Erythropoietin and the use of a transgenic model of erythropoietin-deficient mice

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Abstract: Despite its well-known role in red blood cell production, it is now accepted that erythropoietin (Epo) has other physiological functions. Epo and its receptors are expressed in many tissues, such as the brain and heart. The presence of Epo/Epo receptors in these organs suggests other roles than those usually assigned to this protein. Thus, the aim of this review is to describe the effects of Epo deficiency on adaptation to normoxic and hypoxic environments and to suggest a key role of Epo on main physiological adaptive functions. Our original model of Epo-deficient (Epo-TAg®) mice allowed us to improve our knowledge of the possible role of Epo in O2 homeostasis. The use of anemic transgenic mice revealed Epo as a crucial component of adaptation to hypoxia. Epo-TAg® mice survive well in hypoxic conditions despite low hematocrit. Furthermore, Epo plays a key role in neural control of ventilatory acclimatization and response to hypoxia, in deformability of red blood cells, in cerebral and cardiac angiogenesis, and in neuro- and cardioprotection.

Keywords: Epo-TAg® mice, mouse model, physiological functions, hypoxia

Introduction

Life is dependent upon transport and utilization of oxygen (O2) for the metabolic conversion of nutrients into energy,1 making O2 homeostasis an essential process for survival. Indeed, an inadequate level of O2 is detrimental for the tissues, and complex mechanisms serve to maintain in vivo the cellular O2 concentration within a physiological range.1 An imbalance between O2 delivery and requirement, such as at high altitude, activates a variety of specific mechanisms at molecular, cellular, and systemic levels. High altitude is accompanied by low atmospheric O2 pressure, which sequentially leads to insufficient O2 uptake and reduced tissue oxygenation. Hypoxic exposure can be intermittent (obstructive sleep apnea) or continuous (high altitude, cardiorespiratory failure), leading to different strategies to address these stresses. Indeed, acute hypoxia triggers rapid and transient compensatory mechanisms, while chronic hypoxia (CHx) leads to more durable changes with gene expression modifications.1 The hypoxia-inducible factor-1 (HIF-1) is the most important protein regulating homeostasis when O2 is lacking.2 Under hypoxia, stabilization of HIF-1 modulates the expression of hypoxia-regulated genes such as vascular endothelial growth factor (VEGF), glucose transporters, or erythropoietin (Epo).1,3 Epo is a hematopoietic growth factor and represents the main regulator of erythropoiesis. In low O2 conditions, the number of erythrocytes increases to sustain O2 delivery.6 This polycythemia, secondary to an increase in Epo release, is one of the key factors of acclimatization to CHx, through
the increase in O₂-carrying capacity.7–9 However, despite this well-known role, it was suggested that Epo plays a key role in other physiological functions. Thus, Epo is also known to be fundamental for embryonic development,10 and it is also largely involved in the normal functioning of most organs, including brain, heart, and muscles by regulating numerous cell functions such as calcium flux or cell survival.11,12 To assess these pleiotropic actions of Epo on organs and cells, transgenic models of mice have been developed.13,14

The aim of this review is to provide a brief presentation of the physiological effects of Epo and describe the effects of Epo deficiency on adaptation to normoxic and hypoxic environments in an original model of Epo-deficient (Epo-TAg) mice. From these observations, we suggest a key role of Epo on multiple physiological adaptive functions, especially in response to hypoxia.

**Epo and Epo-receptor expression in organs**

Epo is a glycoprotein that is synthesized by peritubular fibroblasts in the kidneys of adults and in hepatocytes of fetal liver.15 Epo was originally believed to play a role that restricted to stimulation of early erythroid precursor proliferation, inhibition of apoptosis, and differentiation of the erythroid lineage. Currently, in addition to its well-known role in red blood cell production, a diverse group of cells has been identified to produce Epo and/or Epo receptor (Epo-R) including endothelial cells, smooth muscle cells, retina tissues, testis, and cells of the central nervous system.16–18 Moreover, Epo mRNA was also detected in lungs, testis, heart, and brain but not in skeletal muscles, intestine, or bone marrow of rodents.20,21

**Epo and the brain**

Epo mRNA is constitutively expressed in the mice brain,22,23 and Epo-R mRNA and protein are also expressed in the brain of rodents.23,24 Indeed, Epo and its receptors are found in the nervous system, including in neurons, astrocytes, and endothelial cells.16,18,22,23,25–27 Epo/Epo-R couple is involved in neuroprotection,28 promotes neural plasticity,29 and could have a potentially antidepressor effect.30,31 Furthermore, Epo/Epo-R pathway is required for normal brain development.32 Indeed, Epo is required for neural progenitor cell proliferation,33 and it avoids neuronal apoptosis by maintaining Bcl-2 and Bcl-xl expression.34,35 Indirectly, Epo improves sensory, cognitive, and endocrine functions of the central nervous system through its erythropoiesis-stimulating effect, because it increases the O₂ supply to the brain. The direct effects of Epo are independent of erythropoiesis.36 Epo has been demonstrated to have neuroprotective effects after ischemic, hypoxic, metabolic, neurotoxic, and excitotoxic stress in the nervous system. Epo operates at several levels within the central nervous system, including limiting the production of reactive O₂ species and glutamate, neurotransmission modulation, promotion of angiogenesis, prevention of apoptosis, reduction in inflammation, and recruitment of stem cells.37–40 Moreover, Epo-R was localized in both brainstem respiratory centers and carotid bodies,34 and it is involved in ventilatory regulations during hypoxic challenges.

**Epo and the heart**

As in the brain, animal models of ischemia and acute myocardial infarction have shown that Epo reduces infarct size and improves left ventricular (LV) function. These effects are mediated mostly through apoptosis inhibition by activating pro-survival pathways in the myocardium, mobilization of endothelial progenitor cells, and inhibition of migration of inflammatory cells as well as potent pro-angiogenic properties.41 Moreover, recent studies reported that the heart could be a site of Epo production42–44 and, in particular, that cardiac tissue reveals Epo gene and protein expression.40,46 Epo-R expression is high in cardiac progenitor cells.47 Epo has angiogenic and antiapoptotic effects in the heart,48,49 and intraperitoneal injection of Epo promotes the differentiation of cardiac progenitor cells into endothelial cells.49 Overall, Epo is known to have a cardioprotective effect through antiapoptotic, anti-inflammatory, and angiogenic effects,50 and it could act on the oxidative stress.51 For example, Epo may enhance protein kinase B or Akt and protect the heart from ischemia–reperfusion injuries.52 This effect of Epo on Akt could regulate cardiomyocyte mitochondrial biogenesis,53 by acting on the Akt/endothelial nitric oxide (NO)-synthase pathway.54 Recently, it has also been shown that acute Epo injection can efficiently improve resuscitation and survival rates in a model of cardiac arrest in pigs.55,56

**Epo and the muscle**

Epo-R has been identified in muscle biopsies suggesting a potential but undefined role of Epo on muscle metabolism or function.57,58 In humans, these effects were suggested by excessive intake of Epo during endurance exercise in order to improve performance.59 In some athletes, Epo increases performance during endurance exercises more than expected on the only basis of an increase in hematocrit. This suggests a potentially direct effect of Epo on muscle metabolism,50 even if this effect remains to be validated.
Epo could induce angiogenesis and accumulation of VEGF in skeletal muscles submitted to ischemia–reperfusion experiments\textsuperscript{61} and act as a promoter of growth factors in skeletal muscles.\textsuperscript{57} In vitro, Epo-R mRNA and protein expression were identified in the primary muscle cells of mammals.\textsuperscript{57,62,63} However, the effects of administration of human recombinant Epo (rhU-Epo) and the role of Epo-R in culture media remain controversial. Some authors showed a positive effect,\textsuperscript{57} and some others showed no effect,\textsuperscript{52,65} on primary myoblast proliferation. In vivo, although mRNA–encoding Epo-R and Epo-R proteins were detected in rats,\textsuperscript{64} skeletal muscle treatment by rhU-Epo have shown controversial results. However, Epo is suspected to induce a shift in muscle fiber metabolism toward a more oxidative phenotype\textsuperscript{65,66} and prevent the impairment of mitochondrial structure and function.\textsuperscript{67} Moreover, some authors observed a positive effect of rhU-Epo on myoblast apoptosis.\textsuperscript{64,68} Proteolysis, glycolysis, and mitochondrial functions.\textsuperscript{69} The authors suggested\textsuperscript{70} that indirect effects of Epo treatment through the amelioration of \( \text{O}_2 \) supply could explain the observed effects of Epo on skeletal muscles during endurance exercises.

**Epo underproduction and clinical disorders**

In adults, insufficient Epo production results mostly from direct damage to Epo-producing cells in the kidneys or to a lesser extent from the suppression of Epo production by inflammatory cytokines.\textsuperscript{71} Indeed, in patients suffering from rheumatoid arthritis, cancer, and acquired immune deficiency syndrome (AIDS), inflammatory cytokines suppress Epo gene expression.\textsuperscript{71–75} In contrast, patients with renal failure generally develop anemia due to the suppression of erythropoiesis and to a moderate reduction in red cell life span.\textsuperscript{71} Furthermore, the main reason of anemia in patients with uremia is an insufficient Epo production.\textsuperscript{71,76,77} Diabetic nephropathy can also lead to Epo deficiency and anemia.\textsuperscript{78} Moreover, exposure to metals such as cadmium or platinum results in a modification of the structure and function of renal proximal tube, resulting in the suppression of Epo production.\textsuperscript{71} Patients with increased plasma viscosity due to monoclonal dysproteinemias have an inappropriate Epo production.\textsuperscript{71,79}

**Mouse model of Epo deficiency**

A mouse model of Epo-TAg\textsuperscript{h} mice was used for the first time for the identification of the renal Epo-producing cells.\textsuperscript{80} We then developed this model in 2006 in our laboratory to assess the potential roles of Epo and/or anemia in the adaptation processes to hypoxia.

The transgenic construct contains an SV40 sequence in the five untranslated region of the mouse Epo gene, which is flanked on each side by 9 and 7.5 kb of DNA from the mouse Epo locus.\textsuperscript{80} Anemia-inducible Epo expression was observed in the kidneys.\textsuperscript{80} Thus, these mice present a severe reduction in Epo gene expression,\textsuperscript{13,80} leading to low plasmatic level of Epo (122±16 pg/mL in wild type [WT] vs 53±18 pg/mL in Epo-TAg\textsuperscript{h} mice) and thus chronic anemia with low hemoglobin concentration (17.1±0.3 g in WT vs 6.9±0.3 in Epo-TAg\textsuperscript{h} mice; Table 1). However, Epo-TAg\textsuperscript{h} mice have a good survival rate in CHx (14 days, 4,500 m) through an increase in ventilation and cardiac output.\textsuperscript{81,82} They also develop cerebrovascular adaptations to chronic anemia and hypoxia.\textsuperscript{83}

**Characteristics of Epo-TAg\textsuperscript{h} mice in normoxic and hypoxic conditions: effect of Epo deficiency and/or anemia**

**Blood characteristics**

Red blood cells are responsible for the transport of \( >98\% \) of the \( \text{O}_2 \) into the blood. In order to deliver \( \text{O}_2 \) to the tissues, red blood cells deform as they enter capillaries. In Epo-TAg\textsuperscript{h} mice, hematocrit and hemoglobin are ~60% lower (Table 1) than those observed in WT mice.\textsuperscript{82} Furthermore, Epo deficiency decreases blood viscosity and slightly reduces red blood cell deformability.\textsuperscript{84} After CHx, hemoglobin concentration remained 60% lower in Epo-TAg\textsuperscript{h} mice as compared with that of WT mice,\textsuperscript{81} despite a proportional increase in Epo concentration as compared with WT animals.\textsuperscript{85} Nevertheless, in Epo-TAg\textsuperscript{h} mice, Epo concentration following acute hypoxia increased to reach only the normoxic WT value\textsuperscript{83} and returned to basal value after CHx.\textsuperscript{20}

**Cardiac characteristics**

Epo-TAg\textsuperscript{h} mice show right ventricular and LV hypertrophy,\textsuperscript{82} leading to increased stroke volume and cardiac output (Table 1). This adaptive process, already described,\textsuperscript{86} allows to offset the fall in arterial \( \text{O}_2 \) content due to anemia. Echocardiographic data confirmed compensatory LV hypertrophy, higher myocardial chamber volumes, and a higher cardiac output,\textsuperscript{20} which could be explained by an increase in preload without change in left ventricle afterload as depicted by unchanged blood pressure. Although cardiac output was increased in Epo-TAg\textsuperscript{h} mice, \( \text{O}_2 \) delivery remained lower than in control WT animals. Furthermore, Epo/Epo-R pathway is known to be involved in the transcription of target genes that mainly involved in the inhibition of apoptosis and cell...
proliferation through the phosphorylation of Jak2 and STAT-5. Because of unchanged P-STAT-5/STAT-5 ratio, we could not confirm the activation of this cardioprotective pathway in response to chronic Epo deficiency. In addition to its angiogenic function, VEGF may also activate pathways associated with NO synthesis and thus induce vasodilation, improving blood supply to cardiac cells. Thus, we demonstrated that chronic Epo deficiency induces a cardiac angiogenesis probably mediated by HIF-1α/VEGF (Figure 1) and Epo-R pathways, which could optimize O₂ supply and limit the consequences of chronic anemia on cardiac cells.

The effects of both chronic Epo deficiency and hypoxia on myocardial contractile function and cardioprotective processes were investigated using Epo-TAg male mice. The initiation of cardioprotective mechanisms was estimated through Epo-R/P-STAT5 signalization as well as by a change in the P-STAT5/STAT5 ratio in response to chronic Epo deficiency and/or hypoxia. When 14 days of CHx were added to Epo deficiency, the expected cardiac hypertrophy was reduced, and cardiac output could not catch up with the O₂ demand. Systolic blood pressure did not increase indicating that systemic afterload was not responsible for the decrease in cardiac output. Moreover, CHx did not significantly affect right ventricular hemodynamics, and Epo-TAg mice did not develop pulmonary hypertension. Therefore, the decrease in cardiac output was not the consequence of right ventricular failure. After CHx, Epo-TAg mice displayed a lower LV hypertrophy than the normoxic anemic mice, which could account for the decrease in cardiac output and O₂ delivery. Furthermore, our data showed a mild alteration of diastolic and systolic LV function. These results suggested that altered myocardial function in Epo-TAg mice exposed to CHx could participate in the failure of cardiac adaptation in this severe condition.

Although hypoxia or Epo-deficiency leads to an overexpression of HIF-1α, VEGF (Figure 1), Epo, and Epo-R, we did not observe a synergic effect of these combined constraints on the heart of Epo-TAg mice exposed to CHx, except for P-STAT5/STAT5 ratio. However, this ratio was lower in Epo-TAg than in WT mice exposed to CHx, suggesting that the activation of the cardioprotective pathways downstream the Epo/Epo-R system may represent a limiting step. As we found a decrease in LV hypertrophy and functional LV adaptation, a depressed HIF-1α/VEGF pathway (Figure 1) as well as a reduced O₂ delivery, we suggested that cardiac adaptive mechanisms that take place with chronic Epo deficiency and hypoxia might require extensive Epo effects (angiogenesis, cardioprotection) on the heart.

### Ventilatory and metabolic characteristics

Our first publication on the Epo-TAg mice model showed a greater ventilation in normoxia in these anemic mice as compared to WT animals. This difference was mainly due to a larger tidal volume. However, more recently, we have not observed a baseline ventilatory adaptation in Epo-TAg male mice in normoxia (Table 1, Figure 2) when compared with that of WT mice. Some explanations could be proposed to explain this difference. First, in the study of Macarlupu et al, the control animals were classical WT animals and not littersmates, and it is well known that the environmental conditions during the first day of life could induce significant changes in ventilatory variables. Second, the method used to measure ventilatory variables can induce slight differences, according to the plethysmograph used, for example. Third,

### Table 1 Characteristics of wild-type and Epo-TAg male mice in normoxic conditions

<table>
<thead>
<tr>
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<th>References</th>
<th>Wild-type mice</th>
<th>Epo-TAg mice</th>
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<tbody>
<tr>
<td>Hemoglobin (g.dL⁻¹)</td>
<td>Macarlupu et al</td>
<td>17±0.3</td>
<td>6.9±0.3</td>
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<tr>
<td>Hematocrit (%)</td>
<td>Macarlupu et al</td>
<td>54±0.8</td>
<td>24±1.6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>El Hasnaoui-Saadani et al</td>
<td>548±27</td>
<td>548±46</td>
</tr>
<tr>
<td>Stroke volume (µL.g⁻¹)</td>
<td>El Hasnaoui-Saadani et al</td>
<td>1.9±0.19</td>
<td>3.2±0.68</td>
</tr>
<tr>
<td>Cardiac output (mL.min⁻¹.g⁻¹)</td>
<td>El Hasnaoui-Saadani et al</td>
<td>0.94±0.14</td>
<td>1.76±0.43</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>El Hasnaoui-Saadani et al</td>
<td>96.7±8.5</td>
<td>94.2±5.8</td>
</tr>
<tr>
<td>Right ventricular weight (mg)</td>
<td>Macarlupu et al</td>
<td>23±1</td>
<td>33±2</td>
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<tr>
<td>Left ventricular and septum weight (mg)</td>
<td>Macarlupu et al</td>
<td>80±2</td>
<td>113±3</td>
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<tr>
<td>Fulton ratio</td>
<td>Macarlupu et al</td>
<td>0.28±0.013</td>
<td>0.297±0.010</td>
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<td>Minute ventilation (mL.min⁻¹.g⁻¹)</td>
<td>Voituron et al</td>
<td>2.26±0.48</td>
<td>2.17±0.53</td>
</tr>
<tr>
<td>Respiratory frequency (c.min⁻¹)</td>
<td>Voituron et al</td>
<td>261±34</td>
<td>284±54</td>
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<tr>
<td>Tidal volume (µL.g⁻¹)</td>
<td>Voituron et al</td>
<td>8.63±1.26</td>
<td>7.62±1.09</td>
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<td>Resting oxygen consumption (mL.min⁻¹.kg⁻¹)</td>
<td>Macarlupu et al</td>
<td>93.3±4.7</td>
<td>96.8±6.5</td>
</tr>
<tr>
<td>Maximal oxygen consumption (mL.min⁻¹.kg⁻¹)</td>
<td>Macarlupu et al</td>
<td>270.7±22.0</td>
<td>210.2±12.3</td>
</tr>
</tbody>
</table>

**Note:** Indicates significant difference between wild-type and Epo-TAg mice. *Data presented as mean±SEM. Data presented as mean±SD.

**Abbreviations:** Epo, erythropoietin; Epo-TAg mice, Epo-deficient mice; SD, standard deviation; SEM, standard error of the mean.
it could be possible that Epo-TAg⁸ mice could have exhibited some epigenetic adjustments between 2006 and 2014. Indeed, more than 30 generations of mice have lived between the first and the last experiment, and it is not impossible that epigenetic changes would improve the whole O₂ transport steps, and thus, ventilatory adaptation could appear in the last generations of mice.⁹²

In female Epo-TAg⁸ mice, we observed a difference in respiratory frequency and minute ventilation with larger values for the Epo-TAg⁸ mice as compared with those of WT mice (unpublished data). There is no change in resting O₂ consumption (VO₂) in Epo-TAg⁸ mice, while VO₂ max is only 30% reduced (Table 1) as compared with that of WT mice,⁹² despite a 60% reduction in hemoglobin concentration. The normal resting VO₂ in anemic Epo-TAg⁸ mice could also be explained by the elevated cardiac output associated with better tissue extraction of O₂,⁸⁶ which could compensate for the decrease in O₂ transport capacity. Severe anemia also generally induces a reduction in physical performance.⁹³–⁹⁵ In Epo-TAg⁸ mice, the reduction in VO₂ max was only moderate, suggesting compensatory mechanisms such as an increase in maximal cardiac output, augmented capillarization, and better O₂ extraction.

The normal ventilatory response to acute hypoxia is characterized in adult mammals by a hyperventilation followed by a relative ventilatory decline named “roll off”⁹⁶,⁹⁷ If hypoxia persists, an increase in ventilation occurs (ventilatory acclimatization to CHx),⁹⁸ which is accompanied by an increase in the sensitivity of the respiratory control system.⁹⁹–¹⁰¹ Epo-TAg⁸ mice displayed neither ventilatory response to acute hypoxia nor ventilatory acclimatization to CHx (Figure 2). However, after 14 days of exposure to chronic hypoxia, Epo-TAg⁸ mice increased their ventilation when exposed acutely to a hypoxic stress (Figure 2;
8% O₂, 5 minutes). These results differ from those previously published. We cannot exclude the fact that our transgenic mice, along generations, developed adaptation strategies to cope with Epo deficiency and/or chronic anemia.

**Brain adaptations**

Epo deficiency in Epo-TAg<sup>h</sup> mice leads to cerebral adaptations (Figures 3 and 4). Indeed, in the brain of these normoxic mice, we observed an increase in the transcript and the protein levels of HIF-1α, VEGF (Figure 3), Epo-R (Figure 4), and P-STAT-5/STAT-5 ratio accompanied with an increase in cerebral capillary density. Taken together, these data suggest that Epo-TAg<sup>h</sup> mice have developed cerebral angiogenesis, probably via the HIF-1α/VEGF pathway (Figure 3), optimizing O₂ diffusion as previously described. Furthermore, the increase in P-STAT-5/STAT-5 ratio in the brain suggests neuroprotective mechanisms and angiogenesis with a decrease in apoptosis and an increase in cell proliferation. Overall, these results illustrate the direct and indirect effects of Epo in terms of O₂ delivery improvement and the activation of neuroprotective mechanism to counteract the lack of Epo in the brain.

**Skeletal muscles**

Our model of transgenic Epo-TAg<sup>h</sup> mice has allowed us to study the role of Epo on skeletal muscle development, angiogenesis, and acclimatization to hypoxia. Our main

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**Figure 3 Cerebral angiogenesis in Epo-TAg<sup>h</sup> mice.**

**Notes:** Immunohistological detection of HIF-1α and VEGF at the sensory cortex level in normoxia (Nx exposed) and after chronic hypoxia exposure (Hx exposed) in WT (1, 3, 5, 7) and Epo-TAg<sup>h</sup> mice (2, 4, 6, 8). Arrowheads and arrow indicate HIF-1α (1) and VEGF (5)-positive cells, respectively. In normoxia, Epo-TAg<sup>h</sup> mice showed an increase in HIF-1α (2) and VEGF (6)-positive cells suggesting an enhancement of cerebral angiogenesis through the HIF-1α/VEGF pathway. In WT mice, chronic hypoxia led to an increase in HIF-1α (3) and VEGF (7), while they led a decrease in Epo-TAg<sup>h</sup> mice (4, 8). Adapted from *Am J Physiol Regul Integr Comp Physiol* Volume 296(3). El Hanaoui-Saadani R, Pichon A, Marchant D, et al. Cerebral adaptations to chronic anemia in a model of erythropoietin-deficient mice exposed to hypoxia. Pages: R801–R811. Copyright 2009.

**Abbreviations:** Epo, erythropoietin; Epo-TAg<sup>h</sup> mice, Epo-deficient mice; HIF-1α, hypoxia-inducible factor-1α; Hx, hypoxia; Nx, normoxia; VEGF, vascular endothelial growth factor; WT, wild type.

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**Figure 4 Effect of Epo deficiency on Epo-R expression in cerebral cortex.**

**Notes:** Quantitative determination of Epo-R in the cerebral cortex of WT and Epo-TAg<sup>h</sup> mice in Nx and following AHx and CHx. Epo-R mRNA ([A](#)) and protein level ([B](#)) are shown next to their corresponding protein bar graphs. Representative Western blot of Epo-receptor (Epo-R) ([C](#)). Values are expressed as mean ± SD. *P < 0.05 vs Nx WT. According to *Am J Physiol Regul Integr Comp Physiol* Volume 296(3). El Hanaoui-Saadani R, Pichon A, Marchant D, et al. Cerebral adaptations to chronic anemia in a model of erythropoietin-deficient mice exposed to hypoxia. Pages: R801–R811. Copyright 2009.

**Abbreviations:** AHx, acute hypoxia; CHx, chronic hypoxia; Epo, erythropoietin; Epo-R, Epo receptor; Epo-TAg<sup>h</sup> mice, Epo-deficient mice; Nx, normoxia; SD, standard deviation; WT, wild type; PC, peptide control.
results displayed a developmental adaptation to Epo deficiency and/or chronic anemia by an improvement of microvessel network (Table 2) in both fast and slow skeletal muscles.83 This adaptation of Epo-TAg mice was not accompanied by any difference in skeletal muscles for contractile structure, metabolism, maximal strength, fatigability, contraction time, and relaxation time. Moreover, we never observed any overexpression of HIF-1α or VEGF protein. The discrepancy between our results and those of Mille-Hamard et al’s team85 could be explained by a genetic developmental adaptation to chronic anemia in our transgenic model. In the Mille-Hamard et al’s transgenic model, Epo deficiency is induced by vaccination of adult mice, so that developmental adaptation has not occurred. When exposed to severe chronic hypobaric hypoxia (4,300 m) during 14 days, skeletal muscles of Epo-TAg mice were not submitted to deconditioning. Neither skeletal muscle phenotype nor skeletal muscle functions were altered compared with those of WT mice.104 In the skeletal muscles of our Epo-TAg model, we did not detect Epo mRNA in both normoxia and hypoxia. Moreover, the hypoxia-induced elevation of circulating Epo was not correlated with an increase in Epo concentration in skeletal muscle. Overall, these results favor the hypothesis of an indirect effect of Epo on skeletal muscles. We clearly show that, in our model, the deficit in oxygenation caused by anemia is responsible for the main change in skeletal muscles such as the improvement of microvessel network (Table 3). Unfortunately, we cannot study the regeneration process of the Epo-TAg model, because in this mutant, Epo has been replaced by antigen T, which has been shown to alter skeletal muscle regeneration.105

### Limits of our models

Experiments were performed on whole-body Epo-TAg mice13 that display a very low hematocrit (20%) and did not develop polycythemia after CHx.81 Therefore, it is important to note that our model of transgenic mice combines the effects of chronic anemia (low O2 content) and the effects of chronic Epo deficiency. To differentiate the respective effect of each constraint is rather difficult since chronic anemia itself is a consequence of chronic Epo deficiency. However, it could be speculated from the activation of HIF/VEGF systems that the reduction in tissue O2 delivery (and therefore chronic anemia) is the main trigger of the observed adaptations.

### Conclusion

For many years, Epo was mainly considered a growth factor for erythropoiesis and a determinant factor for the acclimatization to CHx only through an increase in O2 transport capacity. It appears from recent studies that it may also participate in the acute and chronic responses to hypoxia (Table 2) through the activation of Epo-Rs in various organs (brain, heart, muscle, chemoreceptors). However, there is still a lot of debates and uncertainties about the presence and functionalities of these receptors. Our model of Epo-TAg mice may help to unravel a possible key role of Epo in O2 homeostasis. Indeed, our studies demonstrate that high levels of Epo are not necessary for survival in chronic moderate hypoxia. Moreover, we showed that Epo could play a key-regulating role in the neural control of ventilatory acclimatization to hypoxia and hypoxic ventilatory response probably via a catalyzing role on the NO central pathway. We also demonstrated that chronic Epo deficiency induced cerebral and cardiac angiogenesis, which could have synergetic effects not only in neuro- and cardioprotection but

### Table 2 Main characteristics of Epo-TAg mice when compared with those of wild-type mice

<table>
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<tr>
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<th>Blood</th>
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<td>Normoxia</td>
<td>Low Epo, low Hb</td>
<td>High CO, high angiogenesis</td>
<td>Low HVR</td>
<td>High angiogenesis</td>
<td>High microvessel network</td>
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<td>Acute hypoxia</td>
<td>Low Epo, low Hb</td>
<td>Low cardioprotection</td>
<td>Low HVR</td>
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<td>Low VAH</td>
<td>High angiogenesis</td>
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</table>

**Abbreviations:** CO, cardiac output; Epo, erythropoietin; Epo-TAg mice, Epo-deficient mice; Hb, hemoglobin; HVR, hypoxic ventilatory response to hypoxia; VAH, ventilatory acclimatization to hypoxia.

### Table 3 Indices of the microvascular supply of the deep region of gastrocnemius muscle fibers in normoxia and after chronic hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Chronic hypoxia</th>
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</tr>
<tr>
<td>Capillary density (cap/mm²)</td>
<td>1699±350</td>
<td>1587±180</td>
</tr>
<tr>
<td>Capillary-to-fiber ratio</td>
<td>2.23±0.29</td>
<td>2.69±0.29*</td>
</tr>
<tr>
<td>Number of capillaries around single fibers</td>
<td>5.20±0.78</td>
<td>6.27±0.54*</td>
</tr>
<tr>
<td>Individual capillary-to-fiber ratio</td>
<td>2.16±0.26</td>
<td>2.46±0.35*</td>
</tr>
</tbody>
</table>

**Notes:** Indicates significant difference between WT and Epo-TAg mice. Data presented as mean ± SD.

**Abbreviations:** Epo, erythropoietin; Epo-TAg mice, Epo-deficient mice; WT, wild type.
also in O2 supply optimization, in order to limit the consequences of chronic anemia on cerebral and cardiac tissues. However, under both constraints (chronic Epo deficiency and hypoxia), angiogenesis, neuroprotective, and cardioprotective pathways along with a functional LV adaptation failed to occur, showing the limits of these adaptive processes in heart and brain, but more importantly suggesting a crucial role of Epo in main physiological functions.

Some future areas of research could focus on the role of Epo as a global regulator of the cardioventilatory adaptations from erythropoietin synthesis, blood hemorheology, blood volume regulation, and ventilatory control.106 Epo seems to be able to protect tissues (cardiomyocytes, lung, neurons) from various aggressions such as hypoxia, ischemia–reperfusion, or inflammation.107 Moreover, there are few data on the possible effect of Epo on the ventilatory response to hypercapnia in contrary to the response to hypoxia. The role of Epo/Epo-R on the oxidative stress needs also to be studied later as this is central on tissue antiapoptosis properties.

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Author contributions
All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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