Important Role of Endogenous Erythropoietin System in Recruitment of Endothelial Progenitor Cells in Hypoxia-Induced Pulmonary Hypertension in Mice

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Background—Recent studies have suggested that endogenous erythropoietin (Epo) plays an important role in the mobilization of bone marrow–derived endothelial progenitor cells (EPCs). However, it remains to be elucidated whether the Epo system exerts protective effects on pulmonary hypertension (PH), a fatal disorder encountered in cardiovascular medicine.

Methods and Results—A mouse model of hypoxia-induced PH was used for study. We evaluated right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary vascular remodeling in mice lacking the Epo receptor (EpoR) in nonerythroid lineages (EpoR−/− rescued mice) after 3 weeks of exposure to hypoxia. Those mice lack EpoR in the cardiovascular system but not in the hematopoietic system. The development of PH and pulmonary vascular remodeling were accelerated in EpoR−/− rescued mice compared with wild-type mice. The mobilization of EPCs and their recruitment to the pulmonary endothelium were significantly impaired in EpoR−/− rescued mice. By contrast, reconstitution of the bone marrow with wild-type bone marrow cells ameliorated PH in the EpoR−/− rescued mice. Hypoxia enhanced the expression of EpoR on pulmonary endothelial cells in wild-type but not EpoR−/− rescued mice. Finally, hypoxia activated endothelial nitric oxide synthase in the lungs in wild-type mice but not in EpoR−/− rescued mice.

Conclusions—These results indicate that the endogenous Epo/EpoR system plays an important role in the recruitment of EPCs and prevents the development of PH during chronic hypoxia in mice in vivo, suggesting the therapeutic importance of the system for the treatment of PH. (Circulation. 2006;113:1442-1450.)

Key Words: hypertension, pulmonary ▪ hypoxia ▪ endothelium ▪ vasculature ▪ remodeling

Erythropoietin (Epo) has long been regarded as a hypoxia-induced hormone that acts exclusively in the proliferation and differentiation of erythroid progenitors. However, recent studies have demonstrated the expression of the Epo receptor (EpoR) in the cardiovascular system, and the therapeutic potential of Epo has been noted in a variety of disorders, including cerebral infarction, myocardial ischemia/reperfusion, and congestive heart failure. We also have recently demonstrated the protective role of endogenous Epo in patients with acute myocardial infarction. However, the potential protective role of the endogenous Epo/EpoR system against pulmonary hypertension (PH) remains to be examined.

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Hypoxia has been considered to increase plasma levels of Epo and hematocrit, resulting in enhanced blood viscosity and PH. However, exogenous Epo does not accelerate hypoxia-induced PH in rats. Rather, Epo exerts direct protective effects on endothelial cells and enhances the mobilization of endothelial progenitor cells (EPCs) and their proliferative and adhesive capacity, thus promoting endothelial repair and postnatal vasculogenesis. Pulmonary endothelium is important in the maintenance of pulmonary vasculature, whereas endothelial dysfunction accelerates pulmonary vascular remodeling in hypoxia-induced PH. Therefore, it is conceivable that Epo has protective...
effects on pulmonary endothelial cells and inhibits the development of PH.

Hypoxia induces vascular endothelial growth factor (VEGF) and Epo, thus promoting mobilization of EPCs and their homing to the ischemic tissue. Recent studies have shown that intravenously injected EPCs home to pulmonary vasculature and ameliorate pulmonary vascular remodeling in monocrotaline-induced PH, demonstrating a potential role of exogenous EPCs as a source of pulmonary endothelial cells in vivo. In the present study, we thus examined our hypothesis that the endogenous Epo/EpoR system plays an important protective role against the development of hypoxia-induced PH. For this purpose, we used EpoR-null mutant mice expressing EpoR exclusively in the erythroid lineage (EpoR-rescued mice).

Methods

Animal Preparation
All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, Sendai, Japan. Because systemic deletion of EpoR is embryo-lethal, we rescued these mice with the EpoR that is exclusively expressed in erythroid progenitor cells under the regulatory domain of globin transcription factor 1 (GATA-1) (EpoR-rescued mice). The detailed procedures used to generate these mice were as follows. First, EpoR cDNA was ligated to a genomic fragment containing the hematopoietic regulatory domain of GATA-1 (GATA-1–HRD), which regulates the expression of EpoR in the hematopoietic progenitors. Second, these GATA-1–HRD-EpoR constructs were applied to produce transgenic mice, which were mated with EpoR-rescued mice to establish the compound mutant EpoR-rescued mice: GATA-1–HRD-EpoR. Finally, EpoR-rescued mice were generated by crossing these compound mutant mice and EpoR-rescued mice. The EpoR was expressed under the control of an erythroid-specific promoter in the EpoR-rescued mice; therefore, the expression was limited to the erythroid-lineage cells. By contrast, endogenous expression of EpoR mRNA was detected in most of the nonerythroid tissues in wild-type mice. Thus, EpoR-rescued mice are characterized by the absence of EpoR in the cardiovascular system but a normal hematopoietic system. In the present study, a total of 120 male wild-type (C57BL/6) and 100 EpoR-rescued male mice (8 weeks old) were used. For the assessment of PH and histological evaluations, wild-type and EpoR-rescued mice were divided into 2 groups each (n=10 each for the 4 groups). One group was maintained in room air (21% O₂), and the other group was exposed to hypoxia (10% O₂) for 3 weeks. For Kaplan-Meier analysis, additional groups of mice were used (n=15 each for the 4 groups).

Bone Marrow Transplantation
Transgenic mice constitutively expressing green fluorescent protein (GFP) under the transcriptional regulation of an endothelial cell–specific promoter (Tie2-GFP mice) were obtained from Jackson Laboratory (Bar Harbor, Me). Bone marrow transplantation was performed as previously described. Briefly, recipient mice were lethally irradiated and received an intravenous injection of 5×10⁶ donor bone marrow cells suspended in 100 μL calcium- and magnesium-free phosphate-buffered saline with 2% fetal bovine serum (FBS). Six weeks after the transplantation, these mice were transferred to hypoxic chambers (10% O₂) and were maintained for 3 weeks as previously described.

Measurements
Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). Right ventricular systolic pressure (RVSP) was measured by insertion of a 25-gauge needle connected to a pressure transducer. Hematocrit was measured by use of an automatic hemocytometer (Nihon-Kohden, Tokyo, Japan). For morphometric analysis, tissue sections were prepared from the formalin-fixed and paraffin-embedded left lung, stained with elastica Masson, and assessed by microscopy. Pulmonary arteries adjacent to an airway distal to the respiratory bronchiole were evaluated as previously reported in a modified protocol. Briefly, the arteries were considered muscularized if they had a distinct double elastic lamina visible for at least half the diameter in the vessel cross section. The percentage of vessels with double elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. In each section, a total of 60 vessels were examined by use of a computer-assisted imaging system (DMX1200 with ACT-1 software, Nikon, Tokyo, Japan). This analysis was performed separately for different categories (external diameters, 25 to 60 μm and 60 to 100 μm). For immunohistological staining, monoclonal antibody to α-smooth muscle actin (aSMA, 1:300, DAKO, Denmark) was used as the primary antibody.

Immunofluorescence Staining
Immunofluorescence staining was performed on 4% paraformaldehyde–fixed frozen sections. The primary antibodies used were anti-EpoR (1:50, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), anti-mouse CD31 (1:400, BD PharMingen, San Diego, Calif), and anti-GFP (1:1000, Molecular Probes). Subsequently, Alexa Fluor 488– or Alexa Fluor 594–labeled secondary antibodies and Prolong antifade reagent with DAPI (Molecular Probes) were used. As a negative control, species- and isotype-matched immunoglobulin G was used in place of the primary antibody. Slides were viewed with a confocal fluorescence microscope (Fluoview FV1000, Olympus, Tokyo, Japan).

Flow Cytometry and EPC Culture Assay
Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Nycoprep Animal 1.077 (Axis-Shield). To quantify the number of Flk-1+CD133+ cells, we used phycoerythrin-labeled anti-mouse Flk-1, FITC-labeled anti-mouse CD133 (eBioscience, San Diego, Calif), and biotinylated anti-mouse lineage antibodies (Mac-1, Gr-1, B220, CD4, CD8, and Ter119, BD PharMingen). Quantitative analysis was performed by a fluorescence-activated cell sorter (FACScalibur, Becton Dickinson, San Jose, Calif). For EPC culture, isolated PBMCs (5×10⁶ cells per well) were suspended in Medium-199 supplemented with 20% FBS, brain pituitary extract, antibiotics, 100 ng/mL VEGF, and 50 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, Minn) and cultured on fibronectin-coated chamber slides (BioCoat, Becton Dickinson) for 7 days. To confirm the incorporation of 1,1'-diocotadecyl-3,3',3''-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiI-AcLDL), cultured cells were incubated in medium containing 2 μg/mL DiI-AcLDL (Molecular Probes) for 3 hours at 37°C. Endothelium-like cells were identified by the uptake of DiI-AcLDL and Flk-1 expression. The expression of EpoR on endothelium-like cells was confirmed by double staining with biotinylated anti-mouse EpoR antibodies (1:50, R&D Systems) and anti-Flk-1 (VEGF receptor-2, 1:200, Santa Cruz Biotechno-

Western Blotting
Cell lysates from lung 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 EpoR (Santa Cruz Biochemicals), endothelial nitric oxide synthase (eNOS, BD Biosciences), and β-actin (Cell Signaling, Danvers, Mass). Signals were visualized by the ECL detection system (Amersham Biosciences, Uppsala, Sweden).
Statistical Analysis

Quantitative results were expressed as mean±SD. Comparisons of parameters among the 2 or 3 groups were made by 1-way analysis of variance, and comparisons of the different oxygen conditions of parameters between the 2 genotypes were made by 2-way analysis of variance, followed by a post hoc analysis using the Bonferroni test. The Kaplan-Meier method was used to make survival curves, and the survival curves were compared by use of the log-rank test. A value of *P*<0.05 was considered to be statistically significant. Statistical analyses were performed by use of the StatView statistical package (StatView 5.0, SAS Institute Inc).

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the article as written.

Results

**EpoR Deficiency Promotes PH and Pulmonary Vascular Remodeling**

Under normoxic conditions, RVSP and right ventricular hypertrophy (RVH) were comparable between wild-type and EpoR−/− rescued mice (Figure 1A–1C). However, after the exposure to hypoxia (10% O₂ for 3 weeks), RVSP was significantly higher and RVH was more accelerated in EpoR−/− rescued mice (Figure 1A through 1C), whereas the hematocrit was comparable between the 2 groups (Figure 1D). In addition, survival was significantly impaired in EpoR−/− rescued mice under chronic hypoxia (Figure 1E). In both groups, most animals that died showed ascites, RVH, and dilatation, suggesting that the main cause of death was right ventricular failure.

Histological examination showed that in wild-type mice, chronic hypoxia caused small-vessel muscularization, whereas in EpoR−/− rescued mice (although the extent of muscularization under normoxia was similar to that found in wild-type mice), the hypoxia-induced muscularization was significantly accelerated (Figures 2 and 3). Immunostaining of lung sections showed that the hyperplastic cell population consisted of smooth muscle cells, inasmuch as they expressed αSMA (Figure 2).

**Impaired Mobilization of EPCs in EpoR−/− Rescued Mice**

To compare the effect of hypoxia on the mobilization of EPCs between wild-type and EpoR−/− rescued mice, fluorescence-activated cell sorter analysis was performed in the peripheral blood. The number of EPCs, when defined as Flk-1+/CD133+ cells in PBMCs, was significantly increased under hypoxic conditions in wild-type mice but not in EpoR−/− rescued mice (Figure 4A). Similarly, hypoxia significantly increased the number of Dil-AcLDL+/Flk-1+ endothelium-like cells cultivated from PBMCs in wild-type mice but not in EpoR−/− rescued mice (Figure 4B). Importantly, cultivated endothelium-like cells from wild-type mice expressed EpoR; however, this was not the case for the cells from EpoR−/− rescued mice (Figure 4C). To further confirm the incorporation of bone marrow–derived endothelium-like cells into the pulmonary vessels, endothelium-like cells cultivated from mononuclear cells were intravenously injected into the wild-type mice that had already been exposed to hypoxia (10% O₂) for 7 days. After additional exposure to hypoxia for 7 days, incorporation of the injected cells into the pulmonary endothelium was assessed by confocal micros-
copy (Figure 4D). Actual incorporation of infused cells from wild-type and EpoR$^{-/-}$ rescued mice into pulmonary endothelial cells was confirmed by immunostaining for CD31. Importantly, the number of the CD31-positive cells migrating to the pulmonary endothelium was significantly less in the cells from EpoR$^{-/-}$ rescued mice than in cells from wild-type mice (Figure 4E).

**Bone Marrow–Derived EPCs Incorporated Into Pulmonary Endothelium Inhibit Development of PH**

To confirm the incorporation of bone marrow–derived EPCs into pulmonary vessels in vivo, wild-type mice were lethally irradiated, transplanted with bone marrow cells from Tie2-GFP transgenic mice, and bred under hypoxic conditions. After 3 weeks of hypoxia, we found that GFP-positive endothelial cells were incorporated into the pulmonary endothelium (Figure 5A). The number of the GFP-positive endothelial cells that were incorporated into the pulmonary endothelium was significantly increased by hypoxic exposure compared with control conditions (Figure 5B).

To further examine whether the impaired mobilization and incorporation of bone marrow–derived EPCs in EpoR$^{-/-}$ rescued mice was involved in the development of severe PH,
EpoR<sup>−/−</sup> rescued mice were lethally irradiated and then transplanted with bone marrow cells from wild-type or EpoR<sup>−/−</sup> rescued mice. As a control, wild-type mice reconstituted with wild-type bone marrow cells were prepared. After 6 weeks, these mice were exposed to hypoxia for 3 weeks. Hemodynamic analysis revealed that the development of PH, when assessed by RVSP and RVH, was partially ameliorated in EpoR<sup>−/−</sup> rescued mice transplanted with wild-type bone marrow cells (Figure 5C). Furthermore, the degree of pulmonary vessel muscularization (diameter, 25 μm to 60 μm) was also ameliorated in this group, whereas hematocrit values were comparable among the 3 groups (Figure 5D).

**Impaired Response of Pulmonary Endothelial Cells in EpoR<sup>−/−</sup> Rescued Mice**

To further elucidate any other mechanism that promotes PH in EpoR<sup>−/−</sup> rescued mice, we focused on the response of pulmonary endothelial cells to hypoxic exposure. The expression of EpoR was absent in pulmonary endothelial cells from EpoR<sup>−/−</sup> rescued mice, whereas the expression was enhanced by hypoxic exposure in wild-type mice (Figure 6). Moreover, because chronic hypoxia has been reported to increase eNOS protein in rat lungs, we evaluated the expression of eNOS. After 3 weeks of hypoxia, lung eNOS protein expression was increased in wild-type mice but not in EpoR<sup>−/−</sup> rescued mice (Figure 7).

**Discussion**

The novel finding of the present study is that the mobilization of EPCs from the bone marrow and their incorporation into the pulmonary endothelium are impaired in EpoR<sup>−/−</sup> rescued mice, with a resultant potentiation of PH and pulmonary vascular remodeling in response to chronic hypoxia. To the best of our knowledge, this is the first study demonstrating that the endogenous Epo/EpoR system exerts protective effects on pulmonary endothelium and its progenitors and inhibits the development of hypoxia-induced PH.

**Epo Mobilizes EPCs and Repairs Pulmonary Endothelium**

In the present study, ex vivo–cultivated peripheral blood–derived EPCs showed the expression of EpoR in wild-type mice but not in EpoR<sup>−/−</sup> rescued mice. The lack of an effect of Epo may involve the progression of pulmonary vascular...
remodeling that is due to impairment in the mobilization of EPCs and their recruitment to the injured pulmonary endothelium. This notion is supported by the present in vivo findings. Indeed, mobilization of EPCs, defined as Flk-1+/CD133+ cells in the peripheral blood and EPC culture assay, was impaired in EpoR−/− rescued mice. Intravenously infused bone marrow–derived EPCs were incorporated into the pulmonary endothelium; this process was significantly impaired in the EPCs from EpoR−/− rescued mice. Moreover, GFP-positive cells were incorporated into the pulmonary endothelium in chimeric mice that were reconstituted with bone marrow cells from Tie2-GFP transgenic mice. The expression of GFP is regulated by the Tie2 promoter and therefore identifies bone marrow–derived differentiated endothelial cells and EPCs. These findings are in line with findings in previous studies showing that circulating EPCs contribute to the endogenous vascular repair process and play an important role in inhibiting vascular remodeling. In addition, Davie et al demonstrated the incorporation of c-kit–positive progenitor cells into the pulmonary vessels under hypoxic conditions. Although the number of bone marrow–derived endothelial cells in the lung field was relatively small (~3% to 5%) compared with that in the hind-limb ischemia or tumor angiogenesis model, bone marrow–derived cells are likely to contribute to pulmonary endothelial repair not only by incorporating into the endothelial cells but also by improving the functions of the resident pulmonary endothelial cells through paracrine effects. EPCs also are reported to express eNOS and produce nitric oxide (NO). Indeed, the concept that bone marrow–derived endothelial cells could repair the pulmonary endothelium and ameliorate PH, especially through paracrine effects, has recently been proposed. By contrast, defective mobilization and recruitment of EPCs in EpoR−/− rescued mice may lead to the potentiation of PH in response to chronic hypoxia. Indeed, it has been recently reported that the number of circulating EPCs is associated with endothelial dysfunction in humans. Taken together, Epo may serve as a cytokine that elicits EPC mobilization, promoting the repair process of the injured pulmonary endothelium and thus inhibiting the development of PH.

Epo Has Direct Protective Effects on Pulmonary Endothelial Cells

Antiinflammatory and antiapoptotic effects on pulmonary endothelial cells have been shown to prevent the development of PH in rats. In line with these findings, we observed that lack of EpoR expression on pulmonary endothelium accelerates pulmonary vascular remodeling in EpoR−/− rescued mice; this occurrence may be partly due to the lack of direct effects of Epo on pulmonary endothelial cells. By contrast, the expression of EpoR was upregulated by hypoxia in wild-type mice. A similar observation has been previously reported with regard to human umbilical vein endothelial cells in vitro, where EpoR expression was induced by Epo and hypoxia. Moreover, parallel increases in eNOS expression and NO production in response to Epo were observed during hypoxia. An increase in the plasma level of Epo under hypoxic conditions combined with an upregulation of EpoR on pulmonary endothelial cells may augment the endothelial responses. In addition, the induction of eNOS and increased endothelial NO production by Epo may allow the pulmonary vasculature to recover blood flow and ameliorate PH. Importantly, these endothelial responses were absent in EpoR−/− rescued mice with accelerated PH and pulmonary vascular remodeling. Taken together, Epo has direct effects on pulmonary endothelial cells in addition to indirect effects on the recruitment of cells from bone marrow. All these effects of Epo may help to protect and maintain endothelial function and to inhibit pulmonary vascular remodeling under chronic hypoxia.
Limitations of the Present Study
Several limitations should be mentioned for the present study. First, the hypoxia-induced PH model may not fully represent primary PH in humans because this model shows a considerably high hematocrit and blood viscosity. Epo has been thought to increase these parameters, with a resultant development of PH. However, in the present study, RVSP and RVH were accelerated in EpoR−/− rescued mice in response to chronic hypoxia compared with wild-type mice, despite the comparable increase in hematocrit between the 2 strains. Furthermore, it has previously been shown that pulmonary arterial pressure and RVH are not increased in Epo-treated rats despite a significant increase in hematocrit and blood viscosity. Taken together, these results suggest that hypoxia-induced PH cannot be explained by simple polycythemia. Second, the mechanisms for the beneficial effects of Epo have been examined in only 1 model (hypoxia-induced PH) by comparing wild-type and EpoR−/− rescued mice. Thus, the importance of the endogenous Epo/EpoR system should be confirmed in other PH models with different etiologies (eg, bone morphogenetic protein receptor-2 [BMPR2]−heterozygous mutant [BMPR2+/−] mice), although the same mechanisms for the protective effects of the Epo/EpoR system may be expected, especially when nonerythropoietic derivatives of Epo are used.

Clinical Implications and Conclusions
In the present study, we were able to demonstrate that the endogenous Epo/EpoR system exerts important protective effects against the development of hypoxia-induced PH through mobilization of bone marrow–derived EPCs and stimulation of preexisting pulmonary endothelial cells. This is in line with our recent finding of the protective role of the endogenous Epo system in patients with acute myocardial infarction. Indeed, recent studies have shown that Epo increases the number of EPCs in humans and that Epo has an ischemia-induced angiogenic potential during retinal angiogenesis in diabetic patients. Therefore, the present findings suggest that the therapeutic use of Epo might be useful for the treatment of human diseases. However, further careful considerations should be made before Epo is applied in the clinical setting.

In conclusion, the present study demonstrates an important protective role of the endogenous Epo/EpoR system in the mobilization and incorporation of EPCs into the pulmonary endothelium and in the maintenance of pulmonary endothelial integrity in the pathogenesis of hypoxia-induced PH.

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Disclosures
None.

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Sato et al. Erythropoietin and Pulmonary Hypertension


**CLINICAL PERSPECTIVE**

Pulmonary hypertension (PH) remains a fatal disorder encountered in cardiovascular medicine. Causes of the disorder include congestive heart failure, congenital heart disease, collagen disease, and primary PH. The increased pulmonary vascular resistance in PH is caused by the combined effects of enhanced pulmonary vascular tone, medial smooth muscle hypertrophy, and intimal thickening. Accumulating evidence indicates that vascular endothelial dysfunction is a key mechanism for the disorder. Erythropoietin (Epo) has long been thought to act exclusively on hematopoietic cells. However, recent studies have suggested that Epo also possesses several biological actions that protect vascular wall cells (including endothelial cells) that express the Epo receptor (EpoR). The endogenous Epo/EpoR system has been considered to play a role in the pathogenesis of PH in conjunction with polycythemia, with a resultant increase in pulmonary vascular resistance. However, our present study provides the novel concept that the pulmonary vascular Epo/EpoR system protects pulmonary endothelial cells directly (through the Epo/EpoR system) and indirectly (through the mobilization of bone marrow–derived endothelial progenitor cells), thus promoting pulmonary endothelial protection and repair. Our present study may have important clinical implications, inasmuch as we were able to demonstrate that the endogenous Epo/EpoR system exerts protective effects in PH and that Epo could be regarded as a new therapeutic tool for the treatment of the disorder. However, further studies are needed to confirm the therapeutic usefulness of Epo for the treatment of PH in humans.
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