Prevention of Erythropoietin Hypertension

Mary S. Lee, John S. Lee, Jong Y. Lee

Abstract—Hypertension is the most significant complication from treatment with erythropoietin (Epo). Can Epo-induced hypertension be eliminated? We examined systemic and local effects of our genetically engineered products, Epo-binding protein (Epo-bp) and anti–Epo-bp antibodies, on randomly assigned Sprague–Dawley rats at midnight, 4 AM, 8 AM, noon, 4 PM, and 8 PM. Blood pressure, hematocrit, and body weight were measured immediately before and after the completion of a 4-week, twice-weekly course of Epo (50 U/kg), Epo-bp, anti–Epo-bp antibodies, or physiological saline injections. Epo treatment increased hematocrit markedly overall as compared with the saline, Epo-bp, and anti–Epo-bp antibody groups (0.616 versus 0.427, 0.439, and 0.441, respectively) and at each of the 6 test times (all \( P < 0.0001 \)). Epo-bp and anti–Epo-bp antibody treatment with Epo had almost no effect on the Epo-induced hematocrit increase (0.616 versus 0.580 or 0.591, respectively). Circadian blood pressures for Epo versus saline, Epo-bp, and anti–Epo-bp antibody groups were 136.2±2.3 versus 116.2±1.7, 118.4±2.1, and 116.6±2.1 mm Hg, respectively (each \( P < 0.0001 \)). Significantly increased blood pressure was detected at noon, 4 PM, 8 PM, and midnight in Epo treatment. When Epo was given with Epo-bp or anti–Epo-bp antibodies, blood pressure was maintained at similar levels as in saline treatment (each \( P < 0.0001 \)) as compared with Epo treatment alone. Overall, body, brain, and heart weights were significantly lower in Epo treatment than those of other groups. Thus, Epo-bp and anti–Epo-bp antibodies eliminate Epo-induced hypertension without affecting hematocrit and blood volume. (Hypertension. 2007;50:439-445.)

Key Words: erythropoiesis ■ erythropoietin-binding protein ■ circadian effects ■ hypertension ■ splenomegaly ■ cardiovascular parameters

Erythropoietin (Epo) and Epo receptor are required for definitive erythropoiesis and progenitor cell maturation. Epo is secreted in response to hypoxia to coordinate erythropoiesis as a primary inducer and regulator of red cell differentiation by suppressing apoptosis and triggering cell division and terminal maturation of blood cell progenitors.\(^1,2\) These effects are mediated through the binding of Epo to specific cell surface receptors.\(^3\)

Epo receptor is a member of the hematopoietic/cytokine/growth factor receptor family, which includes several other growth factor receptors, interleukin-3, -4, and -6 receptors; the granulocyte macrophage colony stimulating factor receptors; and the prolactin and growth hormone receptors.\(^4\) The mechanism of Epo interaction with its receptor in the regulation of erythropoiesis or thrombopoiesis remains obscure, because characterization of the Epo receptor has been difficult because of the extremely small quantities of naturally obtainable Epo receptor.\(^5\) Recently, the mechanism involved in erythropoiesis has become of great interest in understanding the role of growth factors and their receptors in leukemogenesis; altered hematopoietic growth factors and their receptors may contribute to tumorigenesis\(^6\) and leukemogenesis.\(^7-9\)

The function of Epo may exist beyond hematopoietic tissues. Epo receptors exist in the paracrine and autocrine, as well as the hormonal systems. Some studies assert that Epo and Epo receptor exist in the human brain, including astrocytes, microglia, and neurons of the central nervous system.\(^10\) Recent studies reported that Epo has multiple effects as a neurotrophic, antiapoptotic, antioxidant, and angiogenic agent.\(^11,12\) Thus, the effects of Epo are likely to extend beyond its role on hematocrit.\(^13\) Furthermore, no species barrier exists between human and mouse Epo receptors.\(^14\)

Hypertension is the most frequent and most significant complication in Epo treatment. A rise in blood pressure or a need for augmentation of antihypertensive medications is demonstrated in approximately one third of Epo-treated patients.\(^12,15-17\) Although the goal of Epo treatment is to increase hematocrit and hemoglobin, it has shown that the greater the increase in hematocrit with recombinant Epo (Epoetin) treatment, the greater the risk of mortality and cardiovascular events.\(^17\) This may be because of increased blood pressure, because the extent of the rise in blood pressure has been shown to correlate with the increased hematocrit. In fact, the Epoetin label warns that patients with uncontrolled hypertension should not be treated with Epoetin.\(^17,18\)

The purpose of this study was to examine systemic and local effects of Epo-binding protein (Epo-bp) and anti-
Epo–bp antibodies (αEpo-bp). The present study examines the effects of Epo, Epo-bp, and αEpo-bp on circadian blood pressure, hematocrit, and other end organs. Genetically engineered protein Epo (Epoetin from Amgen Co) has been widely used in various patient populations after its initial approval in 1989 by the Food and Drug Administration. Because numerous serious adverse effects of Epo use have been reported, including uncontrollable blood pressure rise, Epo-bp was genetically engineered to examine any reverse effect on the adversity of Epo without affecting the original purpose of hematopoiesis. We also developed antibodies against Epo-bp to test its effects on the adverse effects of Epo.

**Experimental Procedures**

**Materials**

Glutathione agarose was purchased from Pharmacia. Isopropylthio-β-d-galactoside was from BRL Gibco. Thrombin, PMSF, diisopropylfluorophosphate, Triton X-100, and 2,7-dichlorofluorescein were from Sigma. Avidin–horseradish peroxidase and IgG purification kits were from Pierce Co. Pure human Epo-bp, sheep αEpo-bp, and Fab αEpo-bp derived from pJYL26 were prepared in our laboratory. All of the other chemicals were of reagent grade.

**Animals**

Adult Sprague–Dawley rats were housed at the university animal facilities with a light cycle from 4:00 AM to 6:00 PM and standard rat chow with freely accessible drinking water. To seek an effective treatment time, 5-week-old Sprague–Dawley rats were randomly assigned to physiological saline as control or various treatment groups, each group consisting of 6 subgroups in 6 test times at assigned to physiological saline as control or various treatment groups of thrombin between the glutathione S-transferase carrier and Epo receptor polypeptide. After cleaving off the foreign polypeptide glutathione S-transferase, Epo-bp was purified by Epo-affinity chromatography and verified on a 12.5% SDS polyacrylamide gel and Western blot. Binding of Epo to Epo-bp was specific in nanomolar concentrations, and preincubated Epo-bp with unlabeled Epo eliminated labeled Epo's β-chain.

**Methods**

Human Epo-receptor recombinant vector pJYL26 expressed in pGEX-2T cloned and then transformed into the Escherichia coli strain JM 109. Epo-bp was purified from a recombinant fusion protein with the thrombin-cleavable Epo receptor extracellular domain (EpoRex-th) produced from pJYL26, which was constructed with the thrombin cleavage site at the site of the cleavage-recognizing amino acid sequences of the site-specific protease thrombin between the glutathione S-transferase carrier and Epo receptor polypeptide. After cleaving off the foreign polypeptide glutathione S-transferase, Epo-bp was purified by Epo-affinity chromatography and verified on a 12.5% SDS polyacrylamide gel and Western blot. Binding of Epo to Epo-bp was specific in nanomolar concentrations, and preincubated Epo-bp with unlabeled Epo eliminated labeled Epo's β-chain.

We developed αEpo-bp in sheep inoculated with Epo-bp every 3 to 4 weeks for 3 months. After the inoculation, serum antibodies were purified. The antibodies were further purified for Fab fractions only, which were fluorescein labeled according to the manufacturer's description. Because anti-Epo-bp antibodies were developed in sheep, Fab fraction was purified to restrict nonspecific multibinding sites of sheep IgG. These materials were used to detect ligand-binding sites in bone marrow cells and/or tissue samples, which were analyzed under an inverted fluorescence microscope. Fab αEpo-bp showed specific binding, and the binding sites were visualized. The Fab αEpo-bp was used in the present study.

**Statistics**

Data were analyzed by 2-tailed Student t test, the cosinor method, and the linear least-square rhythmometry, allowing variation as a function of the data. Data are expressed as mean±SEM. A P<0.05 was considered statistically significant.

**Results**

As shown in Table 1, before treatment, the intergroup differences for blood pressure, hematocrit, and BW in all of the treatment groups were not statistically significant. The reference circadian mean blood pressure in Epo versus the saline, Epo-bp, and αEpo-bp (Fab αEpo-bp) groups before treatment was not statistically significant (87±2.8 versus 88.8±3.4, 88.7±2.5, and 84.3±2.3 mm Hg, respectively). The reference circadian hematocrit fractions averaged in the range of 0.36 to 0.37, and the mean BW of the rats was ~80 g in each group. Overall, BW was lowered by Epo as compared with the saline group (295 versus 313 g; P<0.01).

After treatment, the circadian mean blood pressure (midline estimating statistic of rhythm [MESOR]) was significantly increased in the Epo-treated group. Epo treatment increased MESOR markedly as compared with the other 5 groups, 136.2±2.3 mm Hg in Epo versus 116.2±1.7 mm Hg in control, 118.4±2.1 mm Hg in Epo-bp, and 116.6±2.1 mm Hg in αEpo-bp treatments (each P<0.0001; Table 1 and Figure 1). When Epo-bp or αEpo-bp was given along with Epo, however, blood pressure was maintained at levels similar to that of the saline control group: 118.3±1.7 mm Hg in the Epo-bp plus the Epo-treated group and 121.0±2.0 mm Hg in the αEpo-bp plus the Epo-treated group, which were significantly lower than that of the Epo-treated group (136.2±2.3 mm Hg; each P<0.0001). Figure 1 shows circadian fluctuations of MESOR, amplitude, and acrophase (peak time) in each treatment group. As described earlier, Epo treatment increased MESOR significantly in comparison with all of the other groups, though all of the group amplitude comparisons were not significantly different. After treatment, the peak time in the Epo-treated group rats was shifted to the daytime, as compared with the control, Epo-bp-, and αEpo-bp–treated groups (7:40 PM versus 4:08 AM, 5:44 AM, and 5:16 AM, respectively). It is an obvious shift change, from a night to daytime peak, with Epo treatment in this nocturnal animal. When Epo-bp or αEpo-bp was given together with Epo, the shift change remained in the same daytime range, as seen in the Epo-alone treatment group (2:48 PM and 7:20 PM, respectively), though MESORs of the Epo-bp plus Epo and αEpo-bp plus Epo groups were similar to that of the control group.

Epo treatment increased hematocrit markedly overall as compared with the control, Epo-bp-, and αEpo-bp–treated groups (0.616 versus 0.427, 0.439, and 0.441, respectively; each P<0.0001; Tables 1 and 2). Epo-bp or αEpo-bp treatment with Epo had almost no effects on the Epo-induced
Epo-bp, and 0.85 in grams overall was 1.58 in Epo versus 0.86 in saline, 0.89 in Epo-bp or 0.591 in Epo plus Epo-bp and 0.591 in Epo plus αEpo-bp treatment), whereas both Epo-bp and αEpo-bp almost eliminated the rise of Epo-induced blood pressure (136.2 mm Hg in Epo versus 116.2 mm Hg in saline, 118.3 mm Hg in Epo plus Epo-bp, and 121.0 mm Hg in Epo plus αEpo-bp treatments). Thus, both Epo-bp and αEpo-bp protected the rats from the blood pressure rise caused by Epo-treatment. Splenomegaly characterized each rat in the Epo-treated group: spleen weight in grams overall was 1.58 in Epo versus 0.86 in saline, 0.89 in Epo-bp, and 0.85 in αEpo-bp (each \(P<0.0001\); Table 1 and Figure 2).

Table 1 summarizes circadian mean weights for the brains and hearts, which were significantly lower in the Epo-treated group as compared with the other groups. In BW-adjusted heart weight comparisons, the overall Epo-treated heart weight was still significantly lower than those of other groups, presented as HW/BW in Table 1. The aorta and kidney weights were similar in each group. Table 2 summarizes the circadian variations of blood pressure, hematocrit, and spleen weight in the 6 subgroups after Epo, Epo-bp, and αEpo-bp treatments. The BW difference between Epo-treated rats and any other current treatment group was not statistically significant in the 6 test time comparisons (data not

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**Table 1. Overall Effects on Circadian Body Weight, Blood Pressure, Hematocrit, and Other End-Organ Systems in Various Treatments**

<table>
<thead>
<tr>
<th>Group (Rx), All Groups n=30</th>
<th>y Before Rx</th>
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<tbody>
<tr>
<td></td>
<td>BW, g</td>
<td>BP, mm Hg</td>
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<tr>
<td>Control (saline) vs</td>
<td>80.1±1.7</td>
<td>88.8±3.4</td>
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<td>Epo</td>
<td>80.2±1.4</td>
<td>87.1±2.8</td>
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<tr>
<td>Epo-bp</td>
<td>81.6±1.5</td>
<td>88.7±2.5</td>
</tr>
<tr>
<td>αEpo-bp</td>
<td>81.2±1.3</td>
<td>84.3±2.3</td>
</tr>
<tr>
<td>Epo+αEpo-bp</td>
<td>81.0±1.0</td>
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<tr>
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<td>79.4±1.5</td>
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<tr>
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<td>321.2±4.9</td>
<td>116.2±1.7</td>
</tr>
<tr>
<td>Epo</td>
<td>294.9±4.2</td>
<td>136.2±2.3§</td>
</tr>
<tr>
<td>Epo-bp</td>
<td>312.1±3.9</td>
<td>118.4±2.1</td>
</tr>
<tr>
<td>αEpo-bp</td>
<td>305.0±4.9</td>
<td>116.6±2.1</td>
</tr>
<tr>
<td>Epo+αEpo-bp</td>
<td>303.4±3.6</td>
<td>118.3±1.7</td>
</tr>
<tr>
<td>Epo+αEpo-bp</td>
<td>298.4±4.4</td>
<td>121.0±2.0</td>
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Rx indicates treatment; n, number of rats (30 rats in each group); y, 24-hour average; HW, heart weight; BP, blood pressure; Hct, hematocrit fraction; SW, spleen weight; AW, aorta weight; R, right; L, left.

\[*P<0.05; †P<0.01; ‡P<0.001; §P<0.0001.\]
A significantly increased blood pressure in the Epo-treated group was detected at noon, 4 PM, 8 PM, and midnight, as compared with the saline, Epo-bp, and αEpo-bp treatment groups. Epo treatment increased hematocrit markedly at each of the 6 test times as compared with the saline, Epo-bp, and αEpo-bp treatment groups (all \( P < 0.0001 \)). The spleen weights were significantly higher in the Epo-treated group rats than those of the saline, Epo-bp, and αEpo-bp groups at all 6 of the test time points, though the BW was somewhat lower at each time comparison.

The overall results in the present study indicate that Epo-bp and αEpo-bp eliminate the Epo-induced blood pressure elevation without affecting the Epo-treated hematocrit increase. Our results also demonstrate that the timing of the Epo treatment in combination with Epo-bp and/or αEpo-bp may be important in clinical use. Cardiovascular parameters should be monitored in Epo treatment, and the Epo dose should be reevaluated to prevent further end-organ damage.

**Discussion**

Epo is known to have effects not only in erythropoiesis and thrombopoiesis\(^{21,22}\) but also on many other cells and tissues, including endothelial cells,\(^{26}\) smooth muscle cells,\(^{27,28}\) cardiomyocytes,\(^{29}\) and kidney cells,\(^{30}\) as well as neuroprotective and suppressive actions in ischemia-induced neuronal cell death as neurogenic and neuroprotective agents via antiapoptotic, neurotrophic, antioxidant, and angiogenic effects.\(^{10,11,31,32}\) Our study, however, showed that brain weight was significantly reduced in the Epoetin-treated group as compared with Epo-bp- and αEpo-bp–treated groups (Table 1). The discrepancy may pertain to the issue of natural versus synthetic recombinant origin of Epoetin. This might explain the increased blood pressure in recombinant Epo (Epoetin) treatment, which was eliminated by Epo-bp and/or αEpo-bp, as shown in our present studies (Tables 1 and 2 and Figure 1).

In our study, the lower range of Epo (50 U/kg of BW) was applied as compared with the Epo dosages used in the Epoetin clinical study (50 to 150 U/kg of BW).\(^{1,12}\) As expected, Epo treatment increased hematocrit markedly as compared with all of the other treatments in the present study (all \( P < 0.0001 \)); however, splenomegaly characterized each rat in Epo treatment (Figure 2). The characteristic phenomenon should be concerning, because the hematologic malignancy manifests as a myeloproliferative disorder, such as...
compared with the saline control group rat (D).

Epo treated with Epo expressed splenomegaly (A, B, and C), as immediately after the sacrifice. As shown in this picture, every spleens. A photographic picture of the fresh spleen was taken settings, the Epo dose should be reevaluated to prevent increase in the number of blood cells and splenomegaly.33 Thus, when Epo is administered repeatedly in various clinical mechanisms of Epo are, nor what the second messenger however, at the present time what the biophysiological end-organ damage. The timing of Epo treatment and concomitant administration of Epo-bp and/or αEpo-bp should also be considered to obtain the benefits of Epo without its adverse effects. Other potential adverse effects of Epo include increased vascular and thrombotic events, such as pulmonary embolism, stroke, and myocardial infarct, especially Epo-associated thrombosis in young healthy athletes.18,34

The Epo receptor has been cloned.35,36 We do not know, however, at the present time what the biophysiological mechanisms of Epo are, nor what the second messenger system involved in the interaction between Epo and the Epo receptor in their binding activities and subsequent processes are. Some studies reported that increased cardiovascular events were associated with a rapid rise in hemoglobin, whereas others proposed that Epo may be involved in a hematocrit-independent, vasoconstriction-stimulated cytoplasmic Ca++ leading to resistance to the vasodilatory action

![Figure 2. Splenomegaly characterized in Epo-treated rat spleens. A photographic picture of the fresh spleen was taken immediately after the sacrifice. As shown in this picture, every rat treated with Epo expressed splenomegaly (A, B, and C), as compared with the saline control group rat (D).](image-url)
of NO, increased endothelin, upregulated renin-angiotensin expression, and possible changes in vascular tissue prostaglandin production. Nevertheless, Epo has potentially beneficial effects on the endothelial and neuroprotection, maybe via antiapoptosis. In our previous study, we demonstrated that the Epo receptor exists in various progenitor cell surfaces and tissues. Using our new products, the ligand-binding sites on bone marrow progenitor cells were visualized by elaborating fluorescein-labeled Epo receptor sites in various blood cells and tissue/cell types, including megakaryocytes, erythroblasts, normoblasts, and myeloblasts. These results may explain the current study results of the effects of Epo-bp and αEpo-bp on blood pressure and multiple end organs.

Some authors suggest that soluble Epo receptor is a contributing factor to resistance to Epo therapy or ineffective erythropoiesis in certain hematologic malignancy. Nevertheless, in the present study, we demonstrated that Epo-bp and its antibody effectively eliminated Epo-induced hypertension. The controversial result may be related to the recombinant materials versus our purified proteins. We speculated that Epo-bp and its antibody might be involved in Epo-Epo receptor–mediated interactions as cleaning house actions to prevent Epo-induced hypertension. Thus, our new products will be helpful in further studies regarding the defects or deficiencies related to Epo or Epo receptor. We also developed test kits using Epo-bp and αEpo-bp, which will be useful tools in differential diagnosis in Epo or Epo receptor–related clinical cases.

Perspectives
Genetically engineered pure human Epo-bp and its antibodies have been developed to observe their effects on the adversity of genetically engineered Epoetin. The adverse effects in Epoetin use have resulted in serious problems, such as uncontrollable blood pressure rise and end-organ damage. Our Epo-bp and αEpo-bp effectively eliminate Epo-associated hypertension.

We speculate that the genetically matched Epo and Epo-bp act in specific binding to reduce this adversity. Epo-bp may bind the specific site responsible for blood pressure elevation. It may be also plausible to produce some harmful materials, such as antibodies, in the repetitive use of synthetic recombinant Epoetin. Although the involvement of αEpo-bp is not clear, it may be possible that αEpo-bp binds to Epo-generated waste materials, including anti-Epo antibodies. If so, the clearing effects of Epo-bp and αEpo-bp will certainly be beneficial in the repetitive use of Epoetin. Several putative mechanisms are summarized in the above Discussion section. However, definite mechanisms in the binding process and fates of the ligand should be further explored. With the availability of pure human Epo-bp and its antibodies, further studies are possible to elucidate the structures and mechanisms in ligand binding and subsequent processes. Future studies should also include examination of their effects on other pathological conditions.

In conclusion, our genetically engineered proteins, Epo-bp and αEpo-bp, effectively eliminate Epo-associated hypertension without affecting hematopoiesis. The new materials should be useful in exploring mechanisms of Epo receptor–ligand interactions and binding processes and differential diagnoses in Epo and/or its receptor-related diseases, as well as in other clinical applications.

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Disclosures
None.

References


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An erratum has been published regarding this article. Please see the attached page for:
/content/50/2/e56.full.pdf
In the *Hypertension* article by Lee et al (Lee MS, Lee JS, Lee JY. Prevention of erythropoietin-associated hypertension. *Hypertension*. 2007;50:439-445), Table 1 was changed after the article was posted on the *Hypertension* web site on June 4, 2007. The correct Table 1 is shown here.

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