Human recombinant erythropoietin protects the striated muscle microcirculation of the dorsal skinfold from postischemic injury in mice

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Abstract

Erythropoietin (EPO) has been proposed as a novel cytoprotectant in ischemia-reperfusion (I/R) injury of the brain, heart, and kidney. However, whether EPO exerts its protection by prevention of postischemic microcirculatory deterioration is unknown. We have investigated the effect of EPO on I/R-induced microcirculatory dysfunctions. We used the mouse dorsal skinfold chamber preparation to study nutritive microcirculation and leukocyte-endothelial cell interaction in striated muscle of the dorsal skinfold by in vivo fluorescence microscopy before 3 h of ischemia and during 5 days of reperfusion. Animals were pretreated with EPO (5,000 U/kg body wt) 1 or 24 h before ischemia. Vehicle-treated I/R-injured animals served as controls. Additional animals underwent sham operation only or were pretreated with EPO but not subjected to I/R. I/R significantly (P < 0.05) reduced functional capillary density, increased microvascular permeability, and enhanced venular leukocyte-endothelial cell interaction during early reperfusion. These findings were associated with pronounced (P < 0.05) arteriolar constriction and diminution of blood […]

Reference


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Human recombinant erythropoietin protects the striated muscle microcirculation of the dorsal skinfold from postischemic injury in mice

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Am J Physiol Heart Circ Physiol 293: H274–H283, 2007. First published March 2, 2007; doi:10.1152/ajpheart.01031.2006.—Erythropoietin (EPO) has been proposed as a novel cytoprotectant in ischemia-reperfusion (I/R) injury of the brain, heart, and kidney. However, whether EPO exerts its protection by prevention of postischemic microcirculatory deterioration is unknown. We have investigated the effect of EPO on I/R-induced microcirculatory dysfunctions. We used the mouse dorsal skinfold chamber preparation to study nutritive microcirculation and leukocyte-endothelial cell interaction in striated muscle of the dorsal skinfold by in vivo fluorescence microscopy before 3 h of ischemia and during 5 days of reperfusion. Animals were pretreated with EPO (5,000 U/kg body wt) 1 or 24 h before ischemia. Vehicle-treated I/R-injured animals served as controls. Additional animals underwent sham operation only or were pretreated with EPO but not subjected to I/R. I/R significantly (P < 0.05) reduced functional capillary density, increased microvascular permeability, and enhanced venular leukocyte-endothelial cell interaction during early reperfusion. These findings were associated with pronounced (P < 0.05) arteriolar constriction and diminution of blood flow during late reperfusion. Pretreatment with EPO induced EPO receptor and endothelial nitric oxide synthase expression at 6 h of reperfusion (P < 0.05). In parallel, EPO significantly (P < 0.05) reduced capillary perfusion failure and microvascular hyperpermeability during early reperfusion and arteriolar constriction and flow during late reperfusion. EPO pretreatment substantially (P < 0.05) diminished I/R-induced leukocytic inflammation by reducing the number of rolling and firmly adhering leukocytes in postcapillary venules. EPO applied 1 h before ischemia induced angiogenic budding and sprouting at 1 and 3 days of reperfusion and formation of new capillary networks at 5 days of reperfusion. Thus our study demonstrates for the first time that EPO effectively attenuates I/R injury by preserving nutritive perfusion, reducing leukocytic inflammation, and inducing new vessel formation.

I/R injury represents a major challenge in cardiovascular medicine and also in trauma and vascular and plastic and reconstructive surgery. The deleterious effects of I/R injury are mediated by microcirculatory dysfunctions, including breakdown of capillary perfusion (27, 28), chemotactic accumulation and venular wall adhesion of activated leukocytes (23, 26, 37), and loss of the integrity of the microvascular endothelial lining (20, 26). In trauma and reconstructive surgery, as well as in transplantation medicine, elective surgical procedures involve prolonged periods of ischemia, which additionally enhance tissue injury during postischemic reperfusion. Although experimental studies have provided evidence that a variety of compounds, such as oxygen radical scavengers and anti-inflammatory drugs (15), reduce I/R injury, pharmacological treatment of reperfusion injury has not been successfully established in clinical practice.

Erythropoietin (EPO) is well known for its stimulatory function on erythrocytic progenitors. However, when administered exogenously, EPO represents a viability and growth factor that exerts neuroprotective (16), cardioprotective (1, 33, 38), and vascular actions (12). Accordingly, it is intensively investigated for its nonhematopoietic pleiotropic actions, which may be responsible for tissue protection (7).

EPO is a renal glycoprotein that is released in response to hypoxia and exerts its effect through its interaction with EPO receptors. Besides an increase of intracellular calcium, the main signaling pathways of hematopoietic cells involve 1) expression of the antiapoptotic protein Bcl-x, 2) activation of MAPK and phosphatidylinositol 3-kinase (PI3K/Akt), and 3) activation of transcription factor 5 for survival, proliferation, and migration of stimulated cells (2, 11, 25, 31).

The effect of EPO on the vascular endothelium is poorly understood. There is some evidence derived from isolated human vessel preparations that EPO modifies endothelial nitric oxide (NO) synthase (eNOS) expression and vascular functions, because it stimulated NO-mediated endothelium-dependent relaxation of arterial vessels (32). EPO may induce endothelial cell responses by EPO receptor and eNOS expression as well as NO release, in particular under hypoxic conditions (6). On the other hand, EPO may have a deleterious effect within the microvasculature, because it mediates prothrombotic mechanisms, such as endothelin-1 (10) and plasminogen activator inhibitor-1 induction (29).

Previous studies have reported that EPO effectively reduces I/R injury in the brain (34), heart (9), and kidney (35). However, whether EPO exerts its protection in I/R injury by preventing postischemic microcirculatory deterioration is unknown. Because it is well known that microcirculatory dysfunction plays a pivotal role in the manifestation of I/R injury (15, 27), we have investigated whether EPO affects I/R-induced microcirculatory deterioration, including capillary perfusion failure, leukocyte-endothelial cell interaction; microvascular permeability; intravital microscopy; angiogenesis; neovascularization.

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Table 1. Hemoglobin, hematocrit, and RBC count after EPO pretreatment and in vehicle-treated controls

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EPO-Control</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>5 Days</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>20.7 ± 1.0</td>
<td>20.6 ± 1.6</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>15.6 ± 1.0</td>
<td>15.2 ± 1.3</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>48.5 ± 3.5</td>
<td>47.5 ± 3.5</td>
</tr>
<tr>
<td>RBC count, 10^6/μl</td>
<td>9.4 ± 0.8</td>
<td>9.5 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6. A single dose of erythropoietin (EPO) did not significantly increase hemoglobin, hematocrit, and red blood cell (RBC) count.

Arterioles and capillaries, microvascular hyperpermeability, and neovascularization.

MATERIALS AND METHODS

Animals. Experiments were performed according to the guiding principles for research involving animals and the German legislation on protection of animals. The experiments were approved by the local governmental animal care committee. A total of 55 C57BL/6J mice (12–24 wk of age, 24–26 g body wt; Charles River Laboratories, Sulzfeld, Germany) were housed in single cages at 22°C and 60–65% relative humidity with a 12:12-h light-dark cycle. The animals were allowed free access to tap water and standard laboratory chow (Altromin, Lage, Germany).

Experimental model. The dorsal skinfold chamber in mice was used for intravital microscopy, as previously described in detail (24).

Briefly, mice were anesthetized intraperitoneally with a mixture of ketamine hydrochloride (90 mg/kg body wt; Ketavet, Parke Davis, Freiburg, Germany) and xylazine hydrochloride (25 mg/kg body wt; Rompun, Bayer, Leverkusen, Germany), and two symmetrical titanium frames were implanted to sandwich the extended double layer of the skin. One layer was removed in a 15-mm-diameter circular area. The remaining layer, consisting of epidermis, subcutaneous tissue, and striated skin muscle, was covered with a glass coverslip incorporated in one of the titanium frames. Animals tolerated the chamber well and showed no signs of discomfort or changes in sleeping and feeding habits. A recovery period of 3 days was allowed before the experiment was started.

I/R. A silicone pad and an adjustable screw were used to apply ~40-mmHg pressure and, thereby, establish a 3-h period of pressure-induced ischemia in a group of 18 animals (36). The pressure was just sufficient to occlude the feeding arterioles and, thus, induce complete cessation of blood flow. In another group of 12 animals, the chamber tissue was not exposed to ischemia; these animals served as nons ischemic sham or EPO controls. An additional group of 12 animals were used for blood sampling to monitor potential effects of EPO on hematocrit and hemoglobin levels.

Intravital fluorescence microscopy. For in vivo microscopic analysis of the microcirculation, the anesthetized mice were placed in the left lateral position on a Plexiglas stage. A modified multifluorescence microscope with a 100-W mercury lamp (Axiotech, Zeiss, Jena, Germany) was attached to a system of ultraviolet light (300- to 390-nm excitation and >430-nm emission), blue (450- to 490-nm excitation and >520-nm emission), and green (530- to 560-nm excitation and >580-nm emission) filters. An epi-illumination technique was used to analyze the stirred intraluminal microcirculation within the chamber tissue. Microscopic images were recorded by a charge-coupled device video camera (model FK 6990, COHU, Pieper, Schwerte, Germany), transferred to a video system (S-VHS Panasonic AG 7350, Matsushita, Tokyo, Japan), and recorded on videotape for subsequent offline analysis. We injected 0.05 ml of FITC-dextran (150,000 mol wt, 50 mg/ml saline; Sigma Chemical, St. Louis, MO) for intravascular contrast enhancement and 0.05 ml of rhodamine 6G (0.1 mg/ml saline; Sigma Chemical) for leukocyte staining in vivo into the tail vein. For quantitative analysis of microvascular perfusion, leukocyte-endothelial cell interaction, and macromolecular extravasation and angiogenic neovascularization, we used long-distance objectives (×4 magnification, 0.16 NA; ×10 magnification, 0.30 NA; ×20 magnification, 0.32 NA; Zeiss) (2).

Quantitative microcirculation analysis. The chamber was scanned for random selection of distinct observation areas, which included four to six second- and third-order arterioles, nine nutritive capillary fields, and four to six draining postcapillary venules. Video printouts were made during videography and initially marked to indicate the exact location for measurements of vessel diameter and RBC velocity. Diameters were measured in micrometers perpendicularly to the vessel path. The line-shift method (Capimage, Zeintl Software, Heidelberg, Germany) with computer assistance was used to analyze centerline RBC velocity. Volumetric blood flow (Q) was calculated from diameter (d) and RBC velocity (v) as follows: $Q = \pi \times (d/2)^2 \times v/1.6$ (pl/s), where 1.6 represents the Baker-Wayland factor, which corrects for the parabolic velocity profile in >20-μm microvessels (5).

The number of permanent adherent leukocytes (i.e., cells that adhered to the venular vessel wall over a 30-s period) was evaluated as the number of cells per square millimeter of endothelial surface (calculated from diameter and length of the vessel segment and with the assumption that the vessel is cylindrical). Rolling leukocytes, defined as cells moving with a velocity less than two-fifths of the

Table 2. Baseline data of microcirculation and leukocyte-endothelial cell interaction

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>EPO-Control</th>
<th>I/R</th>
<th>I/R + EPO 1 h</th>
<th>I/R + EPO 24 h</th>
</tr>
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<tbody>
<tr>
<td><strong>Arterioles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, μm</td>
<td>44.3 ± 12.2</td>
<td>34.0 ± 2.1</td>
<td>49.6 ± 3.4</td>
<td>39.9 ± 4.0</td>
<td>39.9 ± 4.0</td>
</tr>
<tr>
<td>RBC velocity, mm/s</td>
<td>1.41 ± 0.44</td>
<td>1.37 ± 0.52</td>
<td>1.49 ± 0.11</td>
<td>1.44 ± 0.37</td>
<td>1.39 ± 0.27</td>
</tr>
<tr>
<td>Blood flow, pl/s</td>
<td>23.6 ± 15.2</td>
<td>14.2 ± 1.45</td>
<td>25.5 ± 10.8</td>
<td>36.1 ± 21.9</td>
<td>18.3 ± 5.8</td>
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<tr>
<td><strong>Venules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Diameter, μm</td>
<td>37.8 ± 8.8</td>
<td>35.5 ± 2.1</td>
<td>44.4 ± 5.6</td>
<td>34.9 ± 7.6</td>
<td>35.3 ± 1.9</td>
</tr>
<tr>
<td>RBC velocity, mm/s</td>
<td>0.50 ± 0.24</td>
<td>0.47 ± 0.13</td>
<td>0.51 ± 0.18</td>
<td>0.35 ± 0.21</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>Blood flow, pl/s</td>
<td>6.9 ± 2.5</td>
<td>6.9 ± 2.5</td>
<td>8.3 ± 2.8</td>
<td>4.5 ± 2.1</td>
<td>3.7 ± 1.1</td>
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<tr>
<td><strong>Capillaries</strong></td>
<td></td>
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<tr>
<td>Functional capillary density, cm⁻¹</td>
<td>207 ± 17.7</td>
<td>221 ± 21</td>
<td>252 ± 31</td>
<td>236 ± 25</td>
<td>245 ± 26</td>
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<tr>
<td><strong>Leukocyte-endothelial cell interaction</strong></td>
<td></td>
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<tr>
<td>Rolling, cells/min</td>
<td>3.8 ± 0.8</td>
<td>1.6 ± 0.9</td>
<td>2.6 ± 0.7</td>
<td>3.1 ± 0.5</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>Adherence, cells/mm²²</td>
<td>113 ± 28</td>
<td>104 ± 12</td>
<td>126 ± 23</td>
<td>109 ± 0.9</td>
<td>116 ± 1.8</td>
</tr>
</tbody>
</table>

Values are means ± SD. Sham, chamber preparation without ischemia-reperfusion (I/R) and EPO; EPO-control, EPO treatment (5,000 U/kg body wt ip) 1 h before measurements without I/R; I/R, I/R and treatment with equivalent volume of vehicle (saline); I/R + EPO 1 h and I/R + EPO 24 h, I/R and EPO treatment 1 h and 24 h before induction of ischemia, respectively. Note no significant differences between groups at baseline.
centerline velocity, are expressed as the number of cells per minute passing a reference point within the microvessel (26).

Macromolecular extravasation was determined as an indicator of microvascular permeability and, thus, endothelial integrity and was assessed by densitometric measurement of gray levels in the tissue directly adjacent to the venular vessel wall (E1), as well as within the marginal cell-free plasma layer of the vessel (E2). Macromolecular extravasation was then calculated as the ratio of E1 to E2 (26).

Experimental protocol. Sham animals (n = 6) underwent chamber preparation without I/R induction and EPO treatment. EPO-control animals (n = 6) received recombinant human EPO (5,000 U/kg body wt ip; Roche, Basel, Switzerland) 1 h before intravital microscopic baseline measurements but were not subjected to I/R. The I/R + EPO 1h group (n = 6) was subjected to I/R and treated with EPO (5,000 U/kg body wt ip) 1 h before induction of ischemia. The I/R + EPO 24h group (n = 6) was subjected to I/R and treated with EPO (5,000 U/kg body wt ip) 24 h before induction of ischemia. I/R animals (n = 6) were subjected to I/R but treated with equivalent volumes of the vehicle (saline). Repetitive intravital microscopic observations in the anesthetized animal were performed at baseline (before I/R) and 2 and 6 h, as well as 1, 3, and 5 days, after onset of reperfusion. Sham and EPO-control animals were studied at corresponding time points. At the end of the experiments, the animals were euthanized by injection of an overdose of the anesthetic. In another group of 12 animals, hematocrit and hemoglobin concentration were assessed and RBCs were counted 5 days after systemic administration of EPO (5,000 U/kg body wt, n = 6) or vehicle (n = 6). One additional animal of each group was killed 6 h after onset of reperfusion and used for immunohistochemical study of EPO receptor and eNOS expression in small arterioles. Four additional animals from the I/R and I/R + EPO 1h groups were used for semiquantitative study of the EPO receptor and eNOS expression pattern.

Immunohistochemical detection and evaluation of EPO receptor and eNOS. For analysis of the pattern and cell type-specific expression of EPO receptor and eNOS, activity of endogenous peroxidase was blocked by incubation of 4-μm sections of the tissue in 0.3% H2O2-methanol, and the slides were exposed to cross-reacting polyclonal rabbit anti-rat EPO receptor (1:50 dilution; StressGen Biotechnologies, Victoria, BC, Canada) or eNOS (1:25 dilution; BD Biosciences, Germany) antibody at 37°C for 2 h. A horseradish peroxidase-conjugated goat anti-rabbit antibody was used as secondary antibody (1:500 dilution; Amersham Biosciences, Freiburg, Germany), and 3,3′-diaminobenzidine was used as chromogen. Slides were counterstained with hematoxylin and examined by light microscopy (model BX 60F, Olympus Optical, Tokyo, Japan). As a negative control, additional sections from each specimen were exposed to appropriate IgG isotype-matched antibody (Sigma Aldrich Chemie), instead of the primary antibodies, under the same conditions to determine the specificity of antibody binding. All the control stains were found to be negative. The intensity of the staining reactions in arterioles was evaluated using a semiquantitative score.

Statistical analysis. Values are means ± SD. For comparison between individual time points, ANOVA for repeated measures was followed by the appropriate post hoc test, including correction of the alpha error according to Bonferroni’s probabilities. For comparison between the groups, ANOVA for comparison of multiple groups was followed by Student-Newman-Keuls test for appropriate post hoc analysis (SigmaStat, Jandel, San Rafael, CA). P < 0.05 was taken to indicate statistically significant differences.

RESULTS

Hematocrit and hemoglobin concentrations, as well as RBC counts, slightly increased 5 days after treatment with EPO (5,000 U/kg body wt), but these values were not significantly different from vehicle-treated control values (Table 1).

Postischemic arteriolar perfusion. Analysis of arteriolar diameters, RBC velocity, and blood flow revealed comparable values at baseline in all groups (Table 2). I/R provoked arteriolar constriction, which was most pronounced (P < 0.05) during the late-reperfusion phase (Fig. 1A). Analysis of arteriolar RBC velocity and arteriolar blood flow showed a reactive hyperemia during the first 6 h of reperfusion followed by a significant decrease (P < 0.05) during the late-reperfusion phase (Fig. 1, B and C). Pretreatment with EPO 1 or 24 h before ischemia completely prevented arteriolar constriction.

![Fig. 1](image-url)
(Fig. 1A) and effectively abrogated \((P < 0.05)\) the decrease of postischemic arteriolar perfusion during the late-reperfusion phase (Fig. 1, B and C).

**Postischemic capillary perfusion.** Analysis of functional capillary density, as well as capillary diameters, RBC velocity, and blood flow, revealed comparable values at baseline in all groups (Table 2). I/R induced a significant decrease \((P < 0.05)\) in functional capillary density to 40% of baseline during early reperfusion that did not completely recover after the 5-day reperfusion period (Fig. 2A). This was associated with a hypemic capillary perfusion of the remaining patent capillaries during early reperfusion followed by a significant \((P < 0.05)\) capillary narrowing and blood flow reduction during the late-reperfusion phase (Fig. 2, B–D). Pretreatment with EPO 1 or 24 h before ischemia significantly \((P < 0.05)\) attenuated the I/R-induced decrease of functional capillary density (Fig. 2A). EPO was able to completely abrogate the decrease of capillary blood flow during the late-reperfusion period and also induced capillary hyperperfusion \((P < 0.05)\), mainly by widening luminal diameter (Figs. 2, B–D).

**Postischemic venular perfusion.** Analysis of venular diameters, RBC velocity, and blood flow revealed comparable values at baseline in all groups (Table 2). I/R did not significantly affect diameters and flow conditions throughout the reperfusion phase (data not shown). Also, EPO pretreatment 1 h or 24 h before ischemia did not significantly change venular flow conditions during postischemic reperfusion (data not shown).

**Postischemic leukocyte-endothelial cell interaction.** Analysis of venular leukocyte rolling and adherence revealed comparable values at baseline in all groups (Table 2). The I/R-induced 5- to 10-fold increase \((P < 0.05)\) of leukocyte rolling and firm adherence during the initial postischemic reperfusion period did not completely recover during the 5-day observation period (Fig. 3). Pretreatment with EPO 1 or 24 h before ischemia significantly \((P < 0.05)\) attenuated leukocyte rolling (Fig. 3A) and also reduced leukocyte firm adherence, resulting in numbers of cells per endothelial surface comparable to that observed at baseline (Fig. 3B).

**Postischemic microvascular permeability.** Analysis of macromolecular extravasation from postcapillary venules, as an indicator of microvascular permeability, revealed comparable values at baseline in all groups (Table 2). The I/R-induced significant increase \((P < 0.05)\) of macromolecular extravasation during the early postischemic reperfusion period did not recover after the 5-day observation period (Fig. 4). Pretreatment with EPO 1 or 24 h before ischemia significantly \((P < 0.05)\) attenuated I/R-induced microvascular hyperpermeability. At 5 days after reperfusion, permeability in animals treated with EPO 1 h before ischemia was comparable to that at baseline (Fig. 4) and in sham-operated controls (Fig. 5).

**Microcirculation and inflammation in sham-operated and nonischemic EPO-control animals.** In animals that were subjected to only sham operation or treated with EPO without I/R, no significant changes were observed in arteriolar, capillary,
and venular perfusion over the 5-day period (Fig. 5, A–C). No leukocytic inflammatory responses or changes in macromolecular extravasation were noted in these animals (Fig. 5, D–F).

**Postischemic angiogenesis and neovascularization.** Despite the reduction of arteriolar and capillary perfusion during the postischemic reperfusion period, I/R was not associated with an angiogenic response and neovascularization (Fig. 6). In contrast, pretreatment with EPO, in particular 1 h before ischemia, resulted in capillary budding and sprouting 1 and 3 days after onset of reperfusion and formation of new capillary networks with perfused microvessels at the end of the 5-day observation period (Fig. 6).

**Postischemic EPO receptor and eNOS expression.** EPO receptor and eNOS expression was markedly enhanced 6 h after reperfusion in animals pretreated with EPO compared with sham-control animals (Fig. 7). Expression of eNOS was distinct in the endoluminal aspects of endothelial cells in sham animals and I/R controls; after EPO pretreatment, however, the entire cytoplasm showed substantial eNOS expression ($P < 0.05$). The staining was observed in endothelial, but not smooth muscle, cells. EPO receptor expression was distinct in all endothelial cells in sham animals. In vehicle-treated I/R controls, a marked expression of EPO receptor was observed compared with sham ($P < 0.05$); after EPO pretreatment, this effect was more pronounced (not significant compared with I/R controls). In smooth muscle cells, marked expression of EPO receptor was observed after EPO treatment (Fig. 8).

**DISCUSSION**

In the present study, we demonstrate for the first time that EPO pretreatment can attenuate I/R-induced microcirculatory dysfunction and leukocyte-endothelial cell interaction in postischemic striated muscle. Our study strongly suggests that, in the presence of hypoxia, EPO pretreatment may exert its protection through an enhanced eNOS expression and may stimulate the proliferation of endothelial cells in critically reperfused striated muscle.

There is insufficient information about the dose of EPO that should be used to achieve nonhematopoietic effects. The first investigators who described nonhematopoietic beneficial actions of EPO used single, high doses of 5,000 U/kg, which have been shown to reduce infarct size in the heart by preventing apoptosis. This effect was achieved when EPO was injected immediately after myocardial ischemia without increasing the risk of thrombosis. At lower doses, EPO has been shown to be somehow effective, but higher doses extended the therapeutic window. Accordingly, for our study of the effect of EPO on microcirculatory dysfunction in I/R, we used the high dose (5,000 U/kg). In future studies, it is necessary to define the relationship between EPO dose and the various effects of EPO, including the antiapoptotic, proangiogenic, vasculoprotective, and anti-inflammatory actions.

Three hours of ischemia caused a classic hyperemic response during initial reperfusion, with enhancement of arteriolar RBC velocity and, thus, volumetric blood flow. This, however, was followed by a severe arteriolar constriction 6 h after the onset of reperfusion that persisted over the entire 5-day observation period. This arteriolar constriction was functionally significant, as indicated by a reduced volumetric blood flow within the striated muscle tissue. EPO pretreatment completely abrogated the I/R-induced arteriolar constriction and,.

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**Fig. 3.** Leukocyte rolling (A) and leukocyte firm adherence (B), expressed as percentage of baseline, in vehicle-treated I/R animals (○) and I/R animals pretreated with EPO 1 h (●) or 24 h (□) before induction of ischemia. Arrow indicates induction of 3 h of ischemia and onset of reperfusion (I/R). Note massive induction of leukocyte rolling and adherence by I/R and significant attenuation of this leukocytic inflammatory response after EPO pretreatment. Values are means ± SD. *$P < 0.05$ vs. BL. **$P < 0.05$ vs. vehicle-treated I/R animals at corresponding time points.

**Fig. 4.** Macromolecular extravasation, an indicator for microvascular permeability, in vehicle-treated I/R animals (○) and I/R animals pretreated with EPO 1 h (●) or 24 h (□) before induction of ischemia. Arrow indicates induction of 3 h of ischemia and onset of reperfusion (I/R). Note increase of microvascular permeability during postischemic reperfusion, which is significantly attenuated after EPO pretreatment. Values are means ± SD. *$P < 0.05$ vs. BL. **$P < 0.05$ vs. vehicle-treated I/R animals at corresponding time points.
thus, blood flow reduction. This action of EPO may be caused by the EPO-associated attenuation of venular leukocyte adherence. The I/R-induced leukocyte accumulation in venules may act in a paracrine manner on the vasomotor control of the feeding arterioles, because the second- and third-order arterioles are running in parallel to the postcapillary and collecting venules. This view is based on observations of a previous study in which abrogation of CD18-dependent leukocyte adherence in postcapillary venules was shown to successfully blunt arteriolar constriction during postischemic reperfusion (40).

These findings are not inconsistent with the assumption that some beneficial effect of EPO is related to NO synthase, because vascular endothelial dysfunctions in reperfused striated muscle have been shown to be associated with decreased NO concentrations and reduced eNOS activity (17). Accordingly, we observed in the present study that EPO pretreatment increased eNOS expression, and this may be responsible for the abrogation of the I/R-induced arteriolar constriction. Consistent with this observation, a recent study, showing that postischemic inflammation depends on eNOS phosphorylation, supports the view that eNOS is involved in EPO-mediated microcirculatory protection bifunctionally: by increasing the NO availability and through an anti-inflammatory effect (21).

In parallel to the reduction of the arteriolar blood flow, I/R induced significant capillary no-reflow, as indicated by a massive diminution of postischemic functional capillary density. It has been suggested that the postischemic capillary no-reflow in striated muscle is caused by an increased extramural tissue pressure due to the I/R-induced venular leukocytic inflammation, the increase in microvascular permeability, and the formation of interstitial edema (18). Indeed, abrogation of venular leukocyte adherence by monoclonal antibodies directed against CD11/CD18 has been shown to reduce postischemic microvascular permeability and, consequently, capillary perfusion failure (18). This ideally parallels the observations in the present study after EPO pretreatment. Thus the protective action of EPO on postischemic microcirculatory dysfunction seems to be primarily based on targeting of the inflammatory leukocytic response in the postcapillary venules. The attenuation of leukocyte-endothelial cell interactions by EPO may be caused by the increased expression of eNOS during postischemic reperfusion, because NO has been

Fig. 5. Arteriolar blood flow, functional capillary density, capillary blood flow, leukocyte rolling, leukocyte adherence, and macromolecular extravasation in sham-operated animals (○) and EPO-controls without I/R (▲). Arrow indicates induction of 3 h of ischemia and onset of reperfusion (I/R). Sham operation and EPO treatment did not significantly affect microcirculatory and inflammatory parameters. Values are means ± SD.
shown to be protective in limiting leukocyte adherence in I/R injury (19).

In the present I/R model, hypoxia-inducible factors (HIFs) may have been upregulated and vascular endothelial growth factor (VEGF) may have been induced, and both may have contributed to the angiogenesis. Theoretically, VEGF as the vascular permeability factor may also have contributed to the increased microvascular permeability. However, because microvascular permeability was increased most after I/R in vehicle controls, whereas angiogenesis was observed only in EPO-treated animals, which showed a significantly reduced microvascular permeability compared with the vehicle-treated controls, it is highly unlikely that the postischemic increase in microvascular permeability is induced in the present model by the action of HIFs and VEGF. In contrast, it is most probable that the increased microvascular permeability is due to the ischemia- and reoxygenation-mediated inflammatory injury (26) and that this injury is reduced after EPO treatment because of anti-inflammatory potentials of EPO.

I/R induced a reactive hyperemia during the initial postischemic reperfusion period but was associated with luminal narrowing and reduction of blood flow in the still-patent capillaries during the late-reperfusion phase. EPO pretreatment, in particular 1 h before ischemia, resulted in a widening of the capillary lumen, which was associated with capillary hyperperfusion. Although we are not aware whether the capillary widening was passive in nature or actively mediated by EPO, others demonstrated in hepatic tissue that I/R is associated with a reduction of capillary (sinusoidal) diameters due to pericyte constriction, which is triggered by an imbalance between endothelin-1 and NO, and that widening of sinusoids can be achieved by modulating the balance in favor of NO (30). Accordingly, the postischemic capillary widening observed after EPO pretreatment in the present study may be caused by the increased release of NO due to the pronounced eNOS expression.

Thus our study provides evidence that EPO can have a direct action on the endothelium in vivo that increases NO bioavailability by upregulation of eNOS under hypoxic conditions. Binding studies with radio-ionated EPO demonstrated 27,000 receptors per endothelial cell on the intraluminal surface (3). Our results suggest that hypoxia increases the

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Fig. 6. A–C: postischemic intravital fluorescence microscopy [contrast enhancement by 5% FITC-dextran (150,000 mol wt)] of angiogenic budding (A, arrows), sprouting (B, arrow), and microvascular network formation (C) in animals pretreated with EPO 1 h before induction of ischemia. D–F: quantitative analysis of capillary budding, capillary sprouting, and microvascular network formation in vehicle-treated I/R animals (open bars) and I/R animals pretreated with EPO 1 h (solid bars) or 24 h (shaded bars) before induction of ischemia. Arrow indicates induction of 3 h of ischemia and onset of reperfusion (I/R). Note lack of angiogenesis and neovascularization after I/R and capillary budding, capillary sprouting, and microvascular network formation after EPO pretreatment, in particular when EPO was given 1 h before ischemia. Values (percent “angiogenesis-positive” fields of all fields analyzed) are means ± SD. *P < 0.05 vs. BL, #P < 0.05 vs. vehicle-treated I/R animals at corresponding time points. †P < 0.05 vs. EPO at 24 h.
capacity of the endothelial cell to produce NO in response to EPO pretreatment by induction of EPO receptors and eNOS.

These results are consistent with the findings of a recent study in which it was shown that endothelial responses may require an increased exposure to EPO combined with upregulation of EPO receptors (6). Without induction of EPO receptors, only a minimal EPO response is achieved, as demonstrated in the present study in EPO-control animals, which revealed negligible changes in microhemodynamics after EPO pretreatment.

Angiogenesis, involving capillary sprouting from preexisting blood vessels, comprises a series of events including degradation of extracellular matrix components, proliferation and migration of endothelial cells, and tube formation. Therapeutic angiogenesis describes the induction and stimulation of neovascularization for treatment or prevention of pathological clinical situations characterized by local hypovascularity, such as postischemic conditions of critically reperfused striated muscle. In the present study, we observed capillary budding and sprouting from small postcapillary venules, in particular when EPO was given 1 h before ischemia, but no angiogenic response in vehicle-treated animals. This suggests that the ischemic stimulus and EPO pretreatment are necessary for effective induction of angiogenesis.

A recent study demonstrated that ischemic tissue viability can be improved by systemic EPO administration. However, the underlying mechanisms remained unclear, although enhanced angiogenesis was assumed (8). EPO has been shown to induce therapeutic angiogenesis with an effectiveness equal to that of VEGF. Compared with the plethora of data from other angiogenic factors such as VEGF or those of the fibroblast growth factor family, data on the angiogenic properties of EPO are rather rare. The angiogenic activity of EPO has been described in vitro and in the rat aortic ring model (10), in estrogen-dependent uterine angiogenesis (39), and in immortalized human umbilical vein endothelial cells (31). In vivo studies in the chicken embryo chorioallantoic membrane assay (13) demonstrated that EPO induces endothelial cell proliferation and migration and stimulates angiogenesis following an intussusceptive microvascular growth mechanism. In a recent study, accelerated wound healing after experimental thermal injury and EPO treatment was associated with substantial neovascularization in traumatized tissue (14). Promising studies have further indicated that EPO might play a role in endothelial progenitor cell recruitment, promoting repair of the endothelium in the setting of tissue injury (4). Here, we have demonstrated for the first time that EPO can induce angiogenesis and functionally intact microvascular networks in I/R injury.
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In conclusion, our data demonstrate that EPO administration in a single dose before onset of postischemic reperfusion offers significant protection against I/R injury in striated muscle by reducing capillary no-reflow and leukocytic inflammation during early reperfusion and by modulating arteriolar vasomotor response and inducing angiogenesis in the later postischemic period.

REFERENCES


