Important Role of Erythropoietin Receptor to Promote VEGF Expression and Angiogenesis in Peripheral Ischemia in Mice

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Abstract*—**We have recently demonstrated that endogenous erythropoietin (Epo)/Epo receptor (EpoR) system plays an important protective role in hypoxia-induced pulmonary hypertension. However, it remains to be examined whether vascular EpoR system contributes to angiogenesis in response to ischemia. We examined angiogenesis in EpoR$^{-/-}$-rescued mice that lack EpoR in most organs including cardiovascular system except erythroid-lineage cells. Two weeks after femoral artery ligation, blood flow recovery, activation of VEGF/VEGF receptor system, and mobilization of endothelial progenitor cells were all impaired in EpoR$^{-/-}$-rescued mice as compared with wild-type (WT) mice. Bone marrow (BM) transplantation with WT-BM cells in EpoR$^{-/-}$-rescued mice partially but significantly improved blood flow recovery after hindlimb ischemia. The extent of VEGF upregulation and the number of BM-derived cells in ischemic tissue were significantly less in EpoR$^{-/-}$-rescued mice compared with WT mice even after BM reconstitution with WT-BM cells. Similarly, the recovery of blood flow was significantly impaired in recipient EpoR$^{-/-}$-rescued mice that had been transplanted with WT-BM or EpoR$^{-/-}$-rescued-BM as compared with recipient WT mice. Furthermore, the Matrigel implantation assay and aortic ring assay showed that microvessel growth in vitro was significantly reduced in EpoR$^{-/-}$-rescued mice as compared with WT mice. These results indicate that vascular EpoR system also plays an important role in angiogenesis in response to hindlimb ischemia through upregulation of VEGF/VEGF receptor system, both directly by enhancing neovascularization and indirectly by recruiting endothelial progenitor cells and BM-derived proangiogenic cells. (Circ Res*. 2007;100:662-669.)**

Key Words: angiogenesis ■ ischemia ■ progenitor cells ■ VEGF ■ erythropoietin

Prognosis of patients with severe peripheral artery disease (PAD) still remains poor when there is no indications of revascularization therapies such as bypass surgery or percutaneous transluminal angioplasty.[1] Angiogenesis is a promising new therapeutic strategy for severe PAD, however, the effects of angiogenic therapies to improve ischemia are not durable or stable.[2–4] Hypoxia inducible factor-1 (HIF-1) is one of the important factors to induce angiogenesis,[5,6] which upregulates both erythropoietin (Epo) and VEGF.[7,8] These angiogenic cytokines play an important role in recruitment of bone marrow (BM)-derived cells to ischemic tissue, enhancing endothelial cell proliferation and migration, synthesis of extracellular matrix and resultant angiogenesis.[9–11]

Epo is a hypoxia-induced hormone that exclusively stimulates proliferation and differentiation of erythroid progenitor cells and endothelial cells.[12–15] Furthermore, systemic administration of Epo mobilizes endothelial progenitor cells (EPCs) and recruits them to ischemic tissue,[16,17] where EPCs produce abundant cytokines including VEGF and promote postnatal vasculogenesis.[18,19]

Although Epo receptor (EpoR) is known to be expressed abundantly not only in BM but also in a variety of organs including cardiovascular system,[20,21] the role of EpoR in ischemic tissue remains to be clarified. Suzuki et al have recently developed EpoR$^{-/-}$-rescued mice that express EpoR only in the erythroid lineage but not in cardiovascular system[21] and demonstrated the important protective role of endogenous Epo/EpoR system in hypoxia-induced pulmonary hypertension.[21,22] However, it remains to be examined whether vascular Epo/EpoR system also plays an important role in ischemia-induced angiogenesis. In the present study, we thus addressed this important issue in EpoR$^{-/-}$-rescued mice.

Materials and Methods

All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University.

Original received August 2, 2006; revision received December 19, 2006; accepted January 25, 2007.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000260179.43672.fe**

Animal Preparation

In the present study, we used 12-week-old wild-type (WT, n=74) and EpoR$^{-/-}$-rescued male mice (n=58) of C57BL/6 background. All of the animals were normotensive.[23] EpoR$^{-/-}$-rescued mice were generated as previously described.[21,22] In those EpoR$^{-/-}$-rescued mice, EpoR is only expressed in the erythroid-lineage cells because EpoR is expressed under control of an erythroid-specific promoter,[21] whereas endogenous expression of EpoR mRNA is detected in most of the nonerythroid tissues in WT mice.[21] EpoR$^{-/-}$-rescued mice are thus characterized by the absence of EpoR in cardiovascular system but with normal hematopoietic functions.[21,22]

Neovascularization After Hindlimb Ischemia

Animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). Hindlimb ischemia was made by left femoral artery ligation, as previously described.[6] We measured blood flow ratio at ischemic limb (left)/nonischemic limb (right) using a laser Doppler blood flowmeter (Laser Doppler Imager, Moor Instruments Ltd, Devon, UK). The average blood flow ratio of 2 measurements was calculated on the basis of colored histogram pixels.[6] Capillary density was determined in 20 μm-thick frozen sections of the proximal thigh adductor muscles.

Histological Assays

Immunohistochemistry was performed using anti-mouse CD31 (1:100; BD PharMingen), anti-mouse VEGF receptor-2 (VEGFR-2/Flk-1/KDR) (1:100; Santa Cruz), anti-mouse VEGF-A (1:100; Santa Cruz) and tetramethylrhodamine isothiocyanate (TRITC)-labeled lectin (1:100; Sigma) antibodies.[22] Slides were viewed with a laser scanning microscope (LSM510 META, Carl Zeiss, Jena, Germany) and the image browser (Carl Zeiss, Jena, Germany) was used to create a pseudo-3D projection from serial Z-sections through the entire thickness.[24]

Endothelium-Like Cell Assay

Endothelium-like cell assay was performed as previously described.[16,19,22] On the day of surgery and day 7, wild-type mice received an intravenous infusion of Cell Tracker CM-DiI (Molecular Probes, Eugene, Ore)-labeled endothelium-like cells (1x10^6 cells/mouse) isolated from WT or EpoR$^{-/-}$-rescued mice as previously described.[22] Control mice were injected with PBS alone. We injected the cells twice to enhance the detection.

Evaluation of Mobilized EPCs

To examine the role of the Epo/EpoR system in EPC mobilization in response to ischemia, we used fluorescein isothiocyanate (FITC) anti-mouse CD133 (NO. 11-1331, eBioscience, Calif) and phycoerythrin (PE) anti-mouse Flk-1 (VEGFR-2, NO. 12-5821, eBioscience, Calif) antibodies. The number of VEGFR-2$^+$/CD133$^+$ cells in peripheral blood mononuclear cells were examined by fluorescence-activated cell sorter (FACS calibur; Becton Dickinson, San Jose, Calif).[16,19,22,25]

Enzyme-Linked Immunosorbent Assay for VEGF

Enzyme-linked immunosorbent assay (ELISA) was performed with a mouse-specific VEGF ELISA kit (Quantikine M, R&D Systems, Minneapolis, Minn), following the manufacturer's protocol. Protein extracted from muscle homogenates with the Tissue Protein Extraction Reagent (T-PER, Pierce) were standardized for total protein content using the BCA Protein Assay kit (Pierce) as previously described.[26]

Western Blotting

Cell lysates from hindlimb tissue homogenates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). Subsequently, the membranes were probed with antibodies to mouse VEGFR-2/Flk-1 (Santa Cruz, Calif), VEGFR-1/Flt-1 (Santa Cruz, Calif), and GAPDH (Santa Cruz, Calif). Signals

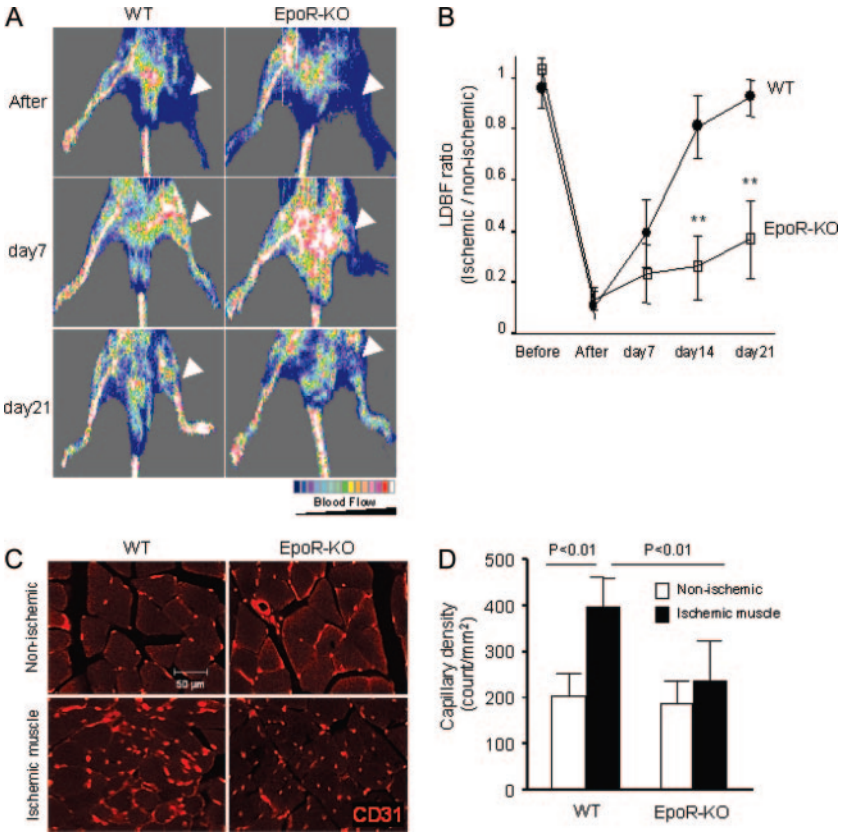

Figure 1. Impaired angiogenic response in ischemic hindlimb of EpoR$^{-/-}$-rescued mice. A, Representative laser Doppler blood flow. Arrows indicate the ischemic left limb. B, Blood flow in ischemic hindlimb was measured before, immediately after, and on days 3, 7, 14, and 21 after the left femoral artery ligation. Results are expressed as a ratio of the left (ischemic) to right (nonischemic) limb perfusion. WT (n=14), and EpoR$^{-/-}$-rescued (n=13). **$P<0.01$ vs WT. C, Representative capillary staining in the adductor muscle sections from WT and EpoR$^{-/-}$-rescued mice at 3 weeks after the hindlimb ischemia. Original magnification, ×200. D, Quantitative analysis of capillary density showing a significantly reduced density in EpoR$^{-/-}$-rescued mice at 3 weeks after the ischemia (n=6 each). Results are expressed as means±SD. WT, wild-type mice; EpoR-KO, EpoR$^{-/-}$-rescued mice.

were visualized by the ECL detection system (Amersham Biosciences, Uppsala, Sweden).[22]

BM Transplantation

BM transplantation was performed as previously described.[18,22] The chimeric rate was more than 95% by FACS analysis.

Skeletal Muscle-Derived Mononuclear Cells From Green Fluorescent Protein-BM Chimeric Mice

Transgenic mice ubiquitously expressing enhanced green fluorescent protein (GFP)[27] were used to generate GFP-BM chimeric mice as previously described.[18,22] Cells among the ischemic and nonischemic tissues were extracted from the thigh adductor muscles at 2 weeks after the ischemic injury.[28] Subsequently, the cells obtained were stained with PE-labeled anti-mouse VEGF receptor (VEGFR)-2 (NO. 12-5821, eBioscience, Calif). Dead cells were stained with propidium iodide (PI).[28] Six weeks after the BM transplantation, the chimeric mice were rendered an hindlimb ischemic injury and the number of GFP^+ cells in the ischemic tissue was counted by confocal microscopy and FACS analysis.[28]

Matrigel Implantation Assay

The Matrigel (Becton Dickinson) implantation assay was performed by injecting 200 μL of growth factor-reduced Matrigel containing VEGF (Invitrogen, 100 ng/mL) plus heparin (20 U/mL) into the abdominal subcutaneous tissue of each mouse.[29] The gels were removed on day 14 and the sections of the gels were stained with FITC-labeled anti-mouse CD31 (1:100; BD PharMingen) and Cy3-labeled α-smooth muscle actin (1:400; C6198, 1A4, Sigma).[29]

Aortic Ring Assay for Angiogenesis

The aortic ring assay was performed as previously described.[30] Briefly, the descending thoracic aorta was isolated and 1-mm long aortic rings were embedded in growth factor-reduced Matrigel supplemented with 20 U/mL heparin. The aortic rings were then cultured in endothelial basal medium (EBM-2, Cambrex Bioscience) supplemented with or without 10 IU/mL human recombinant Epo (Kirin Brewery Co, Tokyo, Japan) and/or 50 ng/mL human recombinant VEGF (Invitrogen) at 37°C.

Statistical Analysis

Quantitative results are expressed as means±SD. Statistical analyses were performed with StatView (StatView 5.0, SAS Institute Inc, Cary, NC). Comparisons of parameters among the 3 groups were made by one-way ANOVA, and comparisons of parameters between the 2 genotypes under different conditions were made by 2-way ANOVA, followed by Bonferroni post-hoc test. A value of $P<0.05$ was considered to be statistically significant.

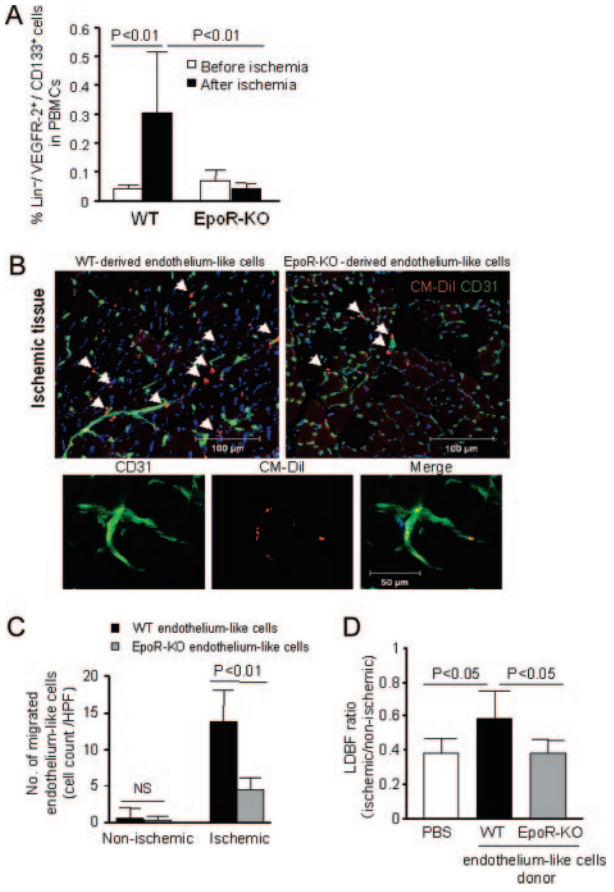

Figure 2. Impaired Endothelium-Like Cell Mobilization in $EpoR^{-/-}$-Rescued Mice. A, Ischemic injury significantly increased the number of $Lin^-/VEGFR\text{-}2^+/CD133^+$ cells in peripheral blood mononuclear cells (PBMCs) on 5 days after the ischemia in WT mice (n=12), but not in $EpoR^{-/-}$-rescued mice (n=13). B, Top 2 photos: CM-DiI–labeled endothelium-like cells cultivated from WT (left) or $EpoR^{-/-}$-rescued (right) mice were intravenously injected into WT mice after the ischemic injury. Numerous $CM\text{-}DiI^+$ cells are migrated to the ischemic tissue (arrows). Bottom 3 photos: CM-DiI–labeled endothelium-like cells were colocalized with CD31-positive endothelial cells C, Number of the $CM\text{-}DiI^+$ endothelium-like cells in the ischemic and nonischemic tissue. HPF, high-power field. D, Quantitative analysis of laser Doppler blood flow images on day 7 after the femoral artery ligation and intravenous administration of PBS alone (n=6), endothelium-like cells cultivated from WT (n=7) or those from $EpoR^{-/-}$-rescued mice (n=7). Results are expressed as a ratio of the left (ischemic) to right (control) limb perfusion, and are reported as means±SD.

Results

Angiogenesis After Femoral Artery Ligation

Immediately after the femoral artery ligation, blood flow in ischemic hindlimb was equally decreased in both strains (Figure 1A and 1B). Although blood flow in ischemic hindlimb was recovered in WT mice until day 14, the recovery was significantly impaired in $EpoR^{-/-}$-rescued mice (Figure 1A and 1B). In addition, histological analysis revealed that the capillary density in ischemic limb was significantly increased in WT mice whereas no such increase was noted in $EpoR^{-/-}$-rescued mice (Figure 1C and 1D). However, the real-time RT-PCR showed that there was no significant difference in mRNA levels of EpoR between the ischemic and nonischemic limbs in wild-type mice (supplemental Figure I in the online data supplement available at http://circres.ahajournals.org). Furthermore, there were no significant differences in Epo or eNOS levels when evaluated by ELISA in the ischemic and nonischemic tissues in wild-type and $EpoR^{-/-}$-rescued mice (supplemental Figure II and III in the online data supplement available at http://circres.ahajournals.org).

$VEGFR\text{-}2^+/CD133^+$ Cell and Endothelium-Like Cell Mobilization

The number of $VEGFR\text{-}2^+/CD133^+$ cells in peripheral blood mononuclear cells was significantly increased after

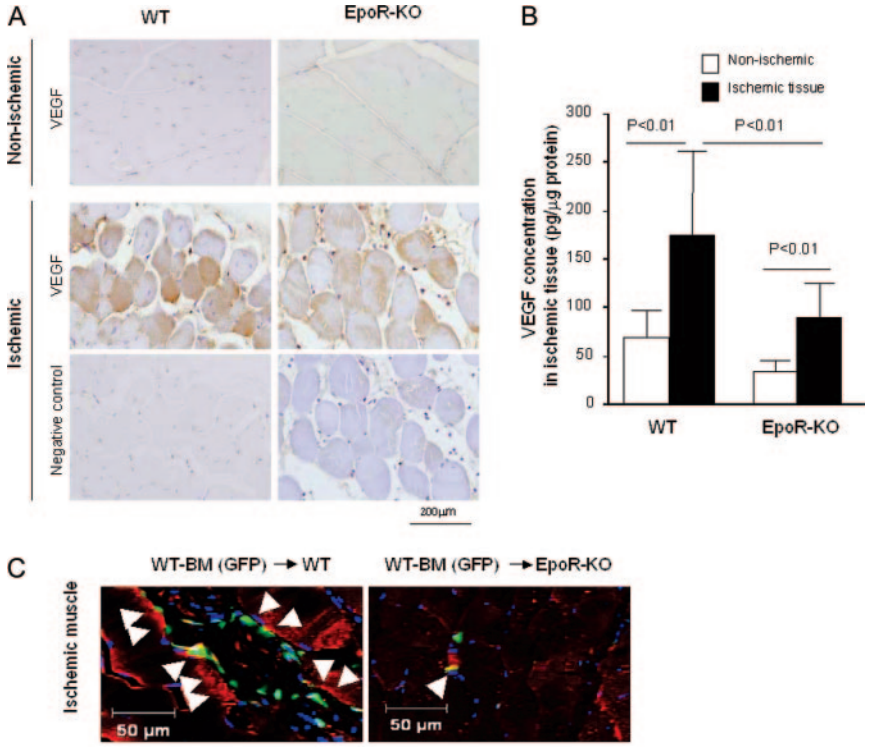

Figure 3. Impaired skeletal muscle VEGF secretion in EpoR$^{-/-}$-rescued mice. A, Representative VEGF immunostaining in ischemic and nonischemic hindlimb tissue. VEGF expression was enhanced in ischemic tissue in WT mice, but not in EpoR$^{-/-}$-rescued mice. B, VEGF concentrations in ischemic and nonischemic tissue evaluated by ELISA (n=9 each). Results are expressed as means ± SD. C, Confocal microscopy showing that the expression of VEGF was significantly enhanced in ischemic tissue in WT mice with WT-BM cells (GFP$^+$). Note that numerous GFP$^+$ cells migrated to the VEGF$^+$ skeletal muscles in WT mice (arrows), but not in EpoR$^{-/-}$-rescued mice.

the hindlimb ischemic injury in WT mice but not in EpoR$^{-/-}$-rescued mice (Figure 2A). Endothelium-like cell assay with CM-DiI-labeling demonstrated that endothelium-like cell migration and homing to the endothelium of the ischemic tissue was significantly impaired in EpoR$^{-/-}$-rescued mice compared with WT mice (Figure 2B and 2C). Furthermore, injection of endothelium-like cells from WT mice, but not those from EpoR$^{-/-}$-rescued mice, improved blood flow recovery after the hindlimb ischemia (Figure 2D).

VEGF Concentrations and Recruitment of BM-Derived Cells

Immunostaining revealed that VEGF expression was enhanced in the ischemic tissue of WT mice compared with EpoR$^{-/-}$-rescued mice (Figure 3A). VEGF concentrations in ischemic tissue was also significantly higher in WT mice than in EpoR$^{-/-}$-rescued mice (Figure 3B), indicating the crucial role of vascular Epo/EpoR system in ischemia-induced VEGF secretion.

To completely exclude the effect of Epo/EpoR system on BM-derived cells, we performed BM transplantation, where BM cells from WT and EpoR$^{-/-}$-rescued mice were reconstituted with those from GFP-transgenic mice. Six weeks after the BM transplantation, confocal microscopy revealed that not only ischemic muscle but also migrated BM-derived cells (GFP$^+$ cells) expressed VEGF (Figure 3C). The VEGF expression was more enhanced in the skeletal muscle close to GFP$^+$ cells (Figure 3C, arrows).

In addition, the number of GFP$^+$ cells was significantly increased in ischemic tissue, especially around the vessels in WT mice, but not in EpoR$^{-/-}$-rescued mice (Figure 4A). Similarly, the number of GFP$^+$ cells detected by FACS analysis was significantly increased in ischemic tissue in WT mice compared with EpoR$^{-/-}$-rescued mice (Figure 4B). Furthermore, ischemia significantly enhanced the expression of VEGFR-2 (Flk-1) in skeletal muscle in WT mice, but not in EpoR$^{-/-}$-rescued mice, although there was no significant change in the expression of VEGFR-1 (Flt-1, Figure 4C).

Furthermore, to evaluate the contribution of BM-derived cells to postischemic angiogenesis, we made the following 4 groups of chimeric mice: (1) WT mice transplanted with BM cells from WT mice; (2) WT mice transplanted with BM cells from EpoR$^{-/-}$-rescued mice; (3) EpoR$^{-/-}$-rescued mice transplanted with BM cells from WT mice; and (4) EpoR$^{-/-}$-rescued mice transplanted with BM cells from EpoR$^{-/-}$-rescued mice (Figure 5A). Two weeks after the hindlimb ischemia, the blood flow ratio was higher in WT recipients, and reduced in of EpoR$^{-/-}$-rescued recipients, regardless of the source of BM (Figure 5A and 5B).

Roles of Vascular Epo/EpoR System in Response to VEGF

To further investigate the role of EpoR in peripheral microvessels, we subcutaneously injected Matrigel with or without VEGF (100 ng/mL) in WT and EpoR$^{-/-}$-rescued mice. On day 14 after the injection, we examined VEGF-induced growth of blood vessels (Figure 6A). The number of the vessels was significantly increased in the Matrigel in response to VEGF in both strains, however, the increase was significantly impaired in EpoR$^{-/-}$-rescued mice compared with WT mice (Figure 6B). Furthermore, in the aortic ring assay, Epo (10 IU/mL) or VEGF (50 ng/mL) increased the number of microvessels sprouting from aortic rings in WT mice compared with EpoR$^{-/-}$-rescued

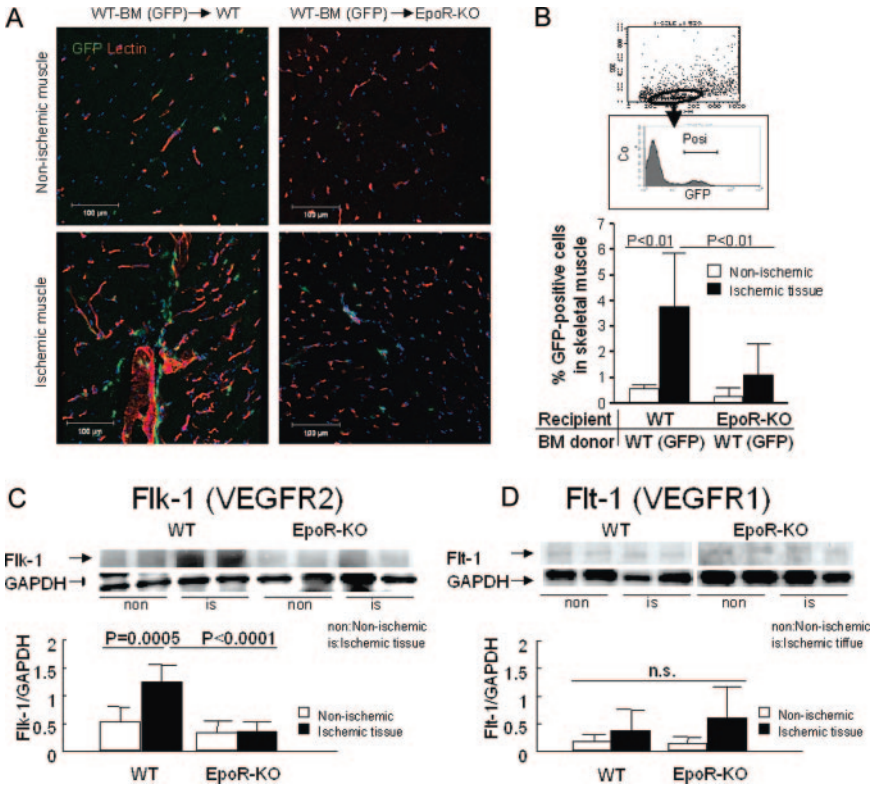

Figure 4. Impaired recruitment of BM-derived cells and reduced enhancement of VEGFR-2 after ischemia in EpoR$^{-/-}$-rescued mice. Left femoral artery ligation was performed in GFP-BM chimeric mice and then tissue samples were extracted from the thigh adductor muscles at 2 weeks after the ischemic injury. A, Three-dimensional study by confocal microscopy revealed that the number of the recruited GFP^{+} cells in ischemic tissue was reduced in EpoR$^{-/-}$-rescued mice compared with WT mice. B, FACS analysis showed that the increase in the number of GFP^{+} cells in ischemic tissue was significantly impaired in EpoR$^{-/-}$-rescued mice (n=6) compared with WT mice (n=10). Results are expressed as means±SD. C, Western blotting analysis showed that the expression of VEGFR-2 (Flk-1) in ischemic tissue was significantly enhanced in WT mice, but not in EpoR$^{-/-}$-rescued mice (n=5, each). D, Western blotting analysis showed that there were no significant differences in the expression of VEGFR-1 (Flt-1, n=5, each). Results are expressed as means±SD.

mice (Figure 7). Epo, when added to VEGF, further enhanced the microvessel sprouting in WT mice but not in EpoR$^{-/-}$-rescued mice (Figure 7).

Discussion

The novel finding of the present study is that vascular Epo/EpoR system plays an important role in ischemia-induced angiogenesis in mice in vivo. To the best of our knowledge, this is the first study that demonstrates the important roles of vascular EpoR system, including induction of postischemic angiogenesis, secretion of VEGF from ischemic muscle and BM-derived cells, enhancement of VEGFR-2 in ischemic tissue, and recruitment of BM-derived cells to ischemic tissue.

Epo and Angiogenesis in Local Ischemia

Tissue ischemia/hypoxia activates HIF-1, which activates Epo and enhances VEGF expression, mobilizes EPCs, and finally contributes to neovascularization.[5,7,18] Administration of exogenous Epo also augments the proliferation of stem/progenitor cells in BM and induces mobilization and proliferation of EPCs.[16,31] Although the enhancing effect of Epo has long been thought to be limited to the kidney, HIF-1-induced production of Epo is also detected in hypoxic retina.[32] However, in the present study, we were unable to detect Epo mRNA in ischemic muscle in WT or EpoR$^{-/-}$-rescued mice, and plasma levels of Epo were comparable after femoral artery ligation between the two genotypes (data not shown). By contrast, both VEGF concentrations and recruitment of BM-derived cells in ischemic muscle were significantly enhanced in WT mice, but were significantly impaired in EpoR$^{-/-}$-rescued mice. These results suggest that EpoR may be important for VEGF secretion, EPC mobilization, and angiogenesis in ischemic tissue as well as Epo in peripheral vasculature.

EpoR and Angiogenesis in Local Ischemia

Although systemic administration of Epo is known to cause angiogenesis, the role of local EpoR remains to be elucidated. However, as demonstrated in the present study (supplemental Figure I and II), hindlimb ischemia in WT mice did not modify Epo/EpoR expressions, probably because both Epo and hypoxia are required to upregulate EpoR.[15] Therefore, we used EpoR$^{-/-}$-rescued mice to evaluate the role of EpoR in hindlimb ischemia. As demonstrated in the present study, vascular EpoR plays a key role to induce angiogenesis in response to ischemia. The VEGF/VEGFR-2 system is also important to mobilize EPCs from BM as an endogenous chemotactic system for BM-derived proangiogenic CXCR4^{+} cells.[33,34] The present study demonstrates that vascular EpoR is important to upregulate the VEGF/VEGFR-2 system and to mobilize EPCs in local ischemic tissue. Even after BM transplantation, the lack of EpoR in peripheral vasculature resulted in the incomplete improvement of VEGF secretion and angiogenesis, probably because some signals through EpoR were required to induce VEGF/VEGFR-2 system in ischemic limb. The VEGF-supplemented Matrigel implantation and aortic ring assays (Figure 6 and 7) also indicate that the lack of EpoR in vasculature is responsible for the impaired angiogenesis even in the presence of VEGF, where the responses to Epo also were significantly impaired. Therefore, we consider that EpoR is, at least partially, contributed to VEGF-induced angiogenesis in the

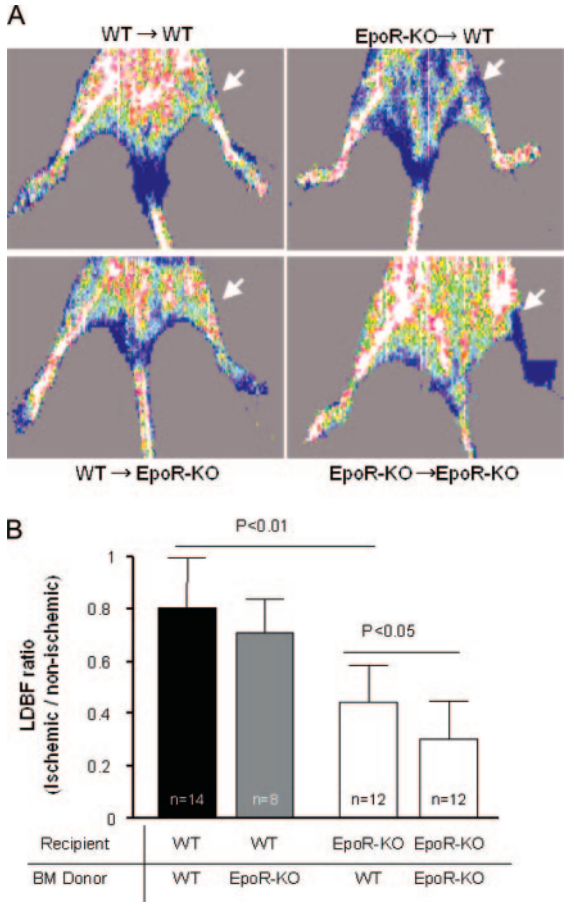

Figure 5. Blood flow recovery after BM reconstitution by WT BM cells in EpoR$^{-/-}$-rescued mice. A, Representative laser Doppler blood flow images of the hindlimbs of chimeric mice. WT→WT, WT mice reconstituted with WT-BM; EpoR$^{-/-}$-rescued→WT, WT mice reconstituted with EpoR$^{-/-}$-rescued BM; WT→EpoR$^{-/-}$-rescued, EpoR$^{-/-}$-rescued mice reconstituted with WT-BM; EpoR$^{-/-}$-rescued→EpoR$^{-/-}$-rescued, EpoR$^{-/-}$-rescued mice reconstituted with EpoR$^{-/-}$-rescued BM. Arrows indicates the ischemic left hindlimb. B, Blood flow in ischemic hindlimb measured at 14 days after femoral artery ligation. Results are expressed as a ratio of the left (ischemic) to right (nonischemic) limb blood flow, and are reported as means±SD.

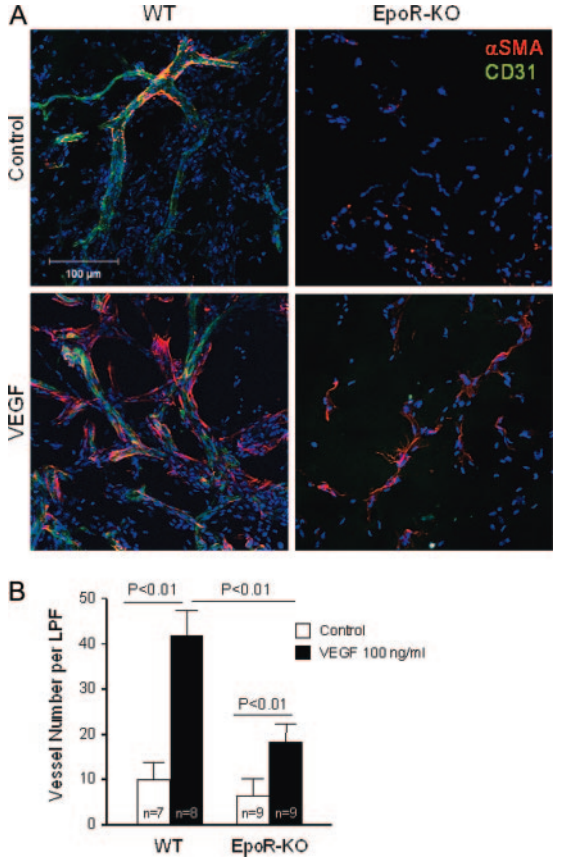

Figure 6. Impaired microvessel sprouting in EpoR$^{-/-}$-rescued mice in Matrigel implantation assay. A, Representative micrographs of vessels in the Matrigel in the presence or absence of 100 ng/mL VEGF. Scale bars, 100 μm. B, Number of sprouting vessels in the Matrigel assay in WT and EpoR$^{-/-}$-rescued mice after 2 weeks. Results are expressed as mean±SD.

present study. These results indicate that local vascular EpoR, as well as Epo, promotes postischemic angiogenesis by enhancing VEGF secretion from ischemic muscle, EPC mobilization, and recruitment of BM-derived proangiogenic cells to the ischemic tissue. The impaired angiogenesis in EpoR$^{-/-}$-rescued mice is not only because of the reduced VEGF secretion and the impaired recruitment of proangiogenic BM-derived cells, but also because of the reduced responsiveness of blood vessels to VEGF in the absence of EpoR.

Clinical Implications

It has been recently demonstrated that administration of recombinant human Epo ameliorates congestive heart failure in humans[35] and protects myocardium from ischemia/reperfusion injury in mice.[35,36] We also have recently demonstrated that the number of apoptotic myocytes is significantly increased after myocardial ischemia/reperfusion injury in EpoR$^{-/-}$-rescued mice[23] and that plasma concentrations of Epo are closely related with cardiac function in patients with acute myocardial infarction.[23,37] In the present study, we were able to demonstrate that local vascular EpoR, in addition to Epo, plays an important role to promote ischemia-induced angiogenesis. Therefore, modulation of vascular EpoR system could be a new therapeutic strategy for the treatment of ischemic cardiovascular diseases, including PAD, although further clinical investigations are needed.

Acknowledgments

We thank Kirin Brewery Co, Ltd, for providing human recombinant Epo, and Drs M. Yamamoto and N. Suzuki at Tsukuba University for providing EpoR$^{-/-}$-rescued mice and Dr N. Minegishi for valuable comments.

Sources of Funding

This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan (16209027, 16659192, 18659218), the Japanese Ministry of Health, Labor, and Welfare, Tokyo, Japan, and the Japan Foundation of Cardiovascular Research, Tokyo, Japan.

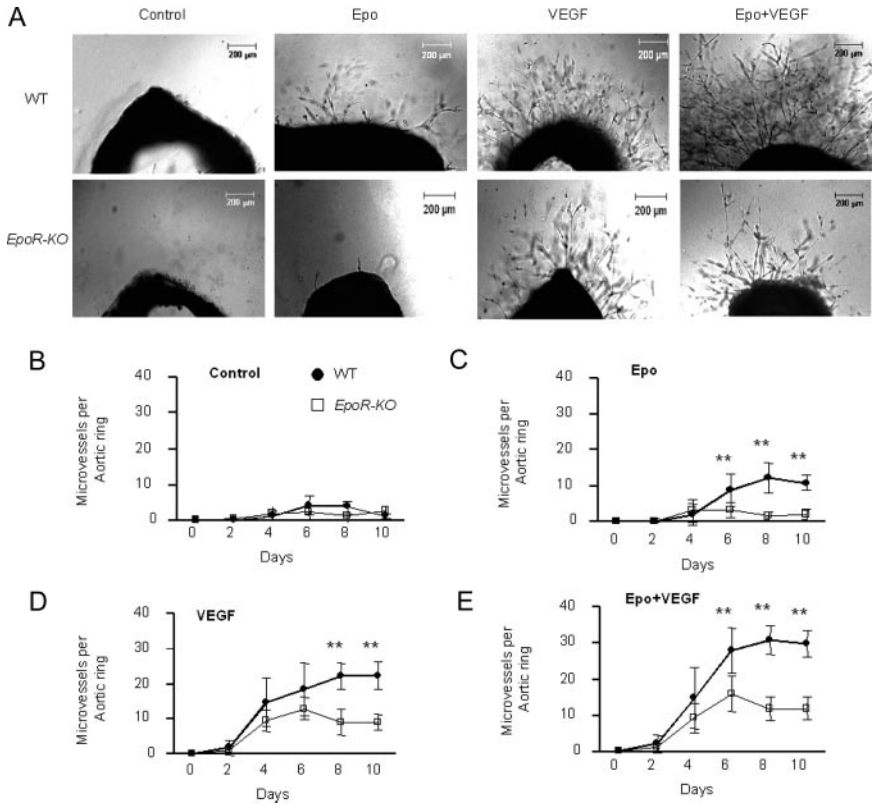

Figure 7. Impaired microvessel sprouting in aortic ring assay in EpoR$^{-/-}$-rescued mice. A, Representative micrographs of sprouting microvessels from aortic ring grown in the presence of 50 ng/mL VEGF and/or 10 IU/mL Epo. Scale bars, 200 μm. B–E, Number of the sprouting microvessels from aortic rings of WT and EpoR$^{-/-}$-rescued mice cultured in the endothelial basal medium with vehicle (control), VEGF, Epo, or VEGF+Epo (n=8 each). $^{**}P<0.01$ vs EpoR$^{-/-}$-rescued. Results are expressed as means±SD.

Disclosures

None.

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Important Role of Erythropoietin Receptor to Promote VEGF Expression and Angiogenesis in Peripheral Ischemia in Mice

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Circ Res. 2007;100:662-669; originally published online February 9, 2007;
doi: 10.1161/01.RES.0000260179.43672.fe
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571

Methods

Real time RT-PCR

Total RNA was isolated from mouse hindlimbs using SV Total RNA isolation System (Promega, WI). RNA was reverse transcribed by standard methods using ExScript RT reagent kit (Takara, Shiga, Japan). Quantitative real-time PCR was conducted using TaqMan gene expression assays (EpoR; Mm00438760_m1, β-actin; Mm00607939, Applied Biosystems, CA), according to the manufacturer's instructions. PCR thermal cycling conditions were 95°C for 10 seconds, and 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds in a total volume of 20 μl/reaction. Data were collected using a 7500 Real Time PCR System and 7500 System Software (Applied Biosystems, CA). All samples were run in triplicate, and the mean values were used for quantification. The expression level of each gene was normalized to the copies of β-actin mRNA from the same sample.

Standard curves for EpoR were generated using four serial dilutions (1/10, $1/10^2$, $1/10^3$, and $1/10^4$) of cDNA from wild type mouse bone marrow cells. Standard curves for β-actin were created from same cDNA.

ELISA Assay for Epo and eNOS

Enzyme-linked immunosorbent assay (ELISA) was performed with a mouse and rat-specific erythropoietin Quantikine kit (R&D) or human eNOS Quantikine kit (R&D), following the manufacturer's protocol. Protein extracted from muscle homogenates with the Tissue Protein Extraction Reagent (T-PER, Pierce) were standardized for total protein content using the BCA Protein Assay kit (Pierce) as previously described.[1]

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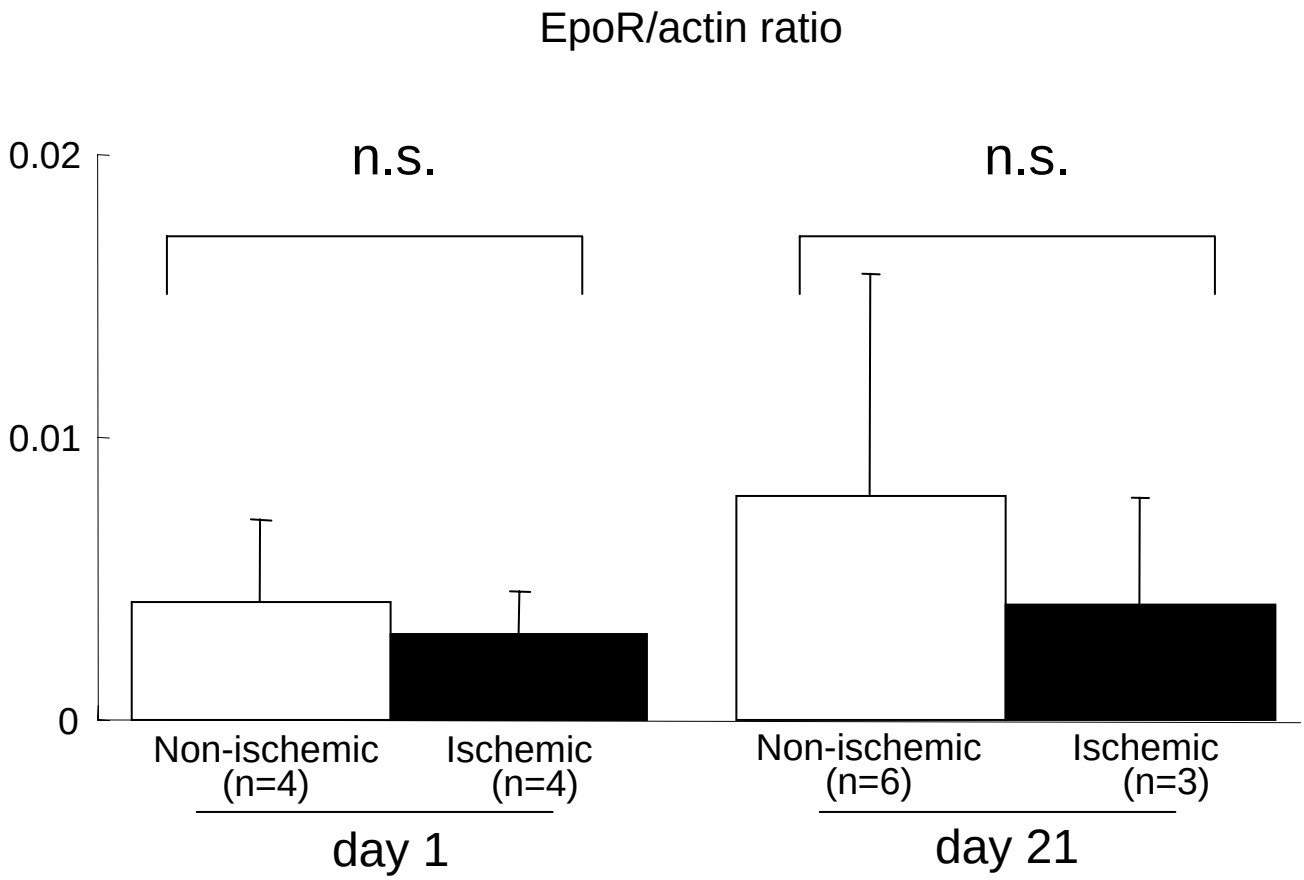
Figure legends

Online Figure I. EpoR induction in ischemic hindlimb of wild-type mice (real time RT-PCR). Hindlimb ischemia did not upregulate mRNA levels of EpoR in wild-type mice at day 1 and 21. Results are expressed as mean ± SD.

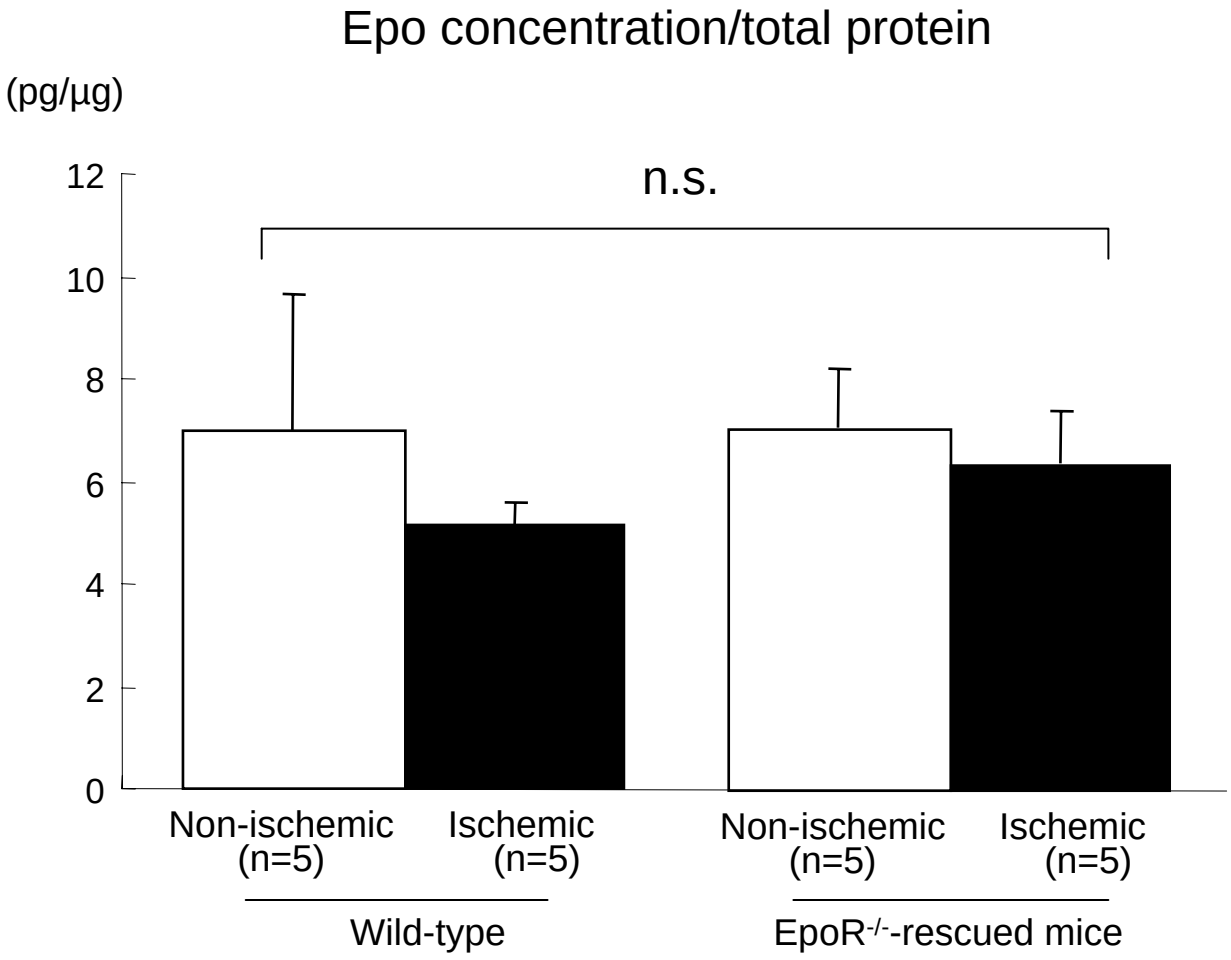
Online Figure II. Epo concentration in ischemic and non-ischemic tissues (ELISA). There were no significant differences of Epo concentrations in ischemic and non-ischemic tissues in wild-type and $EpoR^{-/-}$-rescued mice. Results are expressed as means ± SD.

Online Figure III. eNOS expression in ischemic and non-ischemic tissues (ELISA). There were no significant differences of eNOS protein levels in ischemic and non-ischemic tissues in wild-type and $EpoR^{-/-}$-rescued mice. Results are expressed as means ± SD.

Online figure I



Online figure II



Online figure III