.\(^28\) A procoagulant condition induced by thrombin is the predominant physiological stimulus for expression and release of both plasminogen activators.\(^29\)

In vitro studies have demonstrated that prolonged hyperthermia of >8 hours downregulates plasminogen activators and stimulates PAI-1 expression in human umbilical vein endothelial cells.\(^30,31\) However, hyperthermic exposure times of <8 hour indicated that both uPA and tPA are upregulated by heat shock in endothelial cells.\(^11\) The present study extends this observation, demonstrating for the first time to our knowledge that 30 minutes of heat shock upregulates uPA and tPA also in vivo, and that the expression is found primarily in arteriolar endothelial cells. Thus, an enhanced lysis of fibrin in platelet emboli by uPA and tPA may be the cause for the observed acceleration of recanalization after heat shock priming.

Fibrinolysis is inhibited by endothelium-derived PAI-1, which binds irreversibly to the active site of uPA and tPA.\(^27,32\) The observed abrogation of recanalization by PAI-1 is in line with these previous reports,\(^32,33\) and confirms the causative role of uPA and tPA in spontaneous recanalization of thromboembolized microvasculature. Our PAI-1 experiments further indicate that the heat shock-mediated upregulation of

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**Figure 4.** Fraction of perfused capillaries in nonheat shock-primed (open circles), sham heat shock-primed (open diamonds) and heat shock-primed (closed circles) muscle (A,B,C), skin (D,E,F), subcutis (G,H,I), and periostum (J,K,L) before and after thromboembolization with microthrombi (B,E,H,K) and microthrombi containing PAI-1 (C,F,I,L). Preparations without thromboembolization served as controls (A,D,G,J). Mean±SEM; *P<0.05 vs nonheat shock-primed controls, #P<0.05 vs baseline.
endogenous uPA and tPA does not only counteract the function of endogenous but also exogenously applied PAI-1.

The increase of endogenously expressed PAI-1 on heat shock induction was markedly less pronounced compared with the increase of uPA and tPA. This is in line with previous observations, demonstrating a stronger expression of uPA and tPA compared with PAI-1 in response to hyperthermia. Although PAI-1 is a major stress-regulated gene, it is only weakly expressed in endothelial cells, and may be further reduced in endothelial cells located close to fibrin clots. The endogenous induction of a local pro-thrombolytic state by heat shock priming offers some advantages compared with the systemic application of plasminogen activators. Plasminogen activators are rapidly cleared from the systemic circulation, mainly by the function of the liver. Additionally, the thrombolytic properties after local heat shock are restricted to the endangered microcirculation. Thus, systemic side effects, such as the assumed neurotoxicity of tPA, adverse bleeding complications, or anaphylactoid reactions related to recombinant tPA, may be avoided.

Heat shock priming may represent a promising tool also in the clinical setting. Of interest, fever in septic conditions has been shown to induce heat shock proteins and to result in better respiratory function, lower blood lactate concentrations, and prolonged survival times. These experimental results support clinical studies, demonstrating protective functions of intracellular heat shock protein-70 expression in patients with severe sepsis. However, the effect of sepsis-associated fever on blood coagulation and thrombus formation remains to be determined.

In conclusion, we herein demonstrate that local heat shock preconditioning induces hyperfibrinolysis and consequently results in an accelerated and improved recanalization of thromboembolized microvasculature. Thus, the efficacy of local heat shock preconditioning should be further evaluated experimentally as a novel treatment strategy in disease states associated with microthromboembolization and infarction.
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Disclosures
None.

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