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, postischemic leukocyte responses were significantly reduced on blockade of the integrin CD11b/Mac-1, which also serves as uPAR receptor. Using a cell transfer technique, postischemic 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 postischemic 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 postischemic 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 postischemic tissue is a key event in the pathogenesis of I/R injury. In this multistep cascade, a diversity of adhesion molecules, chemoattractants, 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-

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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 described[10,11] 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 (20×/numeric aperture 0.5 and 10×/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 leukocyte 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 mg kg^{-1} in 50 μL saline; i.p.; WILEX, Munich, Germany) is a competitive inhibitor of murine[16] 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 (PRYQHIGLVAMFRQNTG; disrupts interaction of CD11b/Mac-1 with uPAR[18]) 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, 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 intrascrotal 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 intrascrotal 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 intrascrotal 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 liver 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 antimouse 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 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 ($14.2\pm0.7/10^4$ μm^2) compared with sham-operated animals ($4.8\pm1.0/10^4$ μm^2). This elevation was significantly diminished in uPA-deficient ($8.6\pm0.9/10^4$ μm^2) but not in uPAR-deficient animals ($18.9\pm2.4/10^4$ μm^2).

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 ($25.9\pm2.0/10^4$ μm^2) compared with sham-operated mice ($7.3\pm1.3/10^4$ μm^2). This increase was significantly attenuated in uPA-deficient ($17.8\pm1.9/10^4\mu m^2$) but not in uPAR-deficient mice ($27.7\pm1.5/10^4$ μm^2). 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).

Role of CD11b/Mac-1 for Postischemic Leukocyte Responses

In further experiments, the role of the integrin CD11b/Mac-1, which, in addition to its role as adhesion molecule and complement receptor, has recently been identified as a receptor of uPA, for postischemic leukocyte responses was analyzed. No significant differences were observed in numbers of rolling leukocytes among experimental groups at all time points of measurement (data not shown). At baseline conditions, the number of firmly adherent (Figure 1D) and transmigrated leukocytes (Figure 1E) was low and did not differ among experimental groups. As described above, after 30 minutes of ischemia and 60 as well as 120 minutes of reperfusion, there was a significant elevation in the number of firmly adherent ($20.9\pm1.2/10^4$ μm^2) and (subsequently) transmigrated ($25.8\pm0.9/10^4$ μm^2) leukocytes compared to sham-operated animals ($4.8\pm1.0/10^4$ μm^2; $7.3\pm1.3/10^4$ μm^2). This elevation was almost completely abrogated in animals treated with a blocking anti-CD11b/Mac-1 mAb ($4.2\pm0.7/10^4$ μm^2; $13.5\pm1.3/10^4$ μm^2).

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 all experimental groups (Figure 2A). In control animals receiving an intrascrotal injection of PBS only, few firmly adherent ($4.7\pm0.9/10^4$ μm^2) and transmigrated ($5.7\pm0.2/10^4$ μm^2) leukocytes were found. In contrast, after stimulation with uPA, there was a dose-dependent increase in numbers of firmly adherent ($10.3\pm3.2/10^4$ μm^2; $15.0\pm1.9/10^4$ μm^2; $22.2\pm7.1/10^4$ μm^2; Figure 2B) and transmigrated leukocytes ($8.8\pm1.5/10^4$ μm^2; $10.4\pm1.6/10^4$ μm^2; $18.2\pm3.4/10^4$ μm^2; 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,

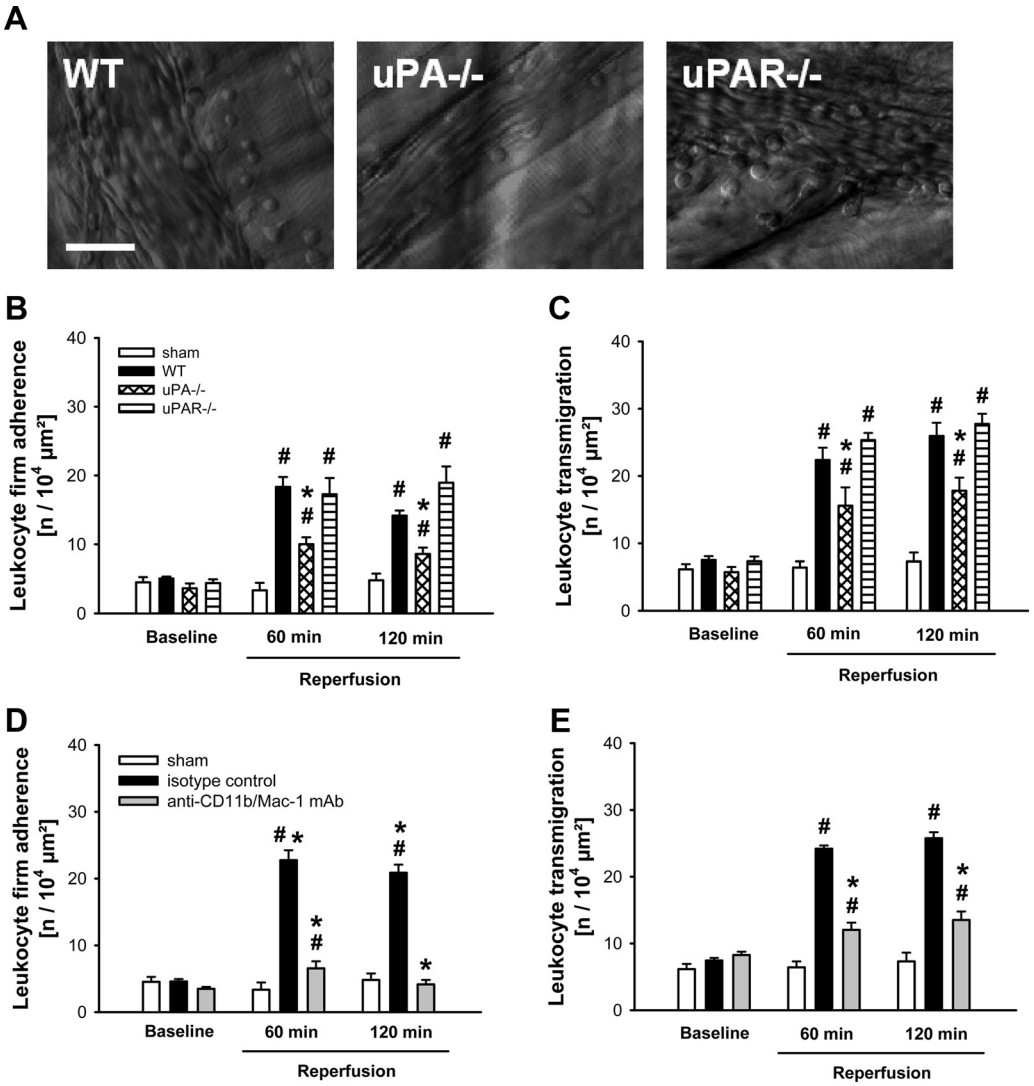

Figure 1. Role of uPA for postischemic leukocyte recruitment. Representative in vivo transillumination microscopy images of postcapillary venules in WT, uPA, and uPAR-deficient mice undergoing I/R (**A**; scale bar: 20 μm). Leukocyte firm adherence and transmigration were quantified in postcapillary venules of the cremaster muscle by using in vivo transillumination microscopy. Panels show results for sham-operated WT mice and for WT, uPA-deficient, and uPAR-deficient mice (**B** and **C**), as well as for WT mice treated with a blocking anti-CD11b/Mac-1 mAb or isotype control (**D** and **E**) undergoing I/R (mean±SEM for n=6 per group; #$P<0.05$ versus sham; *$P<0.05$ versus WT/isotype control). WT indicates wild type; uPA-/-, urokinase-type plasminogen activator deficient; and uPAR-/-, urokinase-type plasminogen activator receptor deficient.

leukocyte rolling was not significantly altered (Figure 2D), whereas firm adherence ($5.7\pm1.7/10^4$ μm^2; Figure 2E) and (subsequent) transmigration ($9.6\pm0.6/10^4$ μm^2; 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 ($20.9\pm1.3/10^4$ μm^2; $20.5\pm1.5/10^4$ μm^2; $20.9\pm2.8/10^4$ μm^2; Figure 2H) and transmigrated ($24.4\pm1.3/10^4$ μm^2; $25.0\pm2.3/10^4$ μm^2; $23.1\pm1.6/10^4$ μm^2) leukocytes (Figure 2I) compared to controls ($4.7\pm0.3/10^4$ μm^2; $5.2\pm0.5/10^4$ μm^2) 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

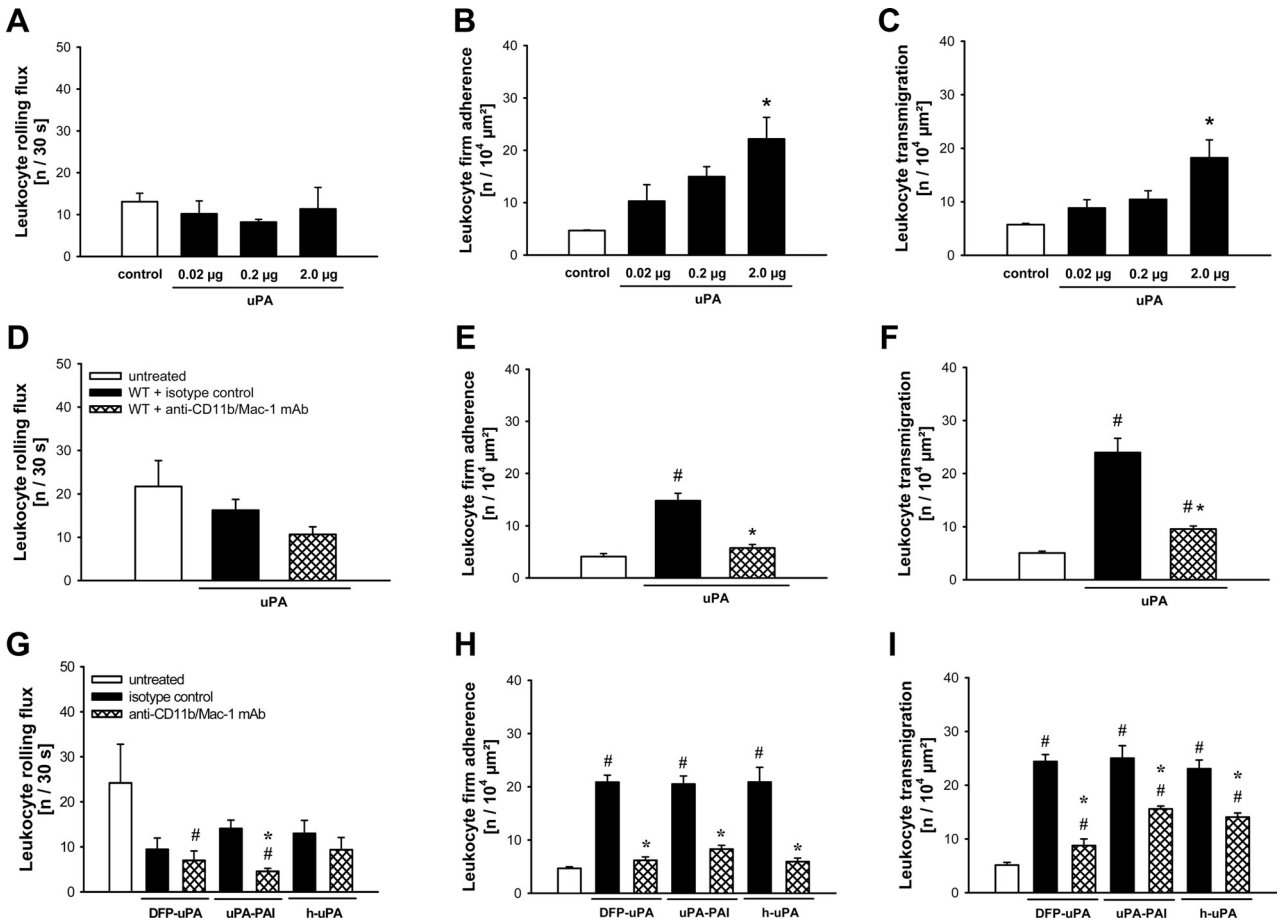

Figure 2. Effect of uPA on rolling, firm adherence, and transmigration of leukocytes. Leukocyte rolling (**A**), firm adherence (**B**), and transmigration (**C**) were quantified in postcapillary venules of the cremaster muscle after 240 minutes of intrascrotal stimulation with varying concentrations 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.

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/high power field; 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

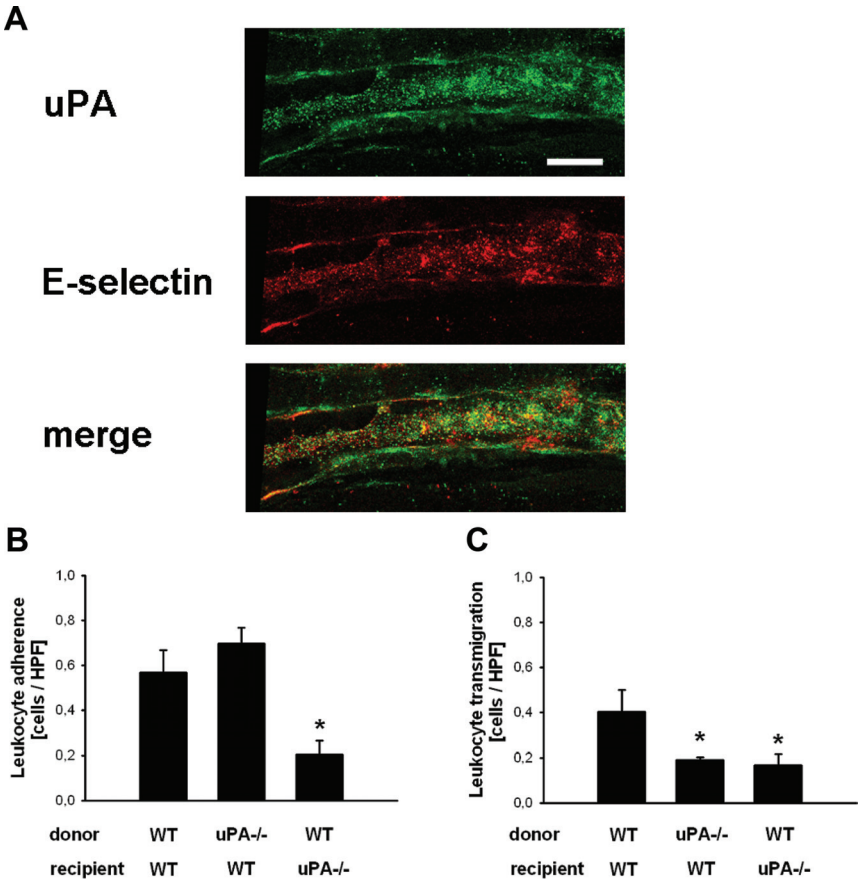

Figure 3. Role of leukocyte versus non-leukocyte uPA for postischemic leukocyte responses. Representative confocal microscopy images of a postcapillary venule in the postischemic cremaster muscle immunostained for uPA (green) and the endothelial cell marker CD62E/E-selectin (red; scale bar: 25 μm; **A**). Adherence (**B**) and transmigration (**C**) of calcein AM-labeled bone marrow leukocytes were quantified in the cremaster muscle after 30 minutes of ischemia and 120 minutes of reperfusion using in vivo fluorescence microscopy. Panels show results for WT mice receiving leukocytes from WT or uPA-deficient donors as well as for uPA-deficient mice receiving leukocytes from WT donors (mean±SEM for n=5 per group; *$P<0.05$ versus WT→WT). uPA indicates urokinase-type plasminogen activator; HPF, high power field; WT, wild type; and uPA-/-, urokinase-type plasminogen activator deficient.

(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 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 ($13.8\pm1.7/10^4$ μm^2; Figure 6A) and transmigrated ($28.9\pm3.2/10^4$ μm^2; Figure 6B) leukocytes compared with sham-operated animals ($4.8\pm1.0/10^4$ μm^2; $7.3\pm1.3/10^4$ μm^2). This increase was significantly diminished in animals treated with WX-340 ($9.4\pm1.5/10^4$ μm^2; $18.0\pm2.0/10^4$ μm^2). 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 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 the uPA inhibitor WX-340 (12.2±1.5/HPF).

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.

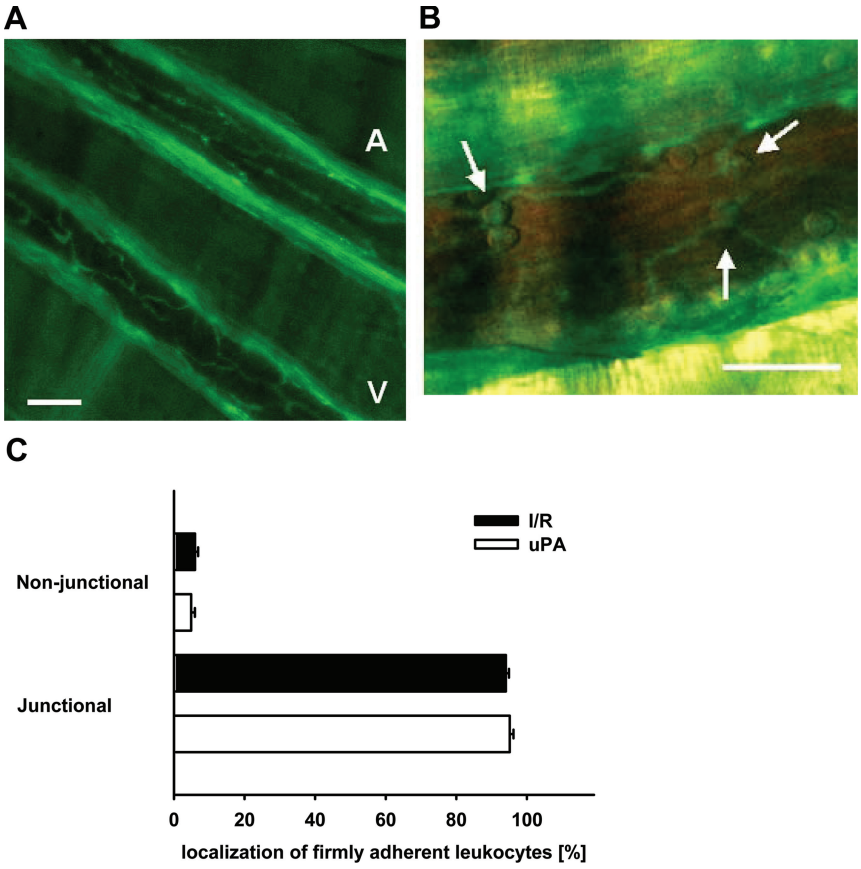

Figure 4. Leukocyte transmigration routes. Representative in vivo microscopy image of an arteriole (A) and of a postcapillary venule (V) in the cremaster muscle immunostained for CD31/PECAM-1 (**A**; scale bar: 20 μm). Arrows indicate firmly adherent leukocytes colocalizing with endothelial junctions of a postcapillary venule in the postischemic 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.

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 postischemic 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

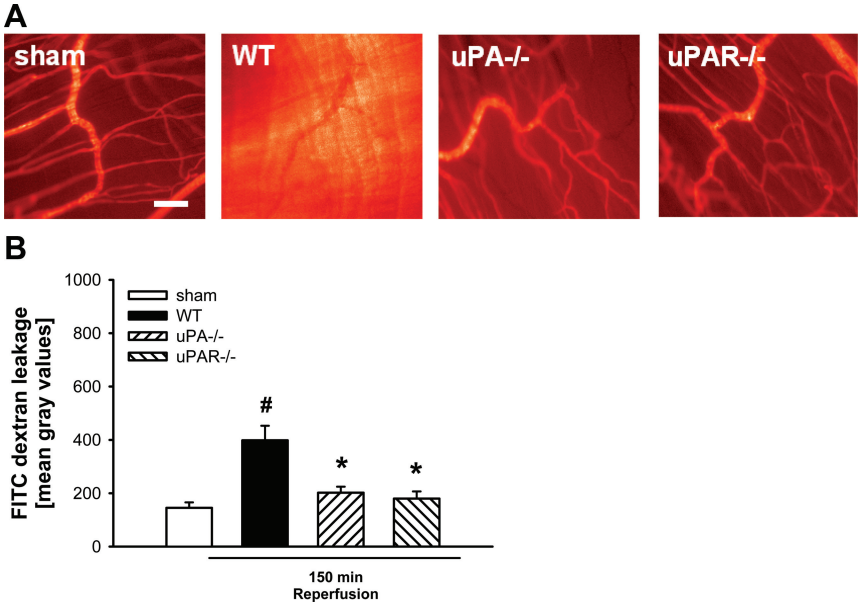

Figure 5. Role of uPA for the regulation of postischemic microvascular permeability**.** As a measure of microvascular permeability, leakage of FITC dextran into the perivascular space was quantified using in vivo fluorescence microscopy (**A**; scale bar: 100 μm). **B**, Results for sham-operated WT 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.

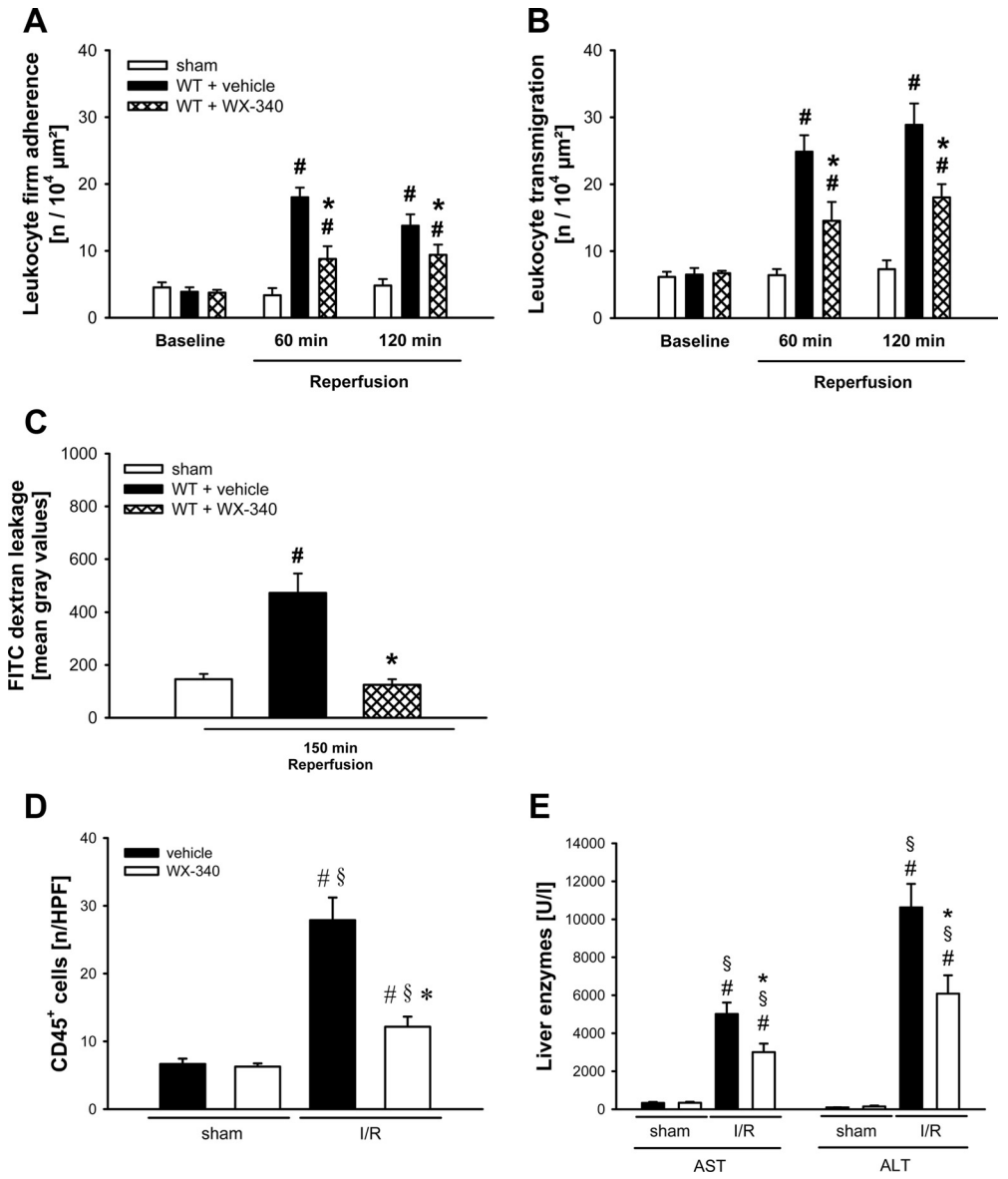

Figure 6. Effect of WX-340 on postischemic leukocyte responses, microvascular leakage, and tissue injury. Leukocyte firm adherence (**A**) and transmigration (**B**) as well as FITC-dextran leakage (**C**) were quantified in postcapillary venules of the cremaster muscle undergoing I/R by using in vivo microscopy. Panels show results for sham-operated WT mice as well as for WT mice treated with the uPA inhibitor WX-340 or vehicle undergoing I/R (mean±SEM for n=6 per group; #$P<0.05$ versus sham; *$P<0.05$ versus WT). In a model of hepatic I/R injury, transmigrated CD45$^+$ cells (representing leukocytes) in paraffin-embedded tissue sections of the liver (**D**) as well as serum levels of aspartate aminotransferase and alanine aminotransferase (**E**) were determined. Panels show results for sham-operated WT mice treated with WX-340 or vehicle as well as for WT mice treated with WX-340 or vehicle undergoing I/R (mean±SEM for n=6 per group; #$P<0.05$ versus sham+vehicle; *$P<0.05$ versus I/R+vehicle; §$P<0.05$ versus sham+WX-340). WT indicates wild type; HPF, high power field; I/R, ischemia-reperfusion; AST, aspartate aminotransferase; and ALT, alanine aminotransferase.

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.[7] 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, subsequently transmigrating neutrophils release reactive oxygen species, cytokines, and proteases, affecting microvascular integrity as well as promoting the postischemic tissue injury.[1–4] Interestingly, transmigrated neutrophils also contribute to tissue healing and regeneration, collectively emphasizing neutrophil recruitment as a hallmark in the pathogenesis of I/R injury.[15,21]

Plasminogen activators such as recombinant uPA are clinically used after thrombembolic events in order to pro-

Table. Intergroup Comparability. Quantification of Inner Vessel Diameters, Blood Flow Velocities, Shear Rates of Analyzed Postcapillary Venules, and Systemic Leukocyte Counts

Stimulus	Strain	Treatment	Inner Diameter, μm	Vmean, mm/s	Shear Rate, Seconds^{-1}	Systemic Leukocyte, Counts 10^3 μL^{-1}
Sham	WT		26.3±1.2	1.4±0.1	2136.2±148.8	3.7±0.5
I/R	WT		26.0±0.7	1.4±0.1	2028.3±139.8	3.6±0.8
I/R	uPA-/-		25.7±1.4	1.3±0.1	2061.2±102.7	4.1±0.5
I/R	uPAR-/-		25.2±1.1	1.4±0.1	2179.8±119.3	4.0±0.8
I/R	WT	IgG2b	25.1±0.6	1.4±0.1	2207.5±54.2	4.8±0.5
I/R	WT	Anti-CD11b mAb	26.2±1.7	1.2±0.1	1804.4±149.7	4.0±0.7
I/R	WT	Vehicle	25.8±0.3	1.3±0.1	1948.5±31.9	4.7±0.7
I/R	WT	WX-340	26.2±0.7	1.3±0.1	1897.7±83.8	3.6±1.1
control (PBS)	WT		24.7±1.0	1.6±0.1	2522.8±142.2	6.3±1.2
uPA (0.02 μg)	WT		25.4±1.0	1.6±0.1	2533.4±101.9	4.4±1.3
uPA (0.2 μg)	WT		24.0±1.5	1.7±0.1	2755.5±33.4	7.2±0.6
uPA (2.0 μg)	WT		24.5±0.9	1.5±0.1	2373.9±256.1	4.9±1.3
control (PBS)	WT		24.2±0.5	1.5±0.1	2427.5±133.4	6.2±0.7
uPA (2.0 μg)	WT	IgG2b	26.4±0.6	1.3±0.1	1903.8±99.1*	5.6±1.0
uPA (2.0 μg)	WT	Anti-CD11b mAb	26.0±1.1	1.4±0.1	2057.2±44.6*	5.5±0.4
control (PBS)	WT		23.9±0.8	1.4±0.1	2302.8±156.9	6.4±0.9
DFP-uPA (2.0 μg)	WT	IgG2b	25.2±0.6	1.2±0.1	1828.2±26.1*	3.3±0.3*
DFP-uPA (2.0 μg)	WT	Anti-CD11b mAb	21.9±1.6	1.2±0.1	2231.8±199.3	3.8±0.5*
uPA-PAI-1 (4.0 μg)	WT	IgG2b	24.2±0.8	1.2±0.1	1901.1±68.7*	3.8±0.2*
UPA-PAI-1 (4.0 μg)	WT	Anti-CD11b mAb	25.2±0.4	1.1±0.1	1780.7±80.2*	3.1±0.8*
h-uPA (2.0 μg)	WT	IgG2b	25.4±0.8	1.1±0.1	1743.2±104.6	4.5±0.8*
h-uPA (2.0 μg)	WT	Anti-CD11b mAb	25.1±0.7	1.1±0.1	1776.5±52.4	4.3±0.4

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.

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.[8] 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 perivenular 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 intraarticular injection of uPA has been reported to cause leukocyte infiltration of the synovial tissue[23] 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

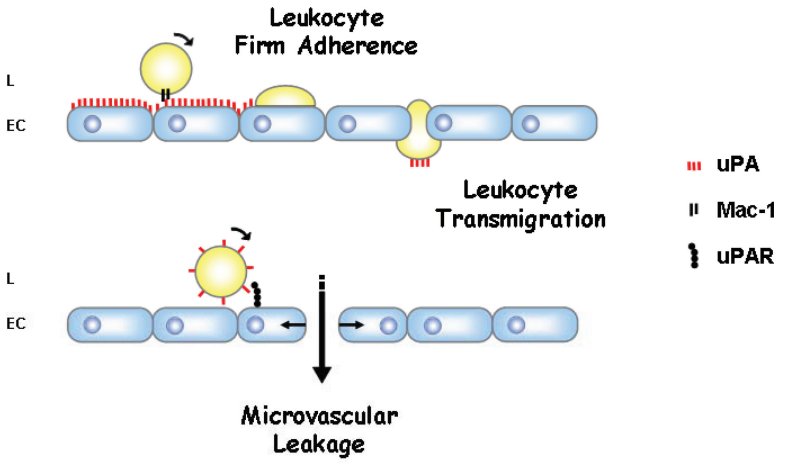

Figure 7. Schematic diagram on the involvement of uPA in the postischemic inflammatory response. Urokinase-type plasminogen activator is thought to mediate postischemic firm adherence and paracellular transendothelial migration of neutrophils via CD11b/Mac-1 and independently of uPAR. In this context, endothelial-presented uPA is thought to promote leukocyte firm adherence 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. L indicates leukocyte; EC, endothelial cells; uPA, urokinase-type plasminogen activator; and uPAR, urokinase-type plasminogen activator receptor.

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.[25] 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[26] 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.[5] 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.[27] In addition, it has been shown that uPA-uPAR interactions are dispensable for other functions of uPA.[28] In animal models of peritonitis[29] or pneumonia,[30] 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 β_2-integrin CD11b/Mac-1, which also serves as complement receptor and as adhesion molecule, as another receptor of uPA.[31] 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 uPAR (which has been reported to be functionally relevant for cell migration[32,33]) 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[31] or indirectly (eg, by induction of release/production of inflammatory mediators[23]) 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.[9] In this context, interaction of uPA with endothelial uPAR is thought to induce retraction of endothelial cells,[34] 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-elicited 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-elicited 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-elicited 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.[8] 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,[35,36] 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[10] and clinical trials did not provide any evidence about prothrombotic side effects of uPA inhibitors,[37] 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.

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Disclosures

None.

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CLINICAL PERSPECTIVE

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.