Urokinase-Type Plasminogen Activator Promotes Paracellular Transmigration of Neutrophils Via Mac-1, But Independently of Urokinase-Type Plasminogen Activator Receptor

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Background—Urokinase-type plasminogen activator (uPA) has recently been implicated in the pathogenesis of ischemia-reperfusion (I/R) injury. The underlying mechanisms remain largely unclear.

Methods and Results—Using in vivo microscopy on the mouse cremaster muscle, I/R-elicited firm adherence and transmigration of neutrophils were found to be significantly diminished in uPA-deficient mice and in mice treated with the uPA inhibitor WX-340, but not in uPA receptor (uPAR)–deficient mice. Interestingly, posts ischemic leukocyte responses were significantly reduced on blockade of the integrin CD11b/Mac-1, which also serves as uPAR receptor. Using a cell transfer technique, posts ischemic adherence and transmigration of wild-type leukocytes were significantly decreased in uPA-deficient animals, whereas uPA-deficient leukocytes exhibited a selectively reduced transmigration in wild-type animals. On I/R or stimulation with recombinant uPA, >90% of firmly adherent leukocytes colocalized with CD31-immunoreactive endothelial junctions as detected by in vivo fluorescence microscopy. In a model of hepatic I/R, treatment with WX-340 significantly attenuated posts ischemic neutrophil infiltration and tissue injury.

Conclusions—Our data suggest that endothelial uPA promotes intravascular adherence, whereas leukocyte uPA facilitates the subsequent paracellular transmigration of neutrophils during I/R. This process is regulated via CD11b/Mac-1, and does not require uPAR. Pharmacological blockade of uPA interferes with these events and effectively attenuates posts ischemic tissue injury. (Circulation. 2011;124:1848-1859.)

Key Words: ischemia ■ leukocytes ■ plasminogen activators ■ reperfusion ■ urokinase

Ischemia-reperfusion (I/R) injury is considered to be the most common cause of organ dysfunction and failure after myocardial infarction, hemorrhagic shock, and transplantation. Leukocyte infiltration of posts ischemic tissue is a key event in the pathogenesis of I/R injury. In this multistep cascade, a diversity of adhesion molecules, chemok attractants, and proteases are involved, regulating intravascular rolling and firm adherence as well as transendothelial migration of leukocytes to the reperfused tissue.1–4

Clinical Perspective on p 1859

Urokinase-type plasminogen activator (uPA) is a serine protease that has been implicated in a variety of physiological and pathophysiological processes. In this context, uPA is known to activate extracellular matrix–degrading enzymes and, through interaction with the urokinase receptor (uPAR; CD87), uPA is thought to induce intracellular signaling pathways, ultimately regulating cell adhesion and migration.5 Moreover, uPA mediates the conversion of plasminogen to plasmin, which, in addition to its fibrinolytic properties, is also able to degrade components of the ECM as well as to activate intracellular signaling mechanisms.6

Plasminogen activators, such as recombinant uPA, are therapeutically used for the activation of the fibrinolytic system during thrombembolic events.7 Interestingly, however, endogenously released uPA has recently been impli-
cated in the pathogenesis of I/R injury.8 The underlying mechanisms remain largely unclear. In particular, the role of uPA for each single step in the extravasation process of leukocytes is not understood.

In addition to leukocyte infiltration, impaired microvascular barrier function is a detrimental consequence of I/R. Previously, uPA has been demonstrated to be critically involved in the regulation of microvascular permeability in diabetic retinopathy.9 The functional relevance of this protease for the control of postischemic microvascular leakage has not yet been explored.

Clinical trials are currently evaluating the curative potential of pharmacological serine protease inhibitors in cancer therapies. The effect of these compounds on the prevention of I/R injury has not yet been studied.

Therefore, the objective of our study was (1) to analyze the functional relevance of uPA for the single steps of the extravasation cascade of leukocytes as well as for the regulation of microvascular permeability during I/R, (2) to characterize the underlying mechanisms, and (3) to evaluate the therapeutic potential of pharmacological uPA inhibition on the prevention of postischemic tissue injury.

Here, we demonstrate that uPA mediates postischemic neutrophil responses independently of uPAR but via the integrin CD11b/Mac-1, which, in addition to its multiple functions, has recently been shown to serve as receptor of uPA. In this context, endothelial-presented uPA is thought to promote intravascular firm adherence via its nonproteolytic properties, whereas leukocyte uPA subsequently facilitates the paracellular transmigration of neutrophils to the reperfused tissue. Concomitantly, postischemic microvascular leakage is triggered by uPA in an uPAR-dependent manner. Pharmacological inhibition of uPA effectively interferes with these events and significantly attenuates neutrophil infiltration, microvascular leakage, and injury of the reperfused tissue.

Methods

Animals

Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). Male uPA-/- and uPAR-/- mice were generated as described10,11 and backcrossed for 10 generations to the C57BL/6 background. All experiments were performed with male mice at the age of 10 to 20 weeks. Animals were housed under conventional conditions with free access to food and water. All experiments were performed according to German legislation for the protection of animals.

M. Cremaster Assay

The surgical preparation of the cremaster muscle of anesthetized mice (ketamine/xylazine) was performed as originally described by Baez with minor modifications.12

In Vivo Microscopy

The setup for in vivo microscopy was centered around an Olympus BX 50 upright microscope (Olympus Microscopy, Hamburg, Germany) equipped for stroboscopic fluorescence epi-illumination microscopy. Microscopic images were obtained with Olympus water immersion lenses (20X/numeric aperture 0.5 and 10X/numeric aperture 0.3) and recorded with an analog black-and-white charge-coupled device (CCD) video camera (Cohu 4920, Cohu, San Diego, CA) and an analog video recorder (AG-7350-E, Panasonic, Tokyo, Japan). Oblique illumination was obtained as described previously.13

Quantification of Leukocyte Kinetics and Microhemodynamic Parameters

Off-line analysis of parameters describing the sequential steps of postischemic extravasation was performed by using the Cap-Image image analysis software (Dr Zeintl, Heidelberg, Germany).13

Quantification of Fluorescent Leukocyte Responses

To investigate the contribution of leukocyte and nonleukocyte uPA to postischemic leukocyte responses, a cell-transfer technique was used as described previously.14

Hepatic I/R Injury

Warm ischemia of the left liver lobe was induced for 90 minutes by reversible clamping of the supplying vessels as described previously.15

Inhibitors and Antibodies

An Alexa Fluor 488-conjugated anti-CD31/PECAM-1 mAb (40 μg in 150 μL of saline; intra-arterially (i.a.); BioLegend, San Diego, CA) was used to delineate endothelial junctions. Recombinant murine high–molecular-weight uPA as well as human uPA (varying doses in 0.4 mL PBS; Molecular Innovations, Novi, MI) was used to analyze the mechanisms underlying uPA-dependent leukocyte responses. Murine uPA was titrated with murine plasminogen activator inhibitor type 1 (PAI-1; Molecular Innovations) or diisopropylfluorophosphate (DFP; Calbiochem, Darmstadt, Germany) so that no proteolytic activity remained. WX-340 (10 μg kg−1 in 50 μL saline; i.p.; WILEX, Munich, Germany) is a competitive inhibitor of murine16 and human uPA.17 Anti-CD11b/Mac-1 mAb, anti-CD54/ICAM-1 mAb, and anti-CD102/ICAM-2 mAb (50 μg in 150 μL saline; i.a.; BioLegend) were used to inhibit interactions with CD11b/Mac-1, CD54/ICAM-1 or CD102/ICAM-2. M25 (PryqHqHlvAMFQRTG; disrupts interaction of CD11b/Mac-1 with uPAR18) and its scrambled peptide scM25 (HQIPGAYVQNFTRML; 250 μg in 150 μL saline; i.a.; Dr G. Arnold, LAFUGA, Ludwig-Maximilians-Universität München, Munich, Germany). SCH 79797 (25 μg/kg body weight in 150 μL saline; i.a.; Tocris Bioscience, Ellisville, MO) was used to inhibit interactions with protease-activated receptor 1.

Experimental Protocols

For the analysis of postischemic leukocyte responses, 3 postcapillary vessel segments in a central area of the spread out cremaster muscle were randomly chosen. After having obtained baseline recordings of leukocyte rolling, firm adhesion, and transmigration in all 3 vessel segments, ischemia was induced by clamping all supplying vessels at the base of the cremaster muscle using a vascular clamp (Martin, Tuttlingen, Germany). In selected experiments, an anti-CD11b/Mac-1 mAb, the uPA inhibitor WX-340, or an isotype control antibody/drug vehicle was applied 5 minutes before the onset of reperfusion. After 30 minutes of ischemia, the vascular clamp was removed and reperfusion was restored for 160 minutes. Measurements were repeated at 60 and 120 minutes after onset of reperfusion.

For the analysis of uPA-dependent leukocyte responses, leukocyte recruitment to the cremaster muscle was induced by intracranial injection of recombinant murine uPA (0.02 μg, 0.2 μg, and 2.0 μg in 0.4 mL PBS). After 240 minutes, 5 vessel segments were randomly chosen in a central area of the spread-out cremaster muscle. In additional experiments, an anti-CD11b/Mac-1 mAb or an isotype control antibody were applied before the intracranial injection of murine uPA, DFP-uPA, uPA-PAI-1 complex, or human uPA.

In further experiments, anti-CD54/ICAM-1 mAb, anti-CD102/ICAM-2 mAb, SCH 79797, or isotype control antibody/drug vehicle were applied before the intracranial injection of murine uPA.

After having obtained recordings of migration parameters, blood flow velocity was determined. In selected experiments, FITC dextran...
was subsequently infused intra-arterially. For the analysis of microvascular permeability (see Microvascular Permeability below). After in vivo microscopy, tissue samples of the cremaster muscle were taken for immunohistochemistry. Blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter AcT Counter (Coulter Corp, Miami, FL). Anesthetized animals were then euthanized by exsanguination.

For the analysis of postischemic tissue injury, warm ischemia of the left leg lobe was induced for 90 minutes. Five minutes prior to reperfusion, the uPA inhibitor WX-340 or drug vehicle were applied. Tissue and blood samples were collected after 120 minutes of reperfusion.

### Microvascular Permeability

Analysis of microvascular permeability was performed as described previously.19

### Leukocyte Transmigration Routes

In separate experiments, assessment of leukocyte transmigration routes was performed in the cremaster muscle as described previously with minor modifications.20

### Confocal Microscopy

For the analysis of uPA expression, cremaster muscles were prepared as described previously.19 After incubation with a primary rabbit anti-uPA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a rat antimonochrome anti-CD62E/E-selectin antibody (Abcam, Cambridge, UK), tissues were incubated with the secondary Alexa Fluor 488-linked goat anti-rat or Alexa Fluor 546-linked goat anti-rabbit antibody (Invitrogen, Carlsbad, CA). Immunostained tissues were mounted in goat anti-rat or Alexa Fluor 546-linked goat anti-rabbit antibody (Invitrogen, Carlsbad, CA). Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA) on glass slides and observed using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany).

### Statistics

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). After testing normality of data (using the Shapiro-Wilk test), the 1-way ANOVA test followed by the Student-Newman-Keuls test (>2 groups) or the t test (2 groups) was used for the estimation of stochastic probability in intergroup comparisons. Mean values and SEM are given. P values <0.05 were considered significant.

### Results

#### Role of uPA and uPAR for Postischemic Leukocyte Responses

In a first set of experiments, rolling, firm adherence, and transendothelial migration of leukocytes were analyzed in the mouse cremaster muscle by using in vivo transillumination microscopy (Figure 1A). Surgical preparation of the cremaster muscle induced leukocyte rolling in postcapillary venules. At baseline conditions before induction of ischemia as well as after 60 and 120 minutes of reperfusion, no significant differences were detected in numbers of rolling leukocytes among all experimental groups (data not shown).

Before ischemia, the number of leukocytes attached to the inner vessel wall of postcapillary venules was low and did not differ among experimental groups (Figure 1B). In contrast, after 30 minutes of ischemia and 120 minutes of reperfusion, there was a significant elevation in numbers of firmly adherent leukocytes (14.2 ± 0.7/10⁴ μm²) compared with sham-operated animals (4.8 ± 1.0/10⁴ μm²). This elevation was significantly diminished in uPA-deficient (8.6 ± 0.9/10⁴ μm²) but not in uPAR-deficient animals (18.9 ± 2.4/10⁴ μm²).

At baseline conditions, only few transmigrated leukocytes were found within the perivenular tissue (Figure 1C). In contrast, the number of transmigrated leukocytes was significantly increased after 120 minutes of reperfusion (25.9 ± 2.0/10⁴ μm²) compared with sham-operated mice (7.3 ± 1.3/10⁴ μm²). This increase was significantly attenuated in uPA-deficient (17.8 ± 1.9/10⁴ μm²) but not in uPAR-deficient mice (27.7 ± 1.5/10⁴ μm²). Similar results for postischemic rolling, firm adherence, and transmigration of leukocytes were obtained already after 60 minutes of reperfusion.

In a further set of experiments, the functional relevance of interaction of uPAR with CD11b/Mac-1 for I/R-elicited leukocyte recruitment was evaluated. We found that administration of M25 (which disrupts interaction of uPAR with CD11b/Mac-1) did not significantly alter postischemic leukocyte responses (online-only Data Supplement Figure I).

### Effect of uPA on Leukocyte Rolling, Firm Adherence, and Transmigration

To directly investigate the effect of uPA on leukocyte rolling, firm adherence, and transmigration, an intrascrotal injection of different concentrations of recombinant murine uPA (0.02, 0.2, or 2.0 μg) was performed. In response to uPA, no significant differences were observed in numbers of rolling leukocytes among experimental groups (Figure 2A). In control animals receiving an intrascrotal injection of PBS only, few firmly adherent (4.7 ± 0.9/10⁴ μm²) and transmigrated (5.7 ± 0.2/10⁴ μm²) leukocytes were found. In contrast, after stimulation with uPA, there was a dose-dependent increase in numbers of firmly adherent leukocytes (10.3 ± 3.2/10⁴ μm²; 15.0 ± 1.9/10⁴ μm²; 22.2 ± 7.1/10⁴ μm²; Figure 2B) and transmigrated leukocytes (8.8 ± 1.5/10⁴ μm²; 10.4 ± 1.6/10⁴ μm²; 18.2 ± 3.4/10⁴ μm²; Figure 2C). Because the highest dose of uPA applied (2.0 μg) induced a significant increase in numbers of firmly adherent as well as transmigrated leukocytes, this dose was used in further experiments. In this context, the role of CD11b/Mac-1 for uPA-dependent leukocyte responses was analyzed. On blockade of CD11b/Mac-1,
leukocyte rolling was not significantly altered (Figure 2D), whereas firm adherence (5.7 ± 1.7 × 10⁴ μm²; Figure 2E) and (subsequent) transmigration (9.6 ± 0.6 × 10⁴ μm²; Figure 2F) of leukocytes were almost completely abolished.

Moreover, stimulation with murine DFP-uPA or with uPA-PAI-1 complex, in which the proteolytic activity of uPA is inhibited, as well as with human uPA, which does not interact with murine uPAR, induced a significant elevation in numbers of firmly adherent (20.9 ± 1.3 × 10⁴ μm²; 20.5 ± 1.5 × 10⁴ μm²; 20.9 ± 2.8 × 10⁴ μm²; Figure 2H) and transmigrated leukocytes (24.4 ± 1.3 × 10⁵ μm²; 25.0 ± 2.3 × 10⁵ μm²; 23.1 ± 1.6 × 10⁵ μm²) leukocytes (Figure 2I) compared to controls (4.7 ± 0.3 × 10⁴ μm²; 5.2 ± 0.5 × 10⁴ μm²) but did not significantly alter leukocyte rolling (Figure 2G). Although DFP-uPA–mediated leukocyte recruitment was almost completely abolished in animals receiving an anti-CD11b/Mac-1 antibody, blockade of CD11b/Mac-1 only partially reduced leukocyte responses elicited by uPA-PAI-1 complex or human uPA. In a further set of experiments, uPA-elicited leukocyte firm adherence and (subsequent) transmigration were significantly reduced on blockade of the potential CD11b/Mac-1 binding partners CD54/ICAM-1 or CD102/ICAM-2 (online-only Data Supplement Figure II) whereas leukocyte rolling was not affected (data not shown). Finally, blockade of protease-activated receptor-1 with compound SCH 79797 did not alter uPA-elicited leukocyte responses (online-only Data Supplement Figure III).

**Role of Leukocyte Versus Nonleukocyte uPA for Postischemic Leukocyte Responses**

Using immunostaining and confocal microscopy, postischemic expression of uPA was primarily detected on
cremasteric microvessels as well as on transmigrated leukocytes (Figure 3A). Expression of uPA by the respective cellular sources was confirmed in isolated endothelial cells and neutrophils using flow cytometry (online-only Data Supplement Figure IV).

To further evaluate the relative contribution of leukocyte versus nonleukocyte uPA for postischemic leukocyte responses, a cell-transfer technique was applied. In postcapillary venules of the reperfused cremaster muscle, the number of adherent (0.21 ± 0.06/HPF; Figure 3B) and transmigrated (0.17 ± 0.06/HPF; Figure 3C) wild-type (WT) donor cells was significantly diminished in uPA-deficient recipient animals compared with WT animals receiving cells from WT donors (0.57 ± 0.1/HPF; 0.40 ± 0.1/HPF). In contrast, postischemic adherence (0.7 ± 0.1/HPF) of uPA-deficient donor cells was not significantly altered in WT recipients whereas their transmigration (0.2 ± 0.01/HPF) was significantly reduced.

Postischemic Transmigration Routes of Leukocytes
Using in vivo immunostaining of endothelial junctions by using an Alexa Fluor 488-antibody directed against the junctional molecule CD31/PECAM-1, a diamond-shaped profile of endothelial junctions in cremasteric arterioles was found, whereas endothelial junctions in postcapillary venules exhibited a cobblestone-like profile (Figure 4A). Leukocytes are thought to either squeeze between endothelial junctions (paracellular transmigration route) or to directly penetrate endothelial cells (transcellular transmigration route) during their transendothelial migration from postcapillary venules.

To assess the transmigration route leukocytes use during I/R, the relative localization of firmly adherent leukocytes to endothelial junctions was determined by combining transillumination and fluorescence in vivo microscopy (Figure 4B). On I/R (95.1 ± 1.1%) as well as after stimulation with uPA (94.0 ± 0.9%), >90% of firmly adherent leukocytes colocalized with endothelial junctions of postcapillary venules (Figure 4C).

Phenotyping Transmigrated Leukocytes
To identify the phenotype of transmigrated leukocytes, immunostaining for CD45 (common leukocyte antigen), Ly-6G

Figure 2. Effect of uPA on rolling, firm adherence, and transmigration of leukocytes. Leukocyte rolling (A), firm adherence (B), and transmigration (C) were quantified in postcapillary venules of the cremaster muscle after 240 minutes of intrascrotal stimulation with varying concentrations of murine uPA using in vivo transillumination microscopy (mean±SEM for n=3 per group; #P<0.05 versus unstimulated control). In further experiments, the role of the alternative uPA receptor CD11b/Mac-1 for rolling, firm adherence, and transmigration of leukocytes was analyzed. Panels show results for unstimulated WT mice receiving an intrascrotal injection of PBS as well as for WT mice treated with a blocking anti-CD11b/Mac-1 mAb or isotype control undergoing stimulation with murine uPA (D, E, and F), murine DFP-uPA, murine uPA-PAI-1 complex, or human uPA (G, H, and I; mean±SEM for n=4–6 per group; #P<0.05 versus unstimulated; *P<0.05 versus isotype control). uPA indicates urokinase-type plasminogen activator; WT, wild type; DFP, diisopropyl-fluorophosphate; PAI, plasminogen activator inhibitor; and h, human.
Role of neutrophils, and F4/80 (monocytes/macrophages) of cremasteric tissue samples was performed. In response to I/R as well as on stimulation with uPA, >80% of transmigrated leukocytes were positive for Ly-6G and 10% to 20% of transmigrated leukocytes were positive for F4/80, respectively (data not shown).

Role of uPA and uPAR for Postischemic Microvascular Leakage

As a measure of microvascular permeability, leakage of the macromolecule FITC dextran into the perivascular tissue was determined by using fluorescence in vivo microscopy on the cremaster muscle (Figure 5A). In response to I/R (30/150 minutes), there was a significant increase in the leakage of FITC dextran (398.5 ± 54.1) compared with sham-operated mice (145.4 ± 20.4; Figure 5B). Interestingly, this increase was completely abolished in both uPA- (201.7 ± 23.0) and uPAR-deficient (180.2 ± 26.9) mice.

Effect of WX-340 on Postischemic Leukocyte Infiltration and Tissue Injury of the Liver

In a model of hepatic I/R, the effect of the uPA inhibitor WX-340 on postischemic leukocyte infiltration as well as on tissue injury was evaluated. On I/R of the liver, a significant increase in numbers of leukocytes (27.9 ± 3.3/HPF; 70% Ly-6G neutrophils) infiltrating the postischemic liver tissue was observed compared with sham-operated animals (6.7 ± 0.8/HPF; Figure 6D). This increase was significantly diminished in animals treated with WX-340 (9.4 ± 1.5/104 μm²; 18.0 ± 2.0/104 μm²). Moreover, I/R (30/150 minutes) caused a significant elevation in the leakage of FITC dextran (471.7 ± 73.8) compared with sham-operated controls (145.4 ± 20.4; Figure 6C). This elevation was significantly reduced in mice treated with WX-340 (124.7 ± 21.2).

Effect of WX-340 on Postischemic Leukocyte Responses and Microvascular Leakage

To analyze the effect of the uPA inhibitor WX-340 on postischemic leukocyte responses and microvascular leakage, an additional set of experiments was performed. In response to I/R (30/120 minutes), there was a significant increase in numbers of firmly adherent (13.8 ± 1.7/104 μm²; Figure 6A) and transmigrated (28.9 ± 3.2/104 μm²; Figure 6B) leukocytes compared with sham-operated animals (4.8 ± 1.0/104 μm²; 7.3 ± 1.3/104 μm²). This increase was significantly diminished in animals treated with WX-340 (9.4 ± 1.5/104 μm²; 18.0 ± 2.0/104 μm²). Moreover, I/R (30/150 minutes) caused a significant elevation in the leakage of FITC dextran (471.7 ± 73.8) compared with sham-operated controls (145.4 ± 20.4; Figure 6C). This elevation was significantly reduced in mice treated with WX-340 (124.7 ± 21.2).

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As a measure of hepatic tissue injury, serum levels of aspartate aminotransferase and alanine aminotransferase were determined. I/R induced a significant elevation in the serum levels of aspartate aminotransferase and alanine aminotransferase (5015.4 ± 602.3 U/L; 10623.4 ± 1241.5 U/L; Figure 6E) compared with sham-operated animals (329.8 ± 56.6 U/L; 97.3 ± 8.0 U/L). This elevation was significantly attenuated in animals treated with WX-340 (3010.5 ± 441.5 U/L; 6088.9 ± 959.5 U/L). It is worth noting that treatment with WX-340 neither induced leukocyte infiltration of the liver, nor an elevation of serum transaminases in sham-operated animals.
Plasma Activity of uPA

In a final set of experiments, uPA activity was measured in plasma samples by zymography. In response to I/R of the right cremaster muscle, uPA activity was not significantly altered compared with sham-operated controls. Moreover, treatment with heparin, which—besides its anticoagulative properties and its inhibitory effect on protein binding to endothelial cells—is thought to exert a broad range of further effects (eg, inhibition of protease activity as well as of ligand binding to adhesion molecules such as CD11b/Mac-1 and selectins), did not affect posts ischemic uPA activity levels (online-only Data Supplement Figure V).

Systemic Leukocyte Counts and Microhemodynamic Parameters

To assure intergroup comparability, quantification of inner vessel diameters, blood flow velocities, and shear rates of the microvasculature was undertaken using in vivo microscopy. A representative image is shown in Figure 4A, which depicts an arteriole (A) and a postcapillary venule (V) immunostained for CD31/PECAM-1 (A; scale bar: 20 μm). Arrows indicate firmly adherent leukocytes colocalizing with endothelial junctions of a postcapillary venule in the posts ischemic cremaster muscle (B; scale bar: 20 μm). C, Results for the relative localization of firmly adherent leukocytes to endothelial junctions (green) in postcapillary venules of WT mice undergoing I/R or stimulation with recombinant murine uPA (mean±SEM for n=3 per group). A indicates arteriole; V, venule; I/R, ischemia-reperfusion; and uPA, urokinase-type plasminogen activator.

Figure 4. Leukocyte transmigration routes. Representative in vivo microscopy image of an arteriole (A) and of a post capillary venule (V) in the cremaster muscle immuno stained for CD31/PECAM-1 (A; scale bar: 20 μm). Arrows indicate firmly adherent leukocytes colocalizing with endothelial junctions of a postcapillary venule in the posts ischemic cremaster muscle (B; scale bar: 20 μm). C, Results for the relative localization of firmly adherent leukocytes to endothelial junctions (green) in postcapillary venules of WT mice undergoing I/R or stimulation with recombinant murine uPA (mean±SEM for n=3 per group). A indicates arteriole; V, venule; I/R, ischemia-reperfusion; and uPA, urokinase-type plasminogen activator.

Figure 5. Role of uPA for the regulation of posts ischemic microvascular permeability. As a measure of microvascular permeability, leakage of FITC dextran into the perivascul ar space was quantified using in vivo fluorescence microscopy (A; scale bar: 100 μm). B, Results for sham-operated WT mice as well as for WT, uPA-deficient, and uPAR-deficient mice undergoing I/R (mean±SEM for n=6 per group; #P<0.05 versus sham; *P<0.05 versus WT). WT indicates wild type; uPA-/-, urokinase-type plasminogen activator deficient; and uPAR-/-, urokinase-type plasminogen activator receptor deficient.

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analyzed postcapillary venules as well as systemic leukocyte counts was performed (Table; online-only Data Supplement Table I). On stimulation with uPA, DFP-uPA, or uPA-PAI-1 complex, shear rates as well as systemic leukocyte counts were significantly altered compared to PBS-treated control animals. Among all other experimental groups, no significant differences were detected.

**Discussion**

Immediate restoration of organ perfusion is the primary goal after myocardial infarction, hemorrhagic shock, and transplantation. Reperfusion of postischemic tissue, however, inevitably induces secondary tissue damage as neutrophils accumulate within the reperfused microvasculature and compromise restoration of the blood flow. In addition, subsequent transmigrating neutrophils release reactive oxygen species, cytokines, and proteases, affecting microvascular integrity as well as promoting the postischemic tissue injury. Interestingly, transmigrated neutrophils also contribute to tissue healing and regeneration, collectively emphasizing neutrophil recruitment as a hallmark in the pathogenesis of I/R injury.

Plasminogen activators such as recombinant uPA are clinically used after thrombembolic events in order to pro-
mote reperfusion of the affected tissue by enhancing fibrinolysis.7 Recently, however, endogenously generated uPA has been implicated in the pathogenesis of I/R injury.6 The underlying mechanisms remain largely unclear.

Using near-infrared transillumination in vivo microscopy, the single steps of the extravasation cascade of leukocytes were analyzed in postcapillary venules of the mouse cremaster muscle. In our experiments, neutrophil infiltration of the postischemic tissue was found to be significantly reduced in animals lacking uPA. These results are in line with previous observations: Leukocyte extravasation has been reported to be significantly attenuated in uPA-deficient animals during different inflammatory conditions.5,22 In this context, proteases including uPA were originally thought to selectively facilitate neutrophil transmigration by cleaving endothelial junctions as well as by proteolytically degrading the perivascular basement membrane. In contrast, our in vivo microscopy data clearly demonstrate that in uPA-deficient animals postischemic neutrophil extravasation is inhibited already on the level of intravascular firm adherence. A possible explanation for these observations might be that uPA, in addition to its classic role as a proteolytic enzyme, might serve as an inflammatory mediator.

To prove this hypothesis, we directly investigated the effect of uPA on rolling, firm adherence, and transmigration of leukocytes. Our data demonstrate that stimulation with recombinant murine uPA induces a dose-dependent increase in numbers of firmly adherent and (subsequently) transmigrated neutrophils whereas leukocyte rolling remains unaltered. In addition, comparable leukocyte responses were measured on stimulation with DFP-uPA or uPA-PAI-1 complex, in which the proteolytic activity of uPA is inhibited. These findings extend previous observations given that intra-articular injection of uPA has been reported to cause leukocyte infiltration of the synovial tissue23 and, moreover, suggest that uPA might act as a neutrophil attractant in the postischemic inflammatory response. In this context, it is interesting that postischemic expression of uPA was primarily detected on cremasteric microvessels. Because glycosaminoglycans have recently been shown to serve as binding partners of uPA,24 these molecules might present uPA—in a manner similar to that of classic chemokines—on the luminal

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<th>Vmean, ( \text{mm/s} )</th>
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<td>uPA (0.02 ( \mu \text{g} ))</td>
<td>WT</td>
<td>IgG2b</td>
<td>25.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>2533.4 ± 101.9</td>
<td>4.4 ± 1.3</td>
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<td>WT</td>
<td>Anti-CD11b mAb</td>
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<td>1.7 ± 0.1</td>
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<td>1.5 ± 0.1</td>
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<td>1.4 ± 0.1</td>
<td>2057.2 ± 44.6*</td>
<td>5.5 ± 0.4</td>
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WT indicates wild type; I/R, ischemia-reperfusion; uPA-/-, urokinase-type plasminogen activator deficient; uPAR-/-, urokinase-type plasminogen activator receptor deficient; IgG, immunoglobulin G; uPA, urokinase-type plasminogen activator; DFP, diisopropylfluorophosphate; PAI-1, plasminogen activator inhibitor type 1; and h-uPA, human urokinase-type plasminogen activator.

The table shows the intergroup comparability, quantification of inner vessel diameters, blood flow velocities, shear rates of analyzed postcapillary venules, and systemic leukocyte counts. The values are given as mean ± standard error of the mean (SEM).
surface of endothelial cells. It is worth noting that uPA has also been detected on the surface of transmigrated leukocytes. According to previous reports, uPA is stored in primary granules of unstimulated neutrophils and rapidly translocated to the cell surface on cell activation.\(^1\) The relative contribution of leukocyte and nonleukocyte uPA to the leukocyte recruitment process, however, is still unknown. In the present study, we demonstrate that leukocyte and nonleukocyte uPA cooperate during leukocyte extravasation. By using a cell-transfer technique, we found that nonleukocyte uPA mediates leukocyte intravascular adherence, whereas leukocyte uPA selectively promotes the transendothelial migration of these inflammatory cells to postischemic tissue. These data lead us to the conclusion that uPA presented on the luminal surface of endothelial cells interacts with its receptor(s) on rolling leukocytes, which in turn further activates these inflammatory cells and promotes their firm adherence to the endothelium. Subsequently, leukocyte uPA is engaged, facilitating the transendothelial migration of leukocytes to the postischemic tissue.

In this process, intravascularly adherent leukocytes might either squeeze between adjacent endothelial cells (paracellular transmigration route) or directly penetrate endothelial cells by using the transcellular transmigration route. Here, we demonstrate that in the early reperfusion phase as well as in response to uPA, >90% of firmly adherent leukocytes colocalize with endothelial junctions of postcapillary venules. Our findings suggest that neutrophils predominantly take the paracellular transmigration route to extravasate to the postischemic tissue and extend recently published observations\(^2\) by demonstrating that uPA is an important mediator in this context.

Following previous in vitro data, uPA is supposed to interact with uPAR, which has been demonstrated to modulate leukocyte-endothelial cell interactions by initiating a variety of intracellular signaling pathways.\(^3\) Surprisingly, deficiency of uPAR did not alter postischemic transmigration of neutrophils, indicating that uPA mediates neutrophil infiltration of the reperfused tissue independently of this protein. These findings are in agreement with previous observations, because uPA-dependent monocyte recruitment did not require uPAR in experimental arteriogenesis.\(^4\) In addition, it has been shown that uPA-uPAR interactions are dispensable for other functions of uPA.\(^2\) In animal models of peritonitis\(^5\) or pneumonia,\(^6\) however, uPAR has been reported to be critically involved in neutrophil migration, collectively suggesting a stimulus- and/or tissue-specific engagement of this receptor.

Interestingly, recent in vitro studies identified the \(\beta_2\)-integrin CD11b/Mac-1, which also serves as complement receptor and as adhesion molecule, as another receptor of uPA.\(^7\) In our experiments, we show that postischemic firm adherence and (subsequent) transmigration of neutrophils were significantly reduced on blockade of CD11b/Mac-1. Furthermore, we found that uPA-elicited neutrophil responses are almost completely dependent on CD11/Mac-1. Consequently, these results indicate that uPA mediates neutrophil recruitment to reperfused tissue via CD11b/Mac-1 and independently of uPAR. This hypothesis is further supported by our data because (1) human uPA, which does not interact with murine uPAR, was able to induce significant CD11b/Mac-1-dependent leukocyte responses and (2) administration of M25, a peptide disrupting interaction of CD11b/Mac-1 with uPA, which has been reported to be functionally relevant for cell migration\(^8\) did not significantly alter postischemic leukocyte recruitment. In this context, however, it cannot clearly be answered whether CD11b/Mac-1 serves as uPA receptor or whether this integrin also mediates uPA-dependent neutrophil responses via its other functional properties. Moreover, it can also not clearly be stated to what extent uPA directly\(^9\) or indirectly (eg, by induction of release/production of inflammatory mediators)\(^10\) promotes the migration of neutrophils to postischemic tissue.

In addition to leukocyte recruitment, microvascular leakage is a key event in the pathogenesis of I/R injury. Using in vivo fluorescence microscopy, I/R-elicited leakage of FITC dextran was found to be significantly attenuated in both uPA- and uPAR-deficient animals. These findings confirm recent observations that pharmacological blockade of uPA-uPAR interaction significantly reduced microvascular leakage in the retina of diabetic mice.\(^11\) In this context, interaction of uPA with endothelial uPAR is thought to induce retraction of endothelial cells,\(^12\) ultimately promoting the postischemic microvascular leakage.

Clinical trials are currently evaluating the curative potential of serine protease inhibitors in cancer. The effect of these
drugs on the prevention of I/R injury, however, has not yet been studied. Our experimental data reveal that WX-340, a novel highly selective uPA inhibitor, effectively diminishes intravascular accumulation and (subsequent) transmigration of neutrophils to the reperfused tissue as well as I/R-elicted leakage of FITC dextran, collectively confirming our previous findings in uPA-deficient animals.

To further evaluate the therapeutic potential of the uPA inhibitor in I/R injury, we employed a different experimental model. On experimental I/R injury of the liver, postischemic levels of serum transaminases were found to be dramatically increased compared with sham-operated controls. Treatment with WX-340, however, significantly attenuated the I/R-elicted increase of serum aspartate aminotransferase and alanine aminotransferase. These effects were accompanied by significantly diminished neutrophil infiltration of the postischemic liver. Consequently, pharmacological blockade of uPA is thought to interfere with uPA-elicted neutrophil recruitment in the early reperfusion phase, which, in turn, effectively ameliorates tissue injury of the reperfused liver. These findings are in line with recent observations, given that protection of the postischemic cerebral microvasculature by moderate hypothermia was associated with reduced tissue activity of uPA. Notably, expression of uPA was found to be down-regulated on renal I/R, and uPA deficiency (but not uPAR deficiency) did not affect postischemic proteinuria, suggesting a tissue-specific involvement of uPA and uPAR in the pathogenesis of I/R injury. Moreover, it is worth to be noted that pharmacological blockade of endogenous plasminogen activation might potentially exert antifibrinolytic side effects, which might be fatal in situations such as myocardial infarction. Because endogenous fibrinolysis is known to be largely dependent on the plasminogen activating action of endogenous tissue-plasminogen activator and clinical trials did not provide any evidence about prothrombotic side effects of uPA inhibitors, targeting endogenously released uPA might emerge as a feasible strategy for the prevention of I/R injury.

In conclusion, our data demonstrate that uPA mediates postischemic firm adherence and paracellular transendothelial migration of neutrophils via CD11b/Mac-1 and independently of uPAR. In this context, endothelial-presented uPA is thought to promote leukocyte firm adherence via its nonproteolytic properties whereas leukocyte uPA is thought to facilitate the subsequent paracellular transmigration of neutrophils to the reperfused tissue. Concomitantly, postischemic microvascular leakage is triggered by uPA in a uPAR-dependent manner (Figure 7). Pharmacological inhibition of uPA interferes with these inflammatory events and attenuates postischemic neutrophil responses, microvascular leakage, and tissue injury. These findings provide novel insights into the pathogenesis of I/R injury and highlight uPA as a promising therapeutic target for the prevention of postischemic tissue injury.

Acknowledgments

The authors thank A. Schropp and G. Adams for technical assistance as well as Dr J. Peters for statistical review of the manuscript. Data presented in this study are part of Dr Uhl’s doctoral thesis.
Ischemia-reperfusion is still the most common cause for organ dysfunction and failure after myocardial infarction, hemorrhagic shock, and transplantation. Neutrophil recruitment to the reperfused tissue as well as postischemic microvascular leakage are critical for the pathogenesis of ischemia-reperfusion injury. Although plasminogen activators such as recombinant urokinase-type plasminogen activator (uPA) are used therapeutically for the activation of the fibrinolytic system in thrombembolic events, endogenously produced uPA has recently been implicated in the progression of ischemia-reperfusion injury. The underlying mechanisms remain poorly understood. In the present study, we found that endogenous uPA promotes intravascular accumulation and paracellular transmigration of neutrophils to the reperfused tissue independently of the uPA receptor via the integrin CD11b/Mac-1, which serves as an alternative receptor of uPA. Endothelial-presented uPA is thought to promote leukocyte intravascular adherence via its nonproteolytic properties whereas leukocyte uPA is thought to trigger the subsequent paracellular transmigration of neutrophils. We further show that postischemic microvascular leakage is mediated by uPA in a uPA receptor–dependent manner. Pharmacological inhibition of uPA interferes with these inflammatory events and attenuates postischemic neutrophil responses, microvascular leakage, and tissue injury. Because fibrinolysis is known to be largely dependent on the plasminogen-activating action of endogenous tissue-plasminogen activator and clinical trials did not provide any evidence of pro-thrombotic side effects relative to uPA inhibitors, pharmacological blockade of endogenously released uPA might emerge as a feasible strategy for the prevention of ischemia-reperfusion injury.
Urokinase-Type Plasminogen Activator Promotes Paracellular Transmigration of Neutrophils Via Mac-1, But Independently of Urokinase-Type Plasminogen Activator Receptor

Christoph A. Reichel, Bernd Uhl, Max Lerchenberger, Daniel Puhr-Westerheide, Markus Rehberg, Johanna Liebl, Andrej Khandoga, Wolfgang Schmalix, Stefan Zahler, Elisabeth Deindl, Stefan Lorenzl, Paul J. Declerck, Sandip Kanse and Fritz Krombach

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). Male uPA-/- and uPAR-/- mice were generated as described \(^1:2\) and backcrossed for 10 generations to the C57BL/6 background. All experiments were performed with male mice at the age of 10 - 20 weeks. Animals were housed under conventional conditions with free access to food and water. All experiments were performed according to German legislation for the protection of animals.

*M. cremaster assay*

The surgical preparation of the cremaster muscle was performed as originally described by Baez with minor modifications \(^3\). Mice were anesthetized using a ketamine/xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine), administrated by i.p. injection. The left femoral artery was cannulated in a retrograde manner for continuous blood pressure monitoring and for administration of microspheres and drugs (see below). The right cremaster muscle was exposed through a ventral incision of the scrotum. The muscle was opened ventrally in a relatively avascular zone, using careful electrocautery to stop any bleeding, and spread over the transparent pedestal of a custom-made microscopic stage. Epididymis and testicle were detached from the cremaster muscle and placed into the abdominal cavity. Throughout the procedure as well as after surgical preparation during *in vivo* microscopy, the muscle was superfused with warm-buffered saline.

*In vivo microscopy*

The setup for *in vivo* microscopy was centered around an Olympus BX 50 upright microscope (Olympus Microscopy, Hamburg, Germany), equipped for stroboscopic fluorescence epi-
illumination microscopy. Light from a 75-W xenon source was narrowed to a near-monochromatic beam of a wavelength of 700 nm by a galvanometric scanner (Polychrome II, TILL Photonics, Graefelfing, Germany) and directed onto the specimen via a FITC filter cube equipped with dichroic and emission filters (DCLP 500, LP515, Olympus). Microscopic images were obtained with Olympus water immersion lenses [20x/numerical aperture (NA) 0.5 and 10x/NA 0.3] and recorded with an analog black-and-white charge-coupled device (CCD) video camera (Cohu 4920, Cohu, San Diego, CA, USA) and an analog video recorder (AG-7350-E, Panasonic, Tokyo, Japan). Oblique illumination was obtained by positioning a mirroring surface (reflector) directly below the specimen and tilting its angle relative to the horizontal plane. The reflector consisted of a round cover glass (thickness, 0.19–0.22 mm; diameter, 11.8 mm), which was coated with aluminum vapor (Freichel, Kaufbeuren, Germany) and brought into direct contact with the overlying specimen as described previously ⁴. For measurement of centerline blood flow velocity, green fluorescent microspheres (2 µm diameter, Molecular Probes, Leiden, The Netherlands) were injected via the femoral artery catheter, and their passage through the vessels of interest was recorded using the FITC filter cube under appropriate stroboscopic illumination (exposure, 1 ms; cycle time, 10 ms; λ=488 nm), integrating video images for sufficient time (>80 ms) to allow for the recording of several images of the same bead on one frame. Beads that were flowing freely along the centerline of the vessels were used to determine blood flow velocity (see below).

Quantification of leukocyte kinetics and microhemodynamic parameters

For off-line analysis of parameters describing the sequential steps of leukocyte extravasation, we used the Cap-Image image analysis software (Dr. Zeintl, Heidelberg, Germany). Rolling leukocytes were defined as those moving slower than the associated blood flow and quantified as described previously. Firmly adherent cells were determined as those resting in the
associated blood flow for more than 30 s and related to the luminal surface per 100 µm vessel length. Transmigrated cells were counted in regions of interest (ROI), covering 75 µm on both sides of a vessel over 100 µm vessel length. By measuring the distance between several images of one fluorescent bead under stroboscopic illumination, centerline blood flow velocity was determined. From measured vessel diameters and centerline blood flow velocity, apparent wall shear rates was calculated, assuming a parabolic flow velocity profile over the vessel cross-section.

Quantification of fluorescent leukocyte responses

To investigate the contribution of leukocyte and non-leukocyte uPA to postischemic leukocyte responses, a cell-transfer technique was used as described previously 5. Briefly, bone marrow leukocytes were isolated from donor mice by flushing the femur and tibia bones with PBS. Cells were then sieved and counted, resuspended in PBS containing BSA (0.25%), and incubated with calcein-AM (10 µM final concentration at 37°C for 30 minutes). After 2 washes, the cells were injected intravenously into recipient mice via the right jugular vein (10^7 cells/mouse) 120 min prior to the surgical preparation. Fluorescent cells were counted in 175 high power fields (HPF) per animal this being equivalent to the total quantifiable area of an exteriorized cremaster muscle in the present studies. Results are shown as the number of adherent or transmigrated calcein-labeled cells/HPF.

Hepatic I/R injury

Warm ischemia of the left liver lobe was induced for 90 min by reversible clamping of the supplying vessels as described previously 6. Tissue and blood samples were taken at the end of the experiment after 120 min of reperfusion. Blood samples were immediately centrifuged at 2000g for 10 min, and stored at −80°C. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured with an automated
analyzer (Hitachi 917, Roche-Boehringer Mannheim Co., Germany) using standardized test systems (HiCo GOT and HiCo GPT, Roche-Boehringer Mannheim Co.).

Experimental groups

Animals were assigned randomly to the following groups: Sham-operated wild-type (WT) mice as well as WT, uPA-/-, and uPAR-/- mice, WT mice treated with the uPA inhibitor WX-340 or with vehicle, WT mice treated with an anti-CD11b/Mac-1 monoclonal antibody (mAb) or with an isotype control antibody, and WT mice treated with M25 or with scM25 undergoing I/R (30/120 min; see below; n=5-6). Moreover, leukocyte responses were analyzed in the cremaster muscle of WT mice receiving fluorescent leukocytes from WT or uPA-/- donors as well of uPA-/- mice receiving fluorescent leukocytes from WT donors undergoing I/R (30/120 min; n=5 each group). A further set of experiments was performed in WT mice with an intrascrotal injection of PBS as well as in WT mice undergoing intrascrotal stimulation with different doses of recombinant murine uPA (see below; n=3 each group). Additionally, experiments were conducted in WT mice treated with an anti-CD11b/Mac-1 mAb or with an isotype control antibody undergoing intrascrotal stimulation with recombinant murine uPA, DFP-uPA, or uPA-PAI-1 complex as well as with human uPA (n=4-6 each group). Furthermore, leukocyte transmigration routes were analyzed in the cremaster muscle of WT animals receiving an Alexa Fluor 488-conjugated anti-CD31/PECAM-1 mAb undergoing I/R (30/60 min) or intrascrotal stimulation with recombinant murine uPA (240 min; n=3 each group). In additional experiments, WT mice undergoing intrascrotal stimulation with recombinant murine uPA were treated with an anti-CD54/ICAM-1 mAb, anti-CD102/ICAM-2 mAb, SCH 79797, or isotype control antibody/drug vehicle. Finally, experiments were performed in sham-operated WT animals as well as WT animals undergoing hepatic I/R treated with the uPA-inhibitor WX-340 or vehicle (5 min prior to the onset of reperfusion; n=6 each group).
Inhibitors and antibodies

An Alexa Fluor 488-conjugated anti-CD31/PECAM-1 mAb (40 µg in 150 µl of saline; i.a.; BioLegend, San Diego, CA, USA) was used to delineate endothelial junctions. Recombinant murine HMW-uPA as well as human uPA (varying doses in 200 µl PBS; Molecular Innovations, Novi, MI, USA) was used to analyze the mechanisms underlying uPA-dependent leukocyte responses. Murine uPA was titrated with murine PAI-1 (Molecular Innovations or produced as described before) or diisopropylfluorophosphate (DFP; Calbiochem, Darmstadt, Germany) so that no proteolytic activity remained.

WX-340 (10 mg kg\(^{-1}\) in 50 µl saline; i.p.; WILEX, Munich, Germany) is a competitive inhibitor of murine and human uPA. An anti-CD11b/Mac-1 mAb, anti-CD54/ICAM-1 mAb, and anti-CD102/ICAM-2 mAb (50 µg in 150 µl saline; i.a.; BioLegend) was used to inhibit interactions with CD11b/Mac-1, CD54/ICAM-1, or CD102/ICAM-2. M25 (PRYQHIGLVAMFRQNTG; disrupts interaction of CD11b/Mac-1 with uPAR) and its scrambled peptide scM25 (HQIPGAYRGVNQRFTML; 250 µg in 150 µl saline; i.a.) were kindly provided by Dr. G. Arnold (LAFUGA, Ludwig-Maximilians-Universität München, Munich, Germany). SCH 79797 (25 µg/kg body weight in 150 µl saline; i.a.; Tocris Bioscience, Ellisville, USA) was used to inhibit interactions with PAR-1.

Experimental protocols

For the analysis of postischemic leukocyte responses, three postcapillary vessel segments in a central area of the spread out cremaster muscle were randomly chosen among those that were at least 150 µm away from neighboring postcapillary venules and did not branch over a distance of at least 150 µm. After having obtained baseline recordings of leukocyte rolling, firm adhesion, and transmigration in all three vessel segments, ischemia was induced by clamping all supplying vessels at the basis of the cremaster muscle using a vascular clamp (Martin, Tuttlingen, Germany). Stagnancy of blood flow was then verified by in vivo
microscopy. In selected experiments, an anti-CD11b/Mac-1 mAb, the uPA inhibitor WX-340, M25, or an isotype control antibody/drug vehicle/scM25 were applied 5 min prior to the onset of reperfusion. After 30 min of ischemia, the vascular clamp was removed and reperfusion was restored for 160 min. Measurements, which took about 5 min, respectively, were repeated at 60 and 120 min after onset of reperfusion.

For the analysis of uPA-dependent leukocyte responses, leukocyte recruitment to the cremaster muscle was induced by intrascrotal injection of recombinant murine uPA (0.02 µg, 0.2 µg, and 2.0 µg in 0.4 ml PBS; Molecular Innovations, Novi, MI, USA). After 240 min, five vessel segments were randomly chosen in a central area of the spread-out cremaster muscle among those that were at least 150 µm away from neighboring postcapillary venules and did not branch over a distance of at least 150 µm. In selected experiments, an anti-CD11b/Mac-1 mAb or an isotype control antibody were applied prior to the intrascrotal injection of murine uPA, DFP-uPA, or uPA-PAI-1 complex as well as of human uPA.

In further experiments, an anti-CD54/ICAM-1 mAb, anti-CD102/ICAM-2 mAb, SCH 79797, or isotype control antibody/drug vehicle were applied prior to the intrascrotal injection of murine uPA.

After having obtained recordings of migration parameters, blood flow velocity was determined as described above. In selected experiments, FITC dextran was subsequently infused intra-arterially for the analysis of microvascular permeability (see below). After in vivo microscopy, tissue samples of the cremaster muscle were taken for immunohistochemistry (see below). Blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter ACT Counter (Coulter Corp., Miami, FL, USA). Anaesthetized animals were then killed by bleeding to death.
For the analysis of postischemic tissue injury, warm ischemia of the left liver lobe was induced for 90 min. Five minutes prior to reperfusion, the uPA inhibitor WX-340 or drug vehicle were applied. Tissue and blood samples were collected after 120 min of reperfusion.

Microvascular permeability
Analysis of microvascular permeability was performed as described previously \(^\text{12}\). Briefly, the macromolecule FITC-dextran (5 mg in 0.1 ml saline, \(M_r\) 150,000, Sigma-Aldrich) was infused intra-arterially after determination of centerline blood flow velocity (see above). Five postcapillary vessel segments as well as the surrounding perivascular tissue were excited at 488 nm, and emission >515 nm was recorded by a CCD camera (Sensicam, PCO, Kelheim, Germany) 30 min after injection of FITC-dextran using an appropriate emission filter (LP 515). Mean gray values of fluorescence intensity were measured by digital image analysis (TILLvisION 4.0, TILL Photonics) in six randomly selected ROIs (50x50 \(\mu m^2\)), localized 50 \(\mu m\) distant from the postcapillary venule under investigation. The average of mean gray values was calculated.

Leukocyte transmigration routes
In separate experiments, assessment of leukocyte transmigration routes was performed in the cremaster muscle as described previously \(^\text{13}\) with minor modifications. After intra-arterial injection of an Alexa Fluor 488-conjugated anti-CD31/PECAM-1 mAb, the relative localization of firmly adherent leukocytes (RLOT \textit{in vivo} microscopy) to anti-CD31/PECAM-1-immunoreactive endothelial junctions (fluorescence \textit{in vivo} microscopy) of postcapillary venules was immediately determined by combining RLOT and conventional fluorescence \textit{in vivo} microscopy using an AxioTech-Vario 100 Microscope (Zeiss MicroImaging GmbH, Göttingen, Germany), equipped with LED excitation light (Zeiss Colibri) for fluorescence epi-illumination.
**Immunohistochemistry**

To determine the phenotype of transmigrated leukocytes, immunostaining of paraffin-embedded serial tissue sections of the cremaster muscle or the liver was performed. Sections were incubated with primary rat anti-mouse anti-Ly-6G, anti-CD45 (BD Biosciences), or anti-F4/80 (Serotec, Oxford, UK) IgG antibodies. Afterwards, the paraffin sections were stained with a commercially available immunohistochemistry kit (Super Sensitive Link-Label IHC detection system, BioGenex, San Ramon, CA, USA), obtaining an easily detectable reddish end product. Finally, the sections were counterstained with Mayer’s hemalaun. The number of extravascularly localized Ly-6G, CD45-, or F4/80-positive cells was quantified by light microscopy (magnification 400x) on six sections (10 observation fields per section) from six individual animals per experimental group in a blinded manner, respectively. The number of transmigrated Ly-6G-positive cells (neutrophils) and F4/80-positive cells (monocytes/macrophages) is expressed as the percentage of total CD45-positive leukocytes.

**Confocal microscopy**

For the analysis of uPA expression, cremaster muscles were fixed in 4 % paraformaldehyde as described previously. Tissues were then blocked and permeabilized in PBS, supplemented with 10 % goat serum (Sigma-Aldrich) and 0.5 % Triton X-100 (Sigma-Aldrich). After incubation with the primary rabbit anti-uPA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rat anti-mouse anti-CD62E/E-selectin antibody (Abcam, Cambridge, UK) at room temperature for 12 h, tissues were incubated with the secondary Alexa Fluor 488-linked goat anti-rat or Alexa Fluor 546-linked goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) for 3 h at room temperature. Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA, USA) on glass slides and observed using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil immersion lens (Leica; 40x/NA 1.25–0.75).
uPA expression on isolated endothelial cells and neutrophils

Lungs from male WT mice were removed, minced, and enzymatically digested. The resulting single cell suspension was double immunostained for VE-cadherin (BD Pharmingen, San Diego, CA; as endothelial marker) and for uPA. Flow cytometric measurements were made in a FacsCalibur (Becton Dickinson, Heidelberg, Germany). VE-cadherin positive cells were gated and evaluated for their fluorescence in FL-2 (uPA staining). Whole blood was drawn from heparinized WT mice and a hypotonic lysis of erythrocytes was performed. After centrifugation and washing, the cells were fixed with 4% buffered paraformaldehyde and immunostained for uPA. Neutrophils were identified by their scatter properties evaluated for their fluorescence in FL-2 (uPA staining). In all experiments, matching isotype controls were performed.

Zymography

In a final set of experiments, plasma samples were taken from sham-operated WT control mice as well as from WT mice treated with heparin (Ratiopharm, Ulm, Germany; 4 mg/kg i.a.; corresponds to 300 USP U/kg) or vehicle undergoing I/R (30 min/120 min) of the right cremaster muscle. Plasminogen activator zymography was performed essentially as described before 15. 2 µl of plasma was mixed with SDS sample buffer and loaded on to a 10% SDS-PAGE. The gel was extensively washed with PBS containing Triton X-100 (2.5% wt/vol). The overlay consisted of low melting agarose (0.8% wt/vol) containing casein (2% wt/vol) and plasminogen (40 µg/ml). The gel was apposed to the overlay and incubated at 37°C for 48 h and photographed.

Statistics

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). After having tested normality of data (using the
Shapiro-Wilk test), the one way ANOVA test followed by the Student-Newman-Keuls test (> 2 groups) or the t-test (2 groups) were used for the estimation of stochastic probability in intergroup comparisons. Mean values and standard error of the mean (SEM) are given. $P$ values 0.05 were considered significant.
Supplemental Tables

Table I

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<td>anti-CD102 mAb</td>
<td>25.1 ± 0.5</td>
<td>1.3 ± 0.1</td>
<td>1923.1 ± 30.2*</td>
<td>4.4 ± 0.4</td>
</tr>
</tbody>
</table>
Supplemental Figures

Figure I

A

Leukocyte firm adherence

![Graph showing leukocyte firm adherence with columns for Baseline, 60 min, and 120 min under Reperfusion conditions, comparing scM25 and M25]

B

Leukocyte transmigration

![Graph showing leukocyte transmigration with columns for Baseline, 60 min, and 120 min under Reperfusion conditions, comparing scM25 and M25]
Figure II

A

Leukocyte firm adherence

B

Leukocyte transmigration

C

Leukocyte firm adherence

D

Leukocyte transmigration

isotype control

anti-CD54/ICAM-1 mAb

uPA

isotype control

anti-CD102/ICAM-2 mAb

uPA
Figure III

A

Leukocyte firm adherence

[n / 10^4 µm^2]

0

10

20

30

40

vehicle

SCH 79797

B

Leukocyte transmigration

[n / 10^4 µm^2]

0

10

20

30

40

uPA

uPA
Figure V

A

B
Figure Legends

Table I. Systemic leukocyte counts and microhemodynamic parameters. Systemic leukocyte counts as well as microhemodynamic parameters including inner vessel diameter, blood flow velocity, and wall shear rate were obtained as detailed in Materials and Methods (mean ± SEM for n = 4-5 per group).

Figure I. Effect of M25 on postischemic leukocyte responses. Leukocyte firm adherence (A) and transmigration (B) were quantified in postcapillary venules of the cremaster muscle by using in vivo transillumination microscopy. Panels show results for WT mice treated with M25 or scrambled peptide (scM25) undergoing I/R (mean±SEM for n=5 per group).

Figure II. Role of ICAM-1 and ICAM-2 for uPA-elicited leukocyte responses. Leukocyte firm adherence (A, C) and transmigration (B, D) were quantified in postcapillary venules of the cremaster muscle by using in vivo transillumination microscopy. Panels show results for WT mice undergoing stimulation with uPA treated with anti-CD54/ICAM-1 mAb, anti-CD102/ICAM-2 mAb, or isotype control antibody (mean±SEM for n=4 per group; *p<0.05, vs. isotype control).

Figure III. Role of PAR-1 for uPA-elicited leukocyte responses. Leukocyte firm adherence (A) and transmigration (B) were quantified in postcapillary venules of the cremaster muscle by using in vivo transillumination microscopy. Panels show results for WT mice undergoing stimulation with uPA treated with SCH 79797 or drug vehicle (mean±SEM for n=4 per group).

Figure IV. Expression of uPA by isolated murine endothelial cells and neutrophils. Relative fluorescence intensity histograms demonstrating expression of uPA (green) on
isolated murine pulmonary endothelial cells (A) and isolated murine neutrophils from the whole blood (B; isotype control in magenta) as assessed by flow cytometry.

**Figure V. Plasma uPA activity.** Zymograms (A) show uPA activity in plasma samples from sham-operated control mice as well as from WT mice treated with heparin or vehicle undergoing I/R of the right cremaster muscle. In panel (B) quantitative results are demonstrated (mean±SEM for n=4 per group).
Supplemental References


**Supplemental video:** Postcapillary venule in the postischemic cremaster muscle as observed by combining transillumination and fluorescence *in vivo* microscopy. The majority of firmly adherent leukocytes co-localizes with CD31-immunoreactive endothelial junctions.