Cerebral adaptations to chronic anemia in a model of erythropoietin-deficient mice exposed to hypoxia

Raja El Hasnaoui-Saadani, Aurélien Pichon, Dominique Marchant, Paul Olivier, Thierry Launay, Patricia Quidu, Michèle Beaudry, Alain Duvallet, Jean-Paul Richalet, and Fabrice Favret

Université Paris 13, EA 2363 “Réponses Cellulaires et Fonctionnelles à l’Hypoxie”, Association pour la Recherche en Physiologie de l’Environnement, Bobigny, France

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control of cerebral circulation could occur to maintain oxygen delivery. Hence, it was of interest to understand the mechanisms underlying acclimatization to hypoxia in the brain of an Epo-deficient animal submitted to both anemic and hypoxic constraints. Moreover, the response of Epo-TAg<sup>b</sup> mice to chronic hypoxia may provide useful information about the role of Epo in acclimatization to hypoxia at the cerebral level. We, therefore, hypothesized that Epo-TAg<sup>b</sup> mice in normoxia could develop cerebral cellular responses and angiogenesis, which could be overexpressed in hypoxia.

**MATERIALS AND METHODS**

**Animals**

All procedures were performed in agreement with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publications No. 85-23, revised 1996) and with the approval of the French “Ministère de l’Agriculture” guidelines (Authorization number: A-93-008-01).

For this study, male wild-type (Bl6/CBA) mice (Charles River, L’Arbresle, France) and male Epo-TAg<sup>b</sup> mice, 6 to 8 weeks old, were divided into six groups: 1) normoxic Epo-TAg<sup>b</sup> (Nx Epo-TAg<sup>b</sup>); 2) normoxic wild-type (Nx WT); 3) acute hypoxic Epo-TAg<sup>b</sup> (AHx Epo-TAg<sup>b</sup>); 4) acute hypoxic wild-type (AHx WT); 5) chronic hypoxic Epo-TAg<sup>b</sup> (CHx Epo-TAg<sup>b</sup>); and 6) chronic hypoxic WT (CHx WT). Mice undergoing acute or chronic hypoxic exposure were housed for 1 or 14 days in a hypoxic chamber connected to a gas pump where air circulated at a pressure of 420 mmHg (−4.2 mN). Hypobaric hypoxia was maintained by a vacuum source at flow rates sufficient to prevent CO<sub>2</sub> buildup. The chamber was returned to sea level (760 mmHg) three times a week for 30 min to clean the cages and to feed and give water to the animals. Normoxic groups were kept outside the hypobaric chamber at 760 mmHg. All animals had free access to food and water at all times. At the end of hypoxic exposure, animals were sacrificed by cervical dislocation. Mice were then decapitated, the brain was rapidly removed, and the cerebral cortex was quickly dissected, frozen in liquid nitrogen, and stored at −80°C until use for protein and mRNA analysis. For the immunohistochemistry method, the whole brain was dissected. All procedures were done on ice to avoid protein degradation. We measured body and cortex weights and hemoglobin concentration (OSM 3, Radiometer, Copenhagen, Denmark).

**Plasma and Brain Mouse Epo Immunoassay**

In addition to the hemoglobin and hematocrit, plasma and brain Epo concentrations were measured to test the Epo-TAg<sup>b</sup> model. The quantitative determination of mouse Epo concentration in plasma and brain extracts was assayed by ELISA using the rat antiswine mouse Epo immunoassay (R&D Systems Europe, Abingdon, UK). Plasma collection and assay procedure were carried out following the manufacturer’s instructions. Briefly, Nx WT (<i>n</i> = 6), Nx Epo-TAg<sup>b</sup> (<i>n</i> = 5), AHx WT (<i>n</i> = 6), and AHx Epo-TAg<sup>b</sup> (<i>n</i> = 5) were used for this experiment. The plasma was collected using heparin as an anti-coagulant, centrifuged at 13,000 g, 15 min at 4°C, and stored at −20°C. Mice were killed by cervical dislocation, the brain was rapidly removed, frozen in liquid nitrogen, and stored at −80°C until used. Brains were homogenized in a very little volume of PBS (1:3 wt/vol) to concentrate total proteins, centrifuged for 10 min at 5,000 g at 4°C and directly stored at −20°C. After two freeze-thaw cycles to break up the cell membranes, brain homogenates were assayed without prior dilution, while plasma samples required a twofold dilution in a calibrator diluent provided in the kit. A standard curve was done in duplicate using a stock solution of Epo. Linear regression was fitted to twofold dilution series ranging from 3,000 to 0 pg/ml. In parallel, 50 μl of sample were added to 50 μl of assay diluent specific for mouse samples into each well of a microplate coated with a monoclonal antibody against mouse Epo and incubated for 2 h on a microplate shaker at room temperature. A positive control consisting of a recombinant Epo solution, provided in the kit, was also loaded. After four washes, 100 μl of a monoclonal antibody against mouse Epo conjugated to horseradish peroxidase (HRP) were added to each well and incubated for 2 h on a microplate shaker at room temperature. After four washes, 100 μl of substrate solution were added and incubated for 30 min at room temperature on the benchtop in the dark. At the end of this time, the reaction was stopped using 100 μl of a stop solution in each well. The optical density was measured at 450 nm. The quantity of Epo (pg/ml) detected in each sample was compared to the Epo standard curve. Finally, data were expressed as the ratio of the quantity of Epo (in pg) to that of total protein (in mg) for brain cortex.

**Cerebral Cortical RNA Extraction and Real-Time RT-PCR**

For mRNA study of HIF-1α, VEGF, EpoR, and for eNOS, iNOS, and nNOS, 5 or 6 animals per group were used, and all conditions were tested. Total RNA was isolated from the isolated cortex of mice by TRI-Reagent (Invitrogen, Carlsbad, CA) and digested with RNase-free DNase I (Invitrogen) for 1 h at 30 min at room temperature to remove any contaminating genomic DNA. cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions [5 μg total RNA/20 μl cDNA synthesis reactions, 50 μM oligo(dT), 10 mM dNTP mix], and RT products were stored at −20°C until used. Before usage, the cDNA was diluted in ultraPure water.

**Real-time RT-PCR.** The Light Cycler FastStart DNA Master SYBR Green I (Roche Biochemicals, Stockholm, Sweden) was used for quantitative analyses of the generated cDNA. cDNA sequences of genes of interest, obtained from GenBank, were used to design gene-specific PCR primers. Primer sequences used in this study are described here from 5′ to 3′: forward: AGCCCTTAGTGCTTG-TGA and reverse: TATCGAGGCGTTGTCGACTG for HIF-1α (Tm = 62°C, NM_014031) forward: CCACGACATAGAGAAGAT-GACG, and reverse: CAAGGCTCACAAGTTGTCTTCTTG for VEGF (Tm = 63°C, NM_008752). forward: CAGCTGCGGCTGTA-GAAGCT and reverse: CATTGGAATGGAAGAATTTG for EpoR (Tm = 62°C, NM_014031) forward: GGGAAACCTCTCG-GAGGAGA and reverse: TGAGGCTGACCCCAAGATAG for nNOS (Tm = 63°C, NM_008712) forward: TGGCAGCCCCCAAGACTTACG and reverse: AGTCCGAAAAATGTCTCCTG for iNOS (Tm = 63°C, NM_008713) forward: GCTCCGGGATG-GACCTCA and reverse: GAGGTCCTGTCGAGGCTACT for eNOS (Tm = 63°C, NM_008713) forward: GCTCCGGGATGGACCTCA and reverse: CAAATGATGACTGAGGCTC for actin (Tm = 62°C, NM_0011149) forward: AGAGGGAATCGTGGCTGACG and reverse: CAATGATGACTGAGGCTC for actin (Tm = 62°C, NM_0011149) PCR amplifications were performed in a total volume of 20 μl, containing 5 μl cDNA sample, 4 μl LightCycler FastStart DNA Master SYBR Green I, 0.5 μM of each primer, and 2.5 mM MgCl<sub>2</sub>. For each reaction, the polymerase was activated by a preincubation at 95°C for 10 min, and amplification was then performed for 45 cycles of switching between 95°C for 10 s, gene-dependent Tm for 5 to 8 s, and 72°C for 8 to 15 s depending on the length of the amplicon and followed by melting point analysis from 65 to 95°C. The results were represented as threshold cycle numbers (C<sub>T</sub> values). Control cDNA of normoxic wild-type mouse cortex was diluted 1:10 to create standard curves by plotting C<sub>T</sub> values vs. the dilution of the cDNA templates. Relative amounts of mRNA, normalized by β-actin were calculated from C<sub>T</sub> values, according to the manufacturer’s description (Roche Biochemicals, Stockholm, Sweden).

**Calculation of relative quantification values.** The relative quantification values were calculated according to the manufacturer’s description (Roche Biochemicals, Stockholm, Sweden). The C<sub>T</sub> represents the PCR cycle at which an increase in fluorescence above a baseline signal can be detected. C<sub>T</sub> value was used to calculate the amount of PCR product compared with the internal control, β-actin.
The β-actin’s C_r value was subtracted from the gene C_r value to evaluate the mean change in C_r in each experimental group.

Cerebral Cortical Protein Extraction and Western Blot Analysis

HIF-1α and EpoR measurements. Cerebral cortex samples of wild-type and Epo-TAg<sup>h</sup> mice exposed in the different oxygenation conditions (six animals in each group) were homogenized in ice-cold buffer (20 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1 mM NaCl, 0.2 mM DTT, 0.5 mM NaVO<sub>4</sub>, pH 7.5) supplemented with a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Homogenates were centrifuged at 10,000 g of protein of each sample studied were loaded on a min at 4°C. Then, Supernatants were centrifuged at 10,000 g for 30 min at 4°C. Then, Supernatants were collected at −20°C until use (7). eNOS, iNOS, and nNOS measurements. Samples (Nx WT n = 7, Nx Epo-TAg<sup>h</sup> n = 7, AhX WT n = 6, AhX Epo-TAg<sup>h</sup> n = 6, ChX WT n = 7, ChX Epo-TAg<sup>h</sup> n = 7) were homogenized in an ice-cold buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, pH 7.2) supplemented with a protease inhibitor cocktail (Sigma Aldrich). Then, homogenates were incubated for 30 min at 4°C before the centrifugation at 13,000 g for 30 min at 4°C. Supernatants were collected and stored at −20°C until used (12).

STAT-5 and phospho-STAT-5 measurements. Cerebral cortex samples (6 animals in each group) were homogenized in an ice-cold lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS and 10% glycerol) supplemented with a protease inhibitor cocktail (Sigma Aldrich). Homogenates were centrifuged at 13,000 g for 10 min at 4°C. Supernatants were collected, 0.05% β-mercaptoethanol were added in each sample and stored at −20°C until used.

Supernatant protein content was determined by Bio-Rad protein assay using BSA as a standard.

To compare protein expression between all studied groups, equal amounts of proteins from animals of the same group were loaded on the same gel. Thus, 100 µg of protein of each sample studied were separated by electrophoresis on 7.5% or 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA). In addition, positive controls were also loaded, and precision prestained standards were used as molecular weight markers (Bio-Rad Laboratories).

Membranes were kept overnight in 5% BSA/TBS-0.5% Tween 20 at 4°C to block nonspecific binding. Membranes were then incubated overnight at 4°C with each primary antibody diluted in 1% BSA/TBS-0.5% Tween 20 (TBS-T). Table 1 summarizes antibodies, positive controls and dilutions used for western blotting and immunohistochemistry. Membranes were washed with TBS-T and incubated for two hours at room temperature with either anti-rabbit, or anti-goat or anti-mouse IgG antibody-horseradish peroxidase conjugate depending on the species of the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and diluted respectively 1:500, 1:1000 and 1:500 in 1% BSA/TBS-0.5% Tween 20. Immunodetection was accomplished using ECL Western blot analysis kit detection (Santa Cruz Biotechnology). Membranes were then probed with anti β-actin (Santa Cruz Biotechnology) as internal control.

Densitometric scanning and image analysis, using ImagePro Plus 4.1 software, were done to quantify the specific protein expression of each sample compared to internal control. The density of each sample was referred to the density of the corresponding β-actin sample. Data were then expressed as the ratio of the control or the experimental density to that of the β-actin density.

Analysis of VEGF Concentration by ELISA

The quantity of VEGF released in the cerebral cortex samples from control and anemic mice following acute and chronic hypoxic exposure (seven animals in each group) was determined using the VEGF ELISA DuoSet Kit (R&D Systems Europe, Abingdon, UK). Supernatants were obtained by the homogenization of the samples in 0.25 M Tris buffer pH = 8 and centrifugation at 13,000 g for 15 min at 4°C. Measurements were performed according to the manufacturer’s instructions. Briefly, a 96-well microplate was coated with 100 µl of the diluted capture antibody (1:180 in PBS) and incubated overnight at room temperature. Then, the microplate was washed in PBS-Tween 0.05%, blocked by adding 300 µl of reagent diluent (1% BSA in PBS) and incubated at room temperature for 2 h. Then, 100 µl of samples or standards (recombinant mouse VEGF) were added into the wells for 2 h, and incubated with the detection antibody (goat anti-mouse VEGF, 1:720 in PBS) for 2 h at room temperature. After washing 3 times with PBS-Tween 0.05%, 100 µl of streptavidin-HRP was added into each well and incubated in the dark for 20 min at room temperature. Finally, 100 µl of substrate solution consisting of tetramethylbenzidine (Sigma Aldrich, St. Louis, MO) was added into each well and kept for 20 min at room temperature. The reaction was stopped by the addition of 50 µl of 2 N H<sub>2</sub>SO<sub>4</sub>. The optical density was measured at 450 nm. The quantity of VEGF (pg/ml) detected in each sample was compared to a VEGF standard curve. Finally, data were expressed as the ratio of the quantity of VEGF (in picograms) to that of total protein (in milligrams).

NO<sub>x</sub> Colorimetric Assay

The total amount of NO in the cerebral cortex samples (Nx WT n = 6, Nx Epo-TAg<sup>h</sup> n = 5, AhX WT n = 6, AhX Epo-TAg<sup>h</sup> n = 5, ChX WT n = 6, ChX Epo-TAg<sup>h</sup> n = 5) was assessed by the Griess reaction using a colorimetric assay kit (nitrates/nitrates colorimetric assay kit; Cayman Chemical, MI) that detects nitrite (NO<sub>x</sub>) and nitrate (NO<sub>3</sub>·), which are stable reaction products of NO. Because the relative proportion of each metabolite may vary, the index of total NO production is best assessed by their sum (NO<sub>x</sub>). Hence, homogenates

Table 1. Antibodies used for Western blotting and immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Source</th>
<th>Positive Controls</th>
<th>Western Blotting Dilutions</th>
<th>Immunohistochemistry Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF 1α (H1a67)</td>
<td>Mouse monoclonal</td>
<td>Novus Biologicals, (Littleton, CO)</td>
<td>K 562 Whole cell lysate</td>
<td>1/200</td>
<td></td>
</tr>
<tr>
<td>iNOS (M 19)</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>RAW LPS/PMCA cell lysate</td>
<td>1/500</td>
<td></td>
</tr>
<tr>
<td>nNOS (R 20)</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>Mouse brain extract</td>
<td>1/500</td>
<td></td>
</tr>
<tr>
<td>eNOS (C 20)</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>Rat brain extract</td>
<td>1/500</td>
<td></td>
</tr>
<tr>
<td>EpoR (M 20)</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>Jurkat whole cell lysate</td>
<td>1/500</td>
<td></td>
</tr>
<tr>
<td>STAT-5</td>
<td>Rabbit monoclonal</td>
<td>Cell signaling Technology (UK)</td>
<td></td>
<td>1/1000</td>
<td></td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Rabbit polyclonal</td>
<td>Biogenesis, (Sandown, NH)</td>
<td></td>
<td>1/100</td>
<td></td>
</tr>
<tr>
<td>HIF 1α</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td></td>
<td>1/200</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Rabbit polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td></td>
<td>1/200</td>
<td></td>
</tr>
<tr>
<td>Actin (I 19)</td>
<td>Goat polyclonal</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td></td>
<td>1/1000</td>
<td></td>
</tr>
</tbody>
</table>

HIF 1α, hypoxia inducible factor 1α; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; EpoR, erythropoietin receptors; GLUT1, glutamate 1; VEGF, vascular endothelial growth factor.

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and assay procedure were carried out following the manufacturer’s instructions. Briefly, cerebral cortex samples were homogenized in 4 volumes (wt/vol) of PBS (pH = 7.6) at 4°C, ultracentrifuged at 100,000 g for 60 min at 4°C. NOX determination was carried out in supernatants. The nitrate reductase reaction was first employed to convert nitrate to nitrite followed by Griess reaction in order to measure metabolites by photometric absorbance using an ELISA plate reader. The optical density was measured at 540 nm. The quantity of NOX (μM) detected in each sample was compared to a nitrite standard curve. Finally, data were expressed as the ratio of the quantity of NOX (in nanomoles) to that of total protein (in milligrams).

Immunohistochemistry

Section preparation. Four mice per group (Nx WT, Nx Epo-TAgsh, CHx WT and CHx Epo-TAgsh) were killed by cervical dislocation, brains were removed, and postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h at room temperature. Then, brains were rinsed in PBS, transferred to 70% ethanol, and embedded in paraffin. Ten-micrometer coronal brain sections were cut and mounted on slides using albumin water. Sections corresponding to plates 41–49 (−1.28 to −2.12 mm from bregma) of the mouse brain atlas were collected for the immunohistochemical studies of HIF-1α and VEGF and for cerebral angiogenesis analysis. Sections were deparaffinized and rehydrated, and antigen retrieval was performed during 10 min in a boiling citrate buffer (0.01 M, pH 6) using a microwave oven for GLUT-1 and VEGF staining and in citrate-EDTA buffer (0.01 M citrate, 2 mM EDTA, 0.05% Tween) for HIF-1α staining. Then, sections were rinsed in TBS-Triton X100 0.5% (TBS-Tx 0.5%) for 15 min at room temperature, and a rabbit polyclonal staining kit (Santa Cruz Biotechnology) was used to perform the staining. Sections were incubated in a peroxidase block solution for 10 min to inhibit endogenous peroxidase; then nonspecific sites were blocked in goat serum for 20 min. Sections were then labeled overnight at room temperature, and a rabbit polyclonal staining kit (Santa Cruz Biotechnology) was used to perform the staining. Sections were incubated in a peroxidase block solution for 10 min to inhibit endogenous peroxidase; then nonspecific sites were blocked in goat serum for 20 min. Sections were then labeled overnight at room temperature, and a rabbit polyclonal staining kit (Santa Cruz Biotechnology) was used to perform the staining. Sections were incubated in TBS-Tx 0.5% without primary antibody.

Cerebral capillary density. Four mice per experimental group were used (Nx WT, Nx Epo-TAgsh, CHx WT, CHx Epo-TAgsh). Capillary density sensory cortex analysis was performed on nine sections per mouse spanning about 120 μm in thickness, including sections for negative controls. Pictures were taken at ×40 magnification, and the number of GLUT-1-positive capillaries was determined using a grid. The area of this grid at ×40 lens was 0.065 mm2. The GLUT-1 positive capillaries inside the grid area were counted in each section alternatively in the right and left hemisphere of the cortex. The obtained values from four mice per group were averaged, and results were expressed as the mean number of capillaries per grid ± SE.

Immunohistochemical studies of HIF-1α and VEGF. Immunohistochemical staining of HIF-1α and VEGF in the cerebral cortex of the normoxic and chronically hypoxic Control and Epo-TAgsh mice was not quantified since quantitative determination of these proteins was performed using Western blot analysis and ELISA methods. All staining was done in duplicate, and the evaluation of the staining was performed by two blinded observers, as previously described (53).

Statistical Analysis

Statistical analysis for RT-PCR, Western blots, VEGF ELISA, NOx colorimetric assay, and immunohistochemistry were performed using a two-way ANOVA for two variables, hypoxia, and anemia. The Newman-Keuls was used as post hoc analysis to compare the mean values between control, hypoxic, and anemic mice. A Student’s t-test was exceptionally used for plasma and brain Epo measurements since half of the Epo-TAgsh mice group was below detection threshold, making impossible to perform a two-way ANOVA. All statistical analysis was done using the Statistica software (StatSoft, Tulsa, OK). A value of P < 0.05 was considered significant.

RESULTS

Body Weight, Cortex Weight, and Hemoglobin Concentration

Table 2 summarizes all the physiological data. Body and cortex weights were similar in all groups with or without hypoxia. Hemoglobin concentration was 60% lower in nonacclimatized Epo-TAgsh mice, demonstrating severe anemia. Exposure to hypoxia during 14 days induced a polycythemia in WT, while hemoglobin concentration in Epo-TAgsh mice did not change when compared to normoxic value.

Plasma and Brain Epo Measurements

In normoxic Epo-TAgsh mice, plasma and cerebral [Epo] of three mice were below detection threshold, while the other three mice showed very low results (<5 pg/ml) demonstrating the strong reduction of plasma and cerebral [Epo] (Table 3). In acute hypoxia, plasma [Epo] was increased in both groups but remained much lower in acute hypoxic Epo-TAgsh mice compared to WT. Brain [Epo] rose by 12-fold in WT and by twofold in Epo-TAgsh mice following acute hypoxia. Furthermore, brain [Epo] was dramatically reduced by 20-fold in hypoxic Epo-TAgsh mice compared with hypoxic WT.

Effect of Anemia in the Cerebral Cortex of Normoxic Epo-TAgsh Mice

In normoxia, Epo-TAgsh mice showed a significant increase in cerebral cortical mRNA and protein expression of HIF-1α and VEGF (Fig. 1, P < 0.05). A strong positive immunostaining was observed in the anemic cerebral cortex (Fig. 2A, pictures 2 and 6) compared with small levels of HIF-1α and VEGF proteins detected in normoxic WT brains (Fig. 2A, pictures 1 and 5). Indeed, a very low level of HIF-1α protein was detected by Western blot analysis in normoxic WT mice.
Table 3. Plasma and brain Epo concentration in WT and Epo-Tagh mice in normoxia and after acute hypoxia

<table>
<thead>
<tr>
<th></th>
<th>WT (Nx)</th>
<th>Epo-Tagh (AHx)</th>
<th>WT (AHx)</th>
<th>Epo-Tagh (CHx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Epo, pg/ml</td>
<td>122±16</td>
<td>460±39*</td>
<td>0.39±0.17</td>
<td>4.68±0.42*</td>
</tr>
<tr>
<td>Brain Epo, pg/mg of total protein</td>
<td>162±25</td>
<td>0.12±0.06*</td>
<td>0.23±0.05*</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05, vs. NX-wild type; &P < 0.05 vs. NX Epo-Tagh; @ P < 0.001 CHx Epo-Tagh vs. CHx-wild type.

Effect of Hypoxia in the Cerebral Cortex of WT Mice

The acute hypoxic control mice demonstrated an increase in gene and protein expressions of all the studied molecules except in the phospho-STAT-5/STAT-5 ratio. In chronic hypoxia, HIF-1α (Fig. 1, A–C, P < 0.05), EpoR, phospho-STAT-5, the phospho-STAT-5/STAT-5 ratio (Fig. 3, A–C, G, P < 0.05), nNOS, and iNOS (Fig. 4) were higher than in normoxic WT. Immunohistochemical studies of both proteins showed no visible differences between normoxia and chronic hypoxia in WT mice. In addition, STAT-5 (Fig. 3D, P < 0.05) and NOx (Fig. 4J, P < 0.05) and returned to basal normoxic values after chronic hypoxia. However, the number of GLUT-1-positive capillaries was significantly increased in the cerebral cortex of acclimatized WT mice (+36%, Fig. 2B, picture 11, P < 0.05).

Effect of Hypoxia in the Cerebral Cortex of Epo-Tagh Mice

HIF-1α mRNA expression was significantly decreased in acute hypoxia (Fig. 1A, P < 0.05), while the protein expression was unchanged when compared with normoxic Epo-Tagh mice (Fig. 1B). Following chronic hypoxia, HIF-1α protein was significantly lower in Epo-Tagh mice compared to both normoxia and acute hypoxia (Fig. 1B, P < 0.05). VEGF protein in chronic hypoxic Epo-Tagh mice was also significantly decreased when compared with normoxic Epo-Tagh mice. In agreement with the Western blot and ELISA results, little positive staining for HIF-1α and VEGF was observed after 14 days of hypoxia, which appeared less important than normoxia. Moreover, no difference was observed in the cerebral capillary density, neither between normoxic and hypoxic Epo-Tagh mice nor between hypoxic WT and Epo-Tagh mice. EpoR mRNA expression was significantly decreased in acute and chronic hypoxia (Fig. 3, A and B, P < 0.05) but without a corresponding change in its protein level. No difference was observed between normoxia and hypoxia for STAT-5 protein expression, while phospho-STAT-5 (Fig. 3E, P < 0.05) and the ratio were dramatically reduced following chronic hypoxia (Fig. 3G, P < 0.05).
Conversely, Epo-TAg$^b$ mice showed an increased mRNA and protein expression of eNOS in acute hypoxia compared with their normoxic counterparts (Fig. 4, G and H, \( P < 0.05 \)), while nNOS and iNOS expression did not vary with hypoxia. In addition, NOx was the only parameter increased in Epo-TAg$^b$ mice with chronic hypoxia (\( \times 240\% \), Fig. 4J, \( P < 0.01 \)) and was much higher than in WT mice (\( \times 85\% \), Fig. 4J, \( P < 0.05 \)).

**DISCUSSION**

The aim of this study was to determine whether chronic anemic mice (Epo deficient) develop cerebral cellular responses and angiogenesis involving HIF-1\( \alpha \) and EpoR signaling pathways in normoxia and hypoxia. The current study demonstrated for the first time that chronic anemia: increased cerebral cortical mRNA and protein expression of HIF-1\( \alpha \), which could lead to VEGF transcription associated with cerebral angiogenesis. Chronic anemia also led to EpoR activation, as demonstrated by the increase in the protein expression of phospho-STAT-5 and in the ratio phospho-STAT-5/STAT-5. As previously shown, exposure to acute hypoxia resulted in an increase in HIF-1\( \alpha \)-activated factors (i.e., Epo, VEGF, iNOS) in WT mice, while these proteins were not higher with longer exposure. However, cerebral capillary density and EpoR pathway, evaluated by the activation of the phospho-STAT-5/STAT-5 ratio, were raised in chronic hypoxic WT mice. In Epo-TAg$^b$ mice, the main results concerned the increase in NOx and the reduction of HIF-1\( \alpha \), VEGF, and of the ratio phospho-STAT-5/STAT-5 in chronic hypoxia, while no change was observed in cerebral capillary density.

A dramatic reduction of plasma and brain [Epo] levels was found in normoxic, as well as in acute hypoxic Epo-TAg$^b$ mice compared to WT mice, resulting in a lower [Hb]. While [Epo] is strongly reduced in Epo-TAg$^b$ mice, EpoR and phospho-STAT-5, as well as the phospho-STAT-5/STAT-5 ratio are increased. This is consistent with a previous report that described the induction of EpoR expression following anemic and ischemic stress (2, 3). These results provide evidence that chronic anemia could activate the JAK/STAT signaling pathway probably to offset the decrease in [Epo], promoting its effects, such as neuroprotection (27, 32) and angiogenesis (2, 32) through the prevention of apoptotic cell death and cell proliferation. In addition, we speculated that JAK2 could also activate NF-\( \kappa \)B, another transcription factor involved in neuroprotection through overexpression of Bcl-2 and Bcl-xL, which also prevents apoptotic cell death (10). However, because Epo concentration is severely reduced in these mice, we...
cannot exclude the possibility that other cytokines, growth factors, or vasoactive peptides could also activate JAK-STAT signaling pathway (13). This study demonstrates that chronic anemia results in an increase in cerebral cortex capillarization in normoxia, potentially mediated by EpoR and HIF-1α signaling pathway, including VEGF. Acute anemia has been shown to upregulate HIF-1α and VEGF proteins in cerebral cortex (35).

Our study shows for the first time that chronic anemia also upregulates proteins and mRNA of these factors. The increase in HIF-1α mRNA suggests that mediators, including growth factors, cytokines, and vascular hormones could contribute to HIF-1α transcription in chronic anemic mice (9, 44). The stabilization and accumulation of HIF-1α lead to overexpression of HIF-1α-responsive genes, including VEGF, which enhance angiogenesis in various conditions, including anemia (11, 43). Surprisingly, iNOS expression (mRNA and protein) is unchanged in spite of HIF-1α accumulation. Recently, Trollmann et al. (53) have also observed a differential response to HIF-1α-responsive genes, with a reduction in iNOS, while VEGF increased in hypoxic neonatal mice. Because iNOS is also modified in response to proinflammatory cytokines, it is difficult to conclude about its lack of change in chronic anemia. A rise of iNOS expression could lead to altered HIF-1α activity (61). It cannot be excluded that iNOS is not increased in order to preserve the other HIF target genes. Although we found an increase in nNOS, as previously described (35), no corresponding change in cerebral NO metabolites was observed.

These results suggest that adaptive mechanisms that take place with acute anemia could be maintained with chronic anemia and could result in angiogenesis involving HIF-1α and VEGF. It cannot be excluded that the activation of the Epo receptor pathway could also contribute to angiogenesis and neuroprotection. It appears that chronic anemia activates some hypoxia-responsive elements to offset the fall in [Epo].

In acute hypoxia, WT mice developed expected physiological responses such as an increase in cerebral NO following a rise in NOS isoform expression, probably allowing a rise in CBF (16, 22, 23). In our study, eNOS expression increased in acute hypoxia and returned to baseline in chronic hypoxia conversely to nNOS and iNOS, which remained elevated. This is inconsistent with reports in which eNOS expression decreased in response to acute hypoxic exposure (54). Serrano et al. (48) have shown that eNOS increased in cerebellum following acute hypoxia, as well as nNOS in the rat cerebral cortex (47). Moreover, prolonged stimulation with rhEpo enhances the expression of eNOS transcript. The increase in
eNOS in acute hypoxia could be explained by the rise in [Epo] (33). In our conditions, this increase in all NOS isoforms may compensate for the reduced NOS activity, which could occur during hypoxia (37).

With longer hypoxic exposure, polycythemia and cerebral angiogenesis take place to enhance cerebral oxygenation, while cerebral NO level returns to basal value (50). The increased expressions of HIF-1α and VEGF with acute hypoxia in WT mice could trigger the increase in cerebral capillary density observed after chronic hypoxia. In addition, HIF-1α remained significantly elevated in rat brains exposed to 14 days of hypoxia in agreement with another study (7). In addition, the increased capillary density with chronic hypoxia is well established in the brain (5), particularly in the rat cerebral cortex (17), and the mechanisms were related to increased expression of HIF-1α and VEGF (21). Because VEGF was upregulated in acute hypoxia and tended to decrease after 14 days of hypoxia, as demonstrated by our quantitative and immunohistochemical data, we suggest that VEGF was necessary for the initiation but not for the maintenance of angiogenesis, as previously proposed (20). In addition, these results are in accordance with a recent study demonstrating a cerebral cortical angiogenesis in different strains of hypoxic mice through the interaction between VEGF and other proangiogenic factors, such as angiopoietins (Ang), which are ligands for the endothelium-specific receptor tyrosine kinase Tie-2 (55). Indeed, hypoxia has been shown to regulate the expression of angiopoietins and Tie-2, suggesting their participation in the angiogenic response to hypoxia (40, 42). Moreover, it has been recently shown that HIF-1α mediates the angiogenic response to hypoxia by activating both the VEGF and Ang/Tie-2 system (58). In our conditions, we could thus reasonably speculate that the Ang/
Tie-2 system participated to the angiogenic response in WT mice, since the role of Ang-2 in angiogenesis is highly dependent on the presence of other angiogenic factors, particularly VEGF. In the presence of VEGF, Ang-2 destabilizes the preexisting vasculature and consequently makes it more responsive to angiogenic stimuli (31).

Because the brain, especially the cerebral cortex, is very sensitive to the hypoxic stress, adaptive mechanisms could occur to protect it from hypoxic injury and cytotoxicity of high NO concentrations (1). We found an enhanced EpoR expression in the cerebral cortex with acute and chronic hypoxia in WT mice, as well as an increase in phospho STAT-5 expression and in phospho STAT-5/STAT-5 ratio in chronic hypoxia. These results are consistent with the fact that hypoxia is a critical stimulator for the activation of STAT proteins (26). Furthermore, they may provide the evidence for the activation of the JAK/STAT pathway in chronic hypoxia to promote neuroprotection (36) from the neurotoxic effects of glutamate and NO (57) through the reversal of Bcl-2 expression and from hypoxia-ischemia (3). It cannot be excluded that the Epo pathway could participate in the maintenance of angiogenesis (32), but the mechanisms remain to be determined (see Perspectives and Significance).

Both events, hypoxia and anemia have been shown to be relevant stimuli for HIF-1α, VEGF, EpoR, and nNOS expressions in the brain tissue (14, 20, 35, 41, 51). However, we did not observe a synergic effect of these stimuli in the cerebral cortex of Epo-TAgb mice except for NOx. Indeed, the response to hypoxia is divergent in WT and anemic mice, raising the question of the mechanisms able to maintain oxygen delivery to the brain in the hypoxic anemic mice. In Epo-TAgb mice, most factors studied did not respond to hypoxia, suggesting an already maximized response due to chronic anemia. Conversely, other factors such as HIF-1α, EpoR mRNA, and phospho-STAT-5 ratio decreased following hypoxic exposure, which could mean that angiogenic and neuroprotective pathways are altered in hypoxic Epo-TAgb mice (see Perspectives and Significance). On the other hand, the NOx level was severely increased in Epo-TAgb mice in chronic hypoxia, with no corresponding changes in NOS isoforms, suggesting no correlation between NOS expression and NO production (28). Normoxic anemic mice might have not increased cerebral NO, since they developed cerebral angiogenesis. However, the rise in NOx observed in chronic hypoxia, could improve cerebral blood flow to offset the lack of angiogenesis and/or cause cerebral injury (1). The increase in NOx associated with a fall in the EpoR signaling pathway could promote brain toxicity of NO (1). Other mechanisms may also help for the survival of these animals, such as a preferential redistribution of cardiac output to the brain (15) and the increase in ventilation (29, 30).

The limitations of the present study should be mentioned. Although CBF was not measured here, previous works have demonstrated a rise of NOS expression and NO production (18, 38, 39) in parallel with an increase in CBF in the brain of hypoxic animals (16, 22, 52), allowing us to speculate that cerebral NO production could be associated with a rise in CBF. We used an anti-Glut-1 antibody, which is known to be upregulated by hypoxia, but the cerebral capillary density was evaluated as an absolute number of Glut-1-positive capillaries per grid and not by the density of the staining, as previously done (17). In addition, we did not perform double immuno-staining studies for HIF-1α and other cerebral cell type markers, since it is well established that hypoxic neurons, astrocytes, and endothelial cells express HIF-1α (7, 53).

**Perspectives and Significance**

The present study provides novel physiological data about cerebral adaptations to chronic anemia. Indeed, the chronically anemic mice activated cerebral hypoxic genes that promote angiogenesis. This study demonstrates that Epo deficiency leads to the activation of mechanisms induced through HIF activation. In addition, the JAK/STAT signaling pathway seems to be activated by chronic anemia and could promote neuroprotection and cell proliferation (27). In this scenario, these mechanisms could thus counteract intracellular hypoxia. Moreover, it might be speculated that these responses are probably able to minimize brain damage that could be induced by chronic anemia (15). However, in hypoxia, while WT mice show expected physiological responses, Epo-TAgb mice did not respond further, except enhanced NO production. In addition, the decrease in neuroprotective pathways, as well as high NO level could suggest that brain cells are damaged and that Epo-TAgb mice failed to aclimatize to chronic hypoxia, but more investigations such as cerebral apoposis description and a survival test are necessary to confirm this hypothesis. Evidence for a role of Epo in regulating angiogenesis has been previously reported in the heart (19), in the uterus (60), and two forms of Epo mRNA have been described in brain capillary endothelial cells (8, 57). In chronic hypoxia, the lack of Epo is the only difference between WT and Epo-TAgb mice, and the cerebral capillary density was not further increased in hypoxic Epo-TAgb. Altogether, these results allow us to speculate that Epo/EpoR system may play an important role in cerebral angiogenesis in hypoxia, which could be tested by an Epo treatment. More investigations are thus needed to settle the role of Epo in cerebral angiogenesis and in aclimatization to hypoxia.

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