Effect of Erythropoietin on Blood Pressure and on the Vascular Endothelial ET-1/ET\textsubscript{B} Receptor System

Marie-Ève Rodrigue\textsuperscript{1,2}, Isabelle Brochu\textsuperscript{3}, Pedro D’orléans-Juste\textsuperscript{3}, Richard Larivièrea\textsuperscript{1,2} and Marcel Lebel\textsuperscript{1,2}

BACKGROUND
Recombinant human erythropoietin (rhEPO) increases blood pressure (BP) and the vascular production of endothelin-1 in renal failure rats. This study was designed to investigate the effect of rhEPO on BP and on the ET\textsubscript{1}/ET\textsubscript{B}R system in rats with normal renal function. To further characterize the effect of rhEPO on the ET\textsubscript{1}/ET\textsubscript{B}R system, we also studied heterozygous (±) ET\textsubscript{B}R knockout (KO) mice.

METHODS
The animals received either the vehicle or rhEPO (100 U/kg subcutaneously three times per week). ET\textsubscript{B}R\textsuperscript{±} mice were compared with ET\textsubscript{B}R\textsuperscript{−/−} and wild-type (WT) mice. In rats, the ET\textsubscript{B}R mRNA expression was assessed in blood vessels as well as the vascular ET\textsubscript{B}R density using immunohistochemistry. In mice, ET-1 concentration was measured in the thoracic aorta.

RESULTS
RhEPO administration increased hematocrit levels in all treated animals. This therapy had no effect on BP in normal rats, but it did increase vascular and renal cortex ET\textsubscript{B}R mRNA expression. Immunohistochemistry confirmed that the ET\textsubscript{B}R density was increased in blood vessel endothelium in these normal rats. In contrast, rhEPO increased BP in ET\textsubscript{B}R\textsuperscript{±} mice and this pressor effect was associated with higher ET-1 concentrations in the thoracic aorta.

CONCLUSIONS
RhEPO exerts a pleotropic effect on the endothelial ET\textsubscript{1}/ET\textsubscript{B}R system. The increase in endothelial ET\textsubscript{B}R expression may contribute to maintaining normal BP during rhEPO administration in normal animals. Conversely, conditions with deficient ET\textsubscript{B}R expression, such as in ET\textsubscript{B}R\textsuperscript{−/−} mice, may lead to hypertension while receiving the same therapy.

INTRODUCTION
Treatment of anemia with recombinant human erythropoietin (rhEPO) in chronic renal failure patients is associated with an increase in blood pressure (BP) resulting in de novo hypertension or exacerbation of existing hypertension.\textsuperscript{1–3} This pressor effect has not been reported when rhEPO was administered to patients with nonrenal anemia\textsuperscript{4} or in healthy volunteers.\textsuperscript{5} Similarly, rhEPO increases BP in rat models of renal failure,\textsuperscript{6,7} whereas control animals receiving the same therapy remain normotensive.\textsuperscript{6} However, hypertension has been observed in normal rats with higher doses of rhEPO,\textsuperscript{8} or using the peritoneal route of injection.\textsuperscript{9}

The precise mechanism underlying the development of hypertension following rhEPO therapy is still unclear. The pressor effect of rhEPO cannot only be accounted for by an increase in hematocrit or erythrocyte mass in renal failure patients\textsuperscript{10} and uremic rats.\textsuperscript{11} Other potential mechanisms include an inappropriate increase in peripheral resistance, nitric oxide (NO) resistance,\textsuperscript{11} enhanced tissue renin activity,\textsuperscript{12} and an increase in angiotensin II receptor expression.\textsuperscript{13} Recent evidence suggests that rhEPO accentuates a pre-existing endothelial dysfunction. Vascular endothelial cells express EPO receptors\textsuperscript{14} and these cells, when stimulated with rhEPO, release endothelin-1 (ET-1).\textsuperscript{15} In vivo studies also show that rhEPO therapy can modulate ET-1 mRNA expression in the renal cortex of uremic rats\textsuperscript{16} and can increase blood vessel and renal cortex ET-1 concentrations as well as ET-1 urinary excretion in hypertensive uremic rats.\textsuperscript{17–20} ET-1 is a 21 amino-acid peptide produced by endothelial cells that acts through the activation of two receptors—ET\textsubscript{A} and ET\textsubscript{B}. ET\textsubscript{A} receptors (ET\textsubscript{A}R) are located on smooth muscle cells of blood vessel media and cause vasoconstriction when stimulated by ET-1. ET\textsubscript{B} receptors (ET\textsubscript{B}R) are predominantly expressed on endothelial cells and mediate vasodilatation upon the release of prostacyclin (PGI\textsubscript{2})\textsuperscript{21} and NO.\textsuperscript{22} ET\textsubscript{B}R is also involved in ET-1 clearance which limits its local accumulation.\textsuperscript{23}

We previously showed that selective ET\textsubscript{A} but not ET\textsubscript{A}/ET\textsubscript{B} receptor blockade prevented the progression of hypertension in rhEPO-treated uremic rats.\textsuperscript{24} This suggested that the ET-1 system is involved in the pathophysiology of rhEPO-induced hypertension and that the ET\textsubscript{A}R and ET\textsubscript{B}R play a differential role. As mentioned earlier, BP as well as vascular ET-1 concentrations remain normal in control rats receiving
rhEPO. These results lead us to hypothesize that, under normal conditions, rhEPO could upregulate ET$_B$R which mediates vasodilatation and ET-1 clearance. This modulation of ET$_B$R could contribute to maintaining normal BP. Conversely, rhEPO could induce hypertension in animals that have an ET$_B$R deficiency.

This study was designed to investigate the effect of rhEPO on BP and on the ET-1/ET$_B$R system in normal rats and in mice with heterozygous (±) knockout (KO) of ET$_B$R.

**METHODS**

**Experiments in normal rats.** This protocol and experimental manipulations were conducted in accordance with the guidelines of the Canadian Council of Protection of animals and approved by the Animal Care committee of Laval University. Male Wistar rats were obtained from Charles River (St-Constant, Quebec, Canada) and allowed to acclimatize for 1 week in our animal facilities with temperature and humidity control and a 12-h day/night cycle before any experimental intervention. Food (standard rat chow) and untreated tap water were available ad libitum. Rats were divided into two groups receiving either the vehicle (saline 0.9%) or rhEPO (Eprex; OrthoBiothe, Don Mills, Ontario, Canada; 100 U/kg three times per week subcutaneously) for 3 weeks. This dosage was commonly used to treat anemia in renal failure patients. Since EPO is cross-reactive between species and since the mouse and human EPO receptors are nearly equivalent, the same dosage was used in animals. We previously demonstrated that rhEPO 100 U/kg three times per week resulted in a similar hematological response in normal and uremic rats. Administration of this dosage for 3 weeks was also adequate to increase BP significantly in uremic rats but had no effect on BP of control rats. Systolic BP was recorded at the end of the treatment using the tail-cuff method after warming and slight restriction using an IITC. BP system fitted with a model 29 pulsar sensor (IITC Life Science, Woodland Hills, CA). BP was recorded using a computerized acquisition system (model MP100; Biopac System, Goleta, CA) and the average of three readings was used for analysis. Finally, the rats were anesthetized with pentobarbital (Somnotol, 50 mg/kg intraperitoneally; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and exsanguinated through abdominal aortic puncture. Blood samples were collected for the measurement of hematocrit. Segments of the thoracic aorta and mesenteric arterial bed were collected for the measurement of hematocrit. Segments of the thoracic aorta and mesenteric arterial bed were quickly frozen and stored at −80 °C for measurement of ET-1.

Hematocrit values were determined in duplicate in Pre-Cal micro-hematocrit tubes (Becton-Dickinson, Parsippany, NJ) after centrifugation at 19,000 r.p.m. for 2 min. Northern blot analysis was performed as described in detail elsewhere. Briefly, total RNA was extracted from the renal cortex using a guanidine isothiocyanate-phenol-chloroform method. RNA samples (25 μg) were run on a 1% agarose gel. The samples were then transferred onto a Hybond-N nylon membrane (GE Health Bio-Sciences, Baie d’Urfe, Quebec, Canada). Hybridization was carried out using a specific 32P-labeled rat ET$_B$ complementary RNA probe or an oligonucleotide probe for the 18S ribosomal RNA which was used for hybridization control. After stringent washing, membranes were exposed to BioMax MS films with a BioMax ME intensifying screen (Eastman Kodak, Rochester, NY).

Immunohistochemistry analysis was performed on paraffin embedded mesenteric arterial beds to determine expression levels of ET$_B$R using an ET$_B$R polyclonal antibody (Maine Biotechnology Services, Portland, ME) and the avidin-biotin-peroxydase technique (Vectastain ABC-AP kit; Vector Laboratories, Burlingame, CA).

Immunoreactive ET-1 concentrations were measured in genetically manipulated mice in a pool of two thoracic aortas using a specific radioimmunoassay in C18 Sep-Pak extracted samples as described previously. Briefly, the recovery of the extraction procedure varied from 75 to 90%. The lower ET-1 detection limit was 1 pg/tube with 50% tracer displacement, around 10 pg/tube on the standard curve. The intra- and interassay coefficients of variation were 7 and 10%, respectively.

**RESULTS**

**Effects of rhEPO in normal rats**

At the end of the protocol, normal rats that received the vehicle (n = 8) had a mean hematocrit and BP of 38 ± 1% and 116 ± 3 mm Hg, respectively. As expected, the group that received rhEPO treatment (n = 8) increased their hematocrit (saline 0.9%) was administered to these mice for 3 weeks. The animals were then anesthetized with ketamine/xylazine (74/9.3 mg/kg, intramuscular) and the right carotid artery was cannulated with a polyethylene catheter m (PE-10) to measure the mean arterial pressure as described previously. Finally, the animals were exsanguinated and blood samples were used for measurement of hematocrit. Segments of the thoracic aorta, from the diaphragm to the aortic arch, were removed and cleaned of adipose tissue. Tissues were then quickly frozen and stored at −80 °C for measurement of ET-1.
to 48 ± 2% \( (P < 0.01 \text{ vs. vehicle}) \). BP for the same group was 119 ± 2 mm Hg (non significant vs. vehicle). The administration of rhEPO increased \( (P < 0.05 \text{ vs. vehicle}) \) the ETBR mRNA expression significantly by 2.14 ± 0.32-folds in the thoracic aorta, by 2.16 ± 0.71-folds in the mesenteric arterial bed (Figure 1), and by 3.5 ± 0.38-folds in the renal cortex (Figure 1). The density of the immunohistochemistry staining of ETBR in the vascular endothelium of rats receiving the vehicle and rhEPO treatment was 5.7 ± 1.2 and 116.2 ± 20.7 (arbitrary units; \( P < 0.01 \text{ vs. vehicle} \)), respectively (Figure 2). No notable immunostaining of ETBR on smooth muscle cells was detectable in either group using that technique.

### Effects of rhEPO in WT, ETBR±, and ETAR± mice

Table 1 shows the hematocrit of WT, ETBR±, and ETAR± mice after 3 weeks of treatment with either the vehicle or rhEPO \( (n = 30–33 \text{ in each group}) \). As expected, hematocrit was similarly increased following rhEPO therapy in each of the three groups \( (P < 0.01 \text{ vs. vehicle}) \). The BP of ETBR± mice treated with the vehicle was higher than in WT and ETAR± mice but it did not reach the statistical difference (Figure 3). RhEPO therapy increased mean arterial BP in ETBR± mice as compared with untreated ETBR± mice \( (P < 0.05) \). In contrast, rhEPO did not change mean arterial pressure in WT or ETAR± mice (Figure 3). As shown in Figure 4, ET-1 concentrations in the thoracic aorta were similar in WT and ETBR± mice while it was lower in ETAR± mice \( (P < 0.05) \). The administration of rhEPO therapy induced a significant increase in vascular ET-1 concentrations in ETBR± mice as compared to their untreated congeners \( (P < 0.05) \). RhEPO had no effect on vascular ET-1 levels in WT and ETAR± mice. However, a positive correlation was observed between mean arterial pressures and thoracic aorta ET-1 concentrations in WT and ETBR± mice whether treated or not with rhEPO (Figure 5).
DISCUSSION

In this study, we documented that rhEPO administration increases vascular and renal ETₐR mRNA expression and endothelial cell protein levels in normal rats. BP remained normal following rhEPO treatment in these rats. In contrast, rhEPO increased mean arterial pressure in ETₐR⁻² mice while having no effect on the same parameter in WT and ETₐR⁻² mice. Thoracic aorta ET-1 concentrations were increased following rhEPO administration solely in ETₐR⁻² mice and there was a positive correlation between arterial pressures and vascular ET-1 levels in ETₐR⁻² and WT mice.

The results concur with our previous work showing that rhEPO, at doses used in this study, does not lead to hypertension in normal rats, although it increases the hematocrit.6,25 Furthermore, vascular ET-1 concentrations remained normal in these animals treated with rhEPO.6,25 The major finding of this study is that rhEPO can modulate the ETₐR expression in the vascular endothelium. This action of rhEPO on the ET-1/ETBR system could contribute to mediating the effect of rhEPO on the blood vessel tone. First, the upregulation of the ETₐR may accelerate the clearance of ET-1 (ref. 23) and account for the normal ET-1 concentrations documented during rhEPO therapy in rats with normal renal function. Second, the increase in ETₐR expression by rhEPO can enhance NO formation. Indeed, it has been shown that EPO induces NO synthase expression in endothelial cells30 and in healthy rats,31 this effect could be mediated through the ETₐR.32 Along the same lines, d’Uschio et al.32 recently reported that EPO administration to endothelial NO synthase–deficient mice induces a significant increase in systolic BP. Conversely, ETₐR blockade in normal animals causes an increase in mean arterial pressure as well as a reduction in ET-1 clearance.33 Another mechanism which might account for the normal level of vascular ET-1 in rhEPO-treated normal animals is the enhanced NO formation resulting from the increase in hematocrit, blood viscosity, and shear stress.34 NO is a powerful inhibitor of ET-1 gene expression.35 However, this mechanism is likely less important in this experimental condition than the NO production mediated through ETₐR stimulation. In this regard, the ETₐR-deficient mice in this study had likely the same increase in NO-mediated blood viscosity, but they developed hypertension, underlining the importance of the ETₐR-NO compensatory mechanism. Thus, the increase in vascular endothelial expression of the ETₐR in normal animals receiving rhEPO may contribute to maintaining normal BP and vascular ET-1 levels.

To further document the effect of rhEPO on the ET-1/ETₐR system and on BP, we studied mice with deficient ETₐR.36 These animals develop mild hypertension which is sensitive to the selective ETₐ antagonist BQ-123 but not to the ETB blocker BQ-788.27 Also, their ETₐ receptor–dependent ET-1 clearance is diminished when compared with ETₐR⁻² or WT mice.27 Therefore, this murine model appears to be highly suitable to substantiate the importance of ETₐR in maintaining normal BP and vascular ET-1 concentrations during rhEPO administration as suggested in the above experiments in normal rats. Basal arterial pressures tended to be higher in ETₐR⁻² mice as compared with ETₐR⁻² and WT mice. This observation is in keeping with Berthiaume et al.27 who reported that basal mean arterial pressure in a larger group of ETₐR⁻² mice was significantly higher than their littermates. Another salient finding of this study is that rhEPO administration induced a significant increase of mean arterial pressure in ETₐR⁻² mice as compared to ETₐR⁻² and WT animals. This observation underscores the importance of intact and functional ETₐR in maintaining adequate ET-1 clearance and normal vascular tone during rhEPO therapy. This may also explain why selective ETₐ but not ETₐ/ETₐ receptor blockade can prevent rhEPO-induced hypertension in renal failure rats,24 a model in which the expression of ETₐR is reduced37,38 and vascular ET-1 concentrations are increased.29

Our study also provides new data on vascular ET-1 concentrations in response to rhEPO in ETₐR and ETₐR genetically manipulated mice. In basal conditions, ETₐR⁻² and WT mice had comparable basal ET-1 levels in the thoracic aorta, whereas ETₐR⁻² mice displayed significantly lower vascular ET-1 concentrations. Following rhEPO administration, ETₐR-deficient animals exhibited a significant increase in thoracic aorta ET-1 concentrations as compared to ETₐR⁻² and WT mice. Genetic deletion of one receptor is usually counterbalanced by either the activity or the expression of other receptors of the same ligand. However, the genetic deletion of either ETₐ or ETₐ receptors for ET-1 cannot be compensated by the other type left intact. Indeed, homozygous KO animal of either gene is lethal at the embryonic stage for the former and through megacolon formation in the latter strain of mice.36,39 The KO mice used in this study have between 40 and 60% of the intact ETₐ or ETₐ-receptor proteins.27 In both cases, the repression of one of the two receptors was sufficient to alter significantly the pharmacological effect of either ET-1 (in ETₐR⁻² mice) or ILR-1620 (in ETₐR⁻² mice).27 This study in ETₐR⁻² mice shows the importance of normally functioning ETₐR in maintaining normal vascular ET-1 concentrations during rhEPO therapy.

Stimulation of erythropoiesis has long been considered the sole physiological function of EPO. More recent work demonstrated pleotropic actions of EPO in nonhematopoietic tissues including blood vessels.40 The stimulation of EPO receptors on endothelial cells leads to the production of several endothelial–derived modulators of vascular tone.13 This study furthers the concept of a novel mechanism by which EPO can modulate the vascular tone via the ET-1/ETₐR system under conditions of anemia/hypoxia and enhanced EPO production. This pleotropic effect may contribute to maintaining normal BP during rhEPO administration in normal animals and patients receiving rhEPO therapy for nonrenal anemia.

This study not only provides a potential explanation as to why rhEPO does not increase BP under normal conditions, but also suggests mechanisms that may account for rhEPO-induced hypertension in chronic renal failure. Indeed, we38 and others37 have shown that the expression of ETₐR is reduced in experimental chronic renal failure as in ETₐR⁻² mice. This may account, at least in part, for the increased vascular and
renal ET-1 concentrations\textsuperscript{17–19} and acquired resistance to NO-mediated vasodilatation\textsuperscript{11} observed in hypertensive uremic animals treated with rEPO. On a therapeutic ground, one might expect that selective ET\textsubscript{A} rather than mixed ET\textsubscript{A}/ET\textsubscript{B} Receptor blockade would be efficacious in treating rEPO-induced hypertension in renal failure patients as documented in experimental animal model of uremia.\textsuperscript{24}

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