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Research Paper

# Brain adaptation to hypoxia and hyperoxia in mice

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ABSTRACT

*Aims:* Hyperoxic breathing might lead to redox imbalance and signaling changes that affect cerebral function. Paradoxically, hypoxic breathing is also believed to cause oxidative stress. Our aim is to dissect the cerebral tissue responses to altered $O_2$ fractions in breathed air by assessing the redox imbalance and the recruitment of the hypoxia signaling pathways.

*Results:* Mice were exposed to mild hypoxia (10%$O_2$), normoxia (21%$O_2$) or mild hyperoxia (30%$O_2$) for 28 days, sacrificed and brain tissue excised and analyzed. Although one might expect linear responses to %$O_2$, only few of the examined variables exhibited this pattern, including neuroprotective phospho- protein kinase B and the erythropoietin receptor. The major reactive oxygen species (ROS) source in brain, NADPH oxidase subunit 4 increased in hypoxia but not in hyperoxia, whereas neither affected nuclear factor (erythroid-derived 2)-like 2, a transcription factor that regulates the expression of antioxidant proteins. As a result of the delicate equilibrium between ROS generation and antioxidant defense, neuron apoptosis and cerebral tissue hydroperoxides increased in both 10%$O_2$ and 30%$O_2$, as compared with 21%$O_2$. Remarkably, the expression level of hypoxia-inducible factor (HIF)–2α (but not HIF-1α) was higher in both 10%$O_2$ and 30%$O_2$ with respect to 21%$O_2$

*Innovation:* Comparing the in vivo effects driven by mild hypoxia with those driven by mild hyperoxia helps addressing whether clinically relevant situations of $O_2$ excess and scarcity are toxic for the organism.

*Conclusion:* Prolonged mild hyperoxia leads to persistent cerebral damage, comparable to that inferred by prolonged mild hypoxia. The underlying mechanism appears related to a model whereby the imbalance between ROS generation and anti-ROS defense is similar, but occurs at higher levels in hypoxia than in hyperoxia.

## 1. Introduction

Despite its relatively small size (2% of total body weight), mammal brain ranks second after the heart as the organ with the highest $O_2$ consumption. As its function strictly depends on continuous oxygenation, any decrease in the $O_2$ supply results into potentially lethal cerebral hypoxia. Brain hypoxia is a dangerous feature in hemorrhage, anemia, trauma, stroke, perinatal encephalopathy, cardiopulmonary failure and high altitude exposure. Hyperoxic oxygenation is therefore a mandatory therapy for brain survival [1,2]. Although necessary to guarantee life, however, excess $O_2$ may become dangerous when the body antioxidant properties become inadequate to deal with higher than physiological levels of $O_2$, a potentially toxic element [3,4]. Brain cells, especially neurons, are known to be highly vulnerable to the deleterious effects of the reactive $O_2$ species (ROS) produced during oxidative stress [5,6]. Because of its high $O_2$ consumption and relatively low antioxidant defense, brain is thus particularly sensitive to ROS [7]. Therefore, although hyperoxia is often used therapeutically in traumatic brain injury and ischemic stroke, it may imbalance the redox status thereby inferring cerebral damage. Comparing the effects driven by mild hypoxia with those driven by mild hyperoxia at the cellular and molecular levels, a mandatory step to focus into the mechanisms underlying the redox imbalance, may thus foster important clinical implications.

The aim of this study is to test whether the molecular pathways of brain adaptation to hypoxia and hyperoxia depend directly on the percent $O_2$ level (%$O_2$). To this purpose, we compare the effects of excess $O_2$ and $O_2$ scarcity on the redox imbalance, the $O_2$-dependent

*Abbreviations:* %O2, percent oxygen level; Akt, Protein kinase B; CD34, Hematopoietic progenitor cell antigen; EPO, erythropoietin; EPO-R, EPO receptor; Hb, hemoglobin; HIF, hypoxia-inducible factor; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOX4, NADPH oxidase subunit 4; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PECAM-1, platelet endothelial cell adhesion molecule (also known as CD31); ROMs, reactive oxygen metabolites; ROS, reactive oxygen species; TdT, terminal deoxynucleotidyl-transferase nuclei; VEGF, Vascular endothelial growth factor; VEGF-R2, KDR/Flk-1 receptor of VEGF

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molecular responses and damage in cerebral tissue. To get a clear picture of the complex interrelationships among the variables in play, we use a chronic (28 days) model that excludes the occurrence of disturbing oxygenation and deoxygenation events to expose animals to three experimental situations that differ only for $\%O_2$ in breathed air in an regularly spaced progression (10–21–$30\%O_2$).

## 2. Materials and methods

### 2.1. Mice and treatments

Seven-week old Foxn1 mice (Harlan, n=19, 27–30 g) were cared in accordance to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). The University of Milan Committee for the Use of Laboratory Animals (OBPA) approved animal handling, training protocol and mode of sacrifice. On day one, mice were randomly transferred into a gas chamber flushed with one of the following mixtures (balance $N_2$): 10% $O_2$ (hypoxia, n=7), $21\%O_2$ (normoxia, n=6), or $30\%O_2$ (hyperoxia, n=6). The duration of the treatments was 28 days for all groups. Mice had free access to water and diet until 24 h before sacrifice. A 12/12 h light/dark cycle was maintained.

To measure the body weight and maintain the chambers, mice were anaerobically transferred into the compensation chamber flushed with the same gas mixture as the gas chamber [8]. At day 28, mice were transferred one-by-one into the compensation chamber, anesthetized by i.p. Na-thiopental (10 mg/100 g body weight) plus heparin (500 units), euthanized by cervical dislocation and taken out of the chamber. Brains were quickly dissected, frozen in liquid nitrogen and stored at −80 °C for analyses. Care was taken to exclude cerebellum tissue from the dissection. Blood hemoglobin concentration was measured by the Drabkin's method, assuming $\varepsilon$=11.05 $cm^{-1}$ $mM^{-1}$.

### 2.2. Western blot

Cytosolic and nuclear extracts, and Western blots were performed for each biopsy as described [9]. The primary antibodies and dilutions were: anti-HIF-1α (Santa Cruz Biotechnology, 1:300), anti-HIF-2α (Abcam, 1:300), anti-VEGF165 (Calbiochem, 1:200), anti-β-actin (Sigma Aldrich, St Louis, MI 1:5000), anti-Akt (Cell Signaling Technology, 1:1000), anti-phospho-Akt-$Ser^{473}$ (Cell Signaling Technology, 1:1000), anti-Nrf2 (Santa Cruz Biotechnology, 1:1000), EPO (Santa Cruz Biotechnology, 1:200), EPO-R (Santa Cruz Biotechnology, 1:200), GAPDH (Sigma Aldrich, 1:15000), NOX4 (Abcam, 1:5000), VEGF Receptor 2 (Cell Signaling technology, 1:100), CD34 (Santa Cruz Biotechnology, 1:500), PECAM-1 (Santa Cruz Biotechnology, 1:600). The secondary antibodies were horse-radish peroxidase-conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA, 1:10000) or anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, 1:10000). Chemiluminescence was detected by incubating the membrane with LiteAblot Chemiluminescent substrate (Lite Ablot, EuroClone, EMPO10004) followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film, Eastman Kodak Company, Rochester, NY). The image was acquired and blots intensity quantified by Gel Doc (Bio-Rad quantitation software Quantity One).

### 2.3. Immunofluorescence

Frozen specimens were treated as described [10] and a triple labeling procedure was performed to distinguish apoptosis in neuronal and non-neuronal cells. To detect apoptosis, we used the Terminal deoxynucleotidyl transferase (TdT) nick end labeling test by the *In Situ Cell Death* detection kit, TMR red (Roche, Mannheim, Germany), where the 3'-OH DNA ends were labeled with TMR red-nucleotides by TdT. After washing in PBS, slides were incubated in 10% normal goat blocking serum for 10 min at room temperature. To identify neuronal cells, we used as primary antibody NeuN [mouse monoclonal antibody (Millipore, Temecula, CA) diluted 1:100 in 1.5% normal goat serum +1.5% Tween20]; slides were incubated for 1 h. The green fluorescein-conjugated secondary antibody was Alexa-Fluor 488 Goat Anti-Mouse IgG (H+L) [(Thermo Fisher Scientific, Rodano, Italy) diluted 1:1000 in PBS (2 h incubation)]. To identify nuclei, we used the blue karyophilic dye Hoechst 33258 (Sigma). After merging the green, red and blue channels (Photoshop v.7.0, Adobe Systems, San Jose, CA), white spots were associated with apoptotic neurons (green+red+blue), while purple spots identified apoptosis in non-neuronal cells (red+blue). Non-neuronal apoptosis was quantified by subtracting TdT-positive neurons from TdT-positive nuclei.

### 2.4. Confocal microscopy

Immunofluorescence analysis was performed as previously reported [11]. Briefly, cryostat coronal sections (15 μm) were collected onto glass slides and processed for immunocytochemistry. Sections were rinsed with PBS (Euroclone), treated with blocking solution (Life-Technologies) and incubated with primary antibodies overnight at 4 °C. After treatment with primary antibodies, sections were washed with PBS and incubated with appropriate secondary antibodies (Alexa Fluor® 488 and 546, Molecular Probes®, Life Technologies) for 2 h at room temperature. After washing, nuclei were stained with DAPI (1 μg/ml final concentration, 10 min at room temperature; Sigma-Aldrich) and then sections were mounted using the FluorSave Reagent (Calbiochem, Merck Chemical, Darmstadt, Germany) and analyzed by confocal microscopy. The following primary antibodies were used: Erythropoietin (1:200; Santa-Cruz), β-Tubulin III (1:150; Covance). Images were acquired and immunofluorescence quantified by using standardized confocal microscopy (Leica SP2 confocal microscope with He/Kr and Ar lasers; Heidelberg, Germany). Images were obtained using the laser same intensity, pinhole, wavelength, and thickness of the acquisition. As a negative reference we used a consecutive section that was stained by omitting primary antibody and replacing it with equivalent concentrations of unrelated IgG of the same subclass. The zero level was adjusted on this reference and used for all the further analysis (we used a new zero reference for each new staining). The fluorescence intensities of three consecutive sections (15 μm thick) were averaged to obtain the mean relative optical density [12].

### 2.5. D-ROMs and Lipotiss tests

To evaluate the oxidative stress, we determined the overall level of oxidant chemical species produced, including ROS, hydrogen peroxide, hypochlorous acid. By attacking organic molecules, these species generate stable Reactive Oxygen Metabolites (ROMs), primarily composed by hydroperoxydes (ROOH). To determine oxidative stress in plasma, we used the photometric D-ROMS test (Diacron International srl, Grosseto, Italy) that evaluates the capacity of in vivo formed ROOH to generate alkoxyl (•R-O) and peroxyl (•R-OO) radicals in the presence of iron released from plasma by an acidic buffer [13]. Data are expressed as Carratelli Units (U CARR). To determine oxidants in brain tissue, we measuresd the lipoperoxide level by a method based on the peroxide capacity to oxidize $Fe^{2+}$ to $Fe^{3+}$, which binds thiocyanate developing a colored complex (Lipotiss test MC040, Diacron International srl, Grosseto, Italy). Briefly, samples (200 mg) were homogenized in 0.5 ml distilled water, centrifuged (5 min at 15000*g*) and washed twice with distilled water. After removing the supernatant, 0.5 ml of the indicator mixture (R1) was added, mixed (5 min) and centrifuged (5 min at 1400*g*). Then, 0.250 ml of supernatant or 2.5 μl of standard (4000 μEq/L terbutilhydroperoxide) diluted in 0.250 ml indicator mixture was added into the 96-well plate, followed by addition of 10 μl $Fe^{2+}$(R2 reagent, diluted 1:4 with R1). After incubation (5 min at 37 °C), the optical density was read at $\lambda$=505 nm and the

**Table 1**
Systemic changes after exposure to 10% $O_2$, 21% $O_2$ and 30% $O_2$ for 28 days. Data expressed as mean ± SEM.

| n | Units | 10%O2<br>7 | 21%O2<br>6 | 30%O2<br>6 | ANOVA P |
|---|---|---|---|---|---|
| Initial body weight | g | 27.31 ± 0.95 | 28.80 ± 0.74 | 29.90 ± 0.79 | ns |
| Final body weight | g | 26.12 ± 0.55[b,a] | 29.50 ± 0.53 | 32.82 ± 1.40 | 0.0003 |
| Blood hemoglobin concentration | g/dL | 11.48 ± 0.53[b,a] | 7.91 ± 0.45 | 6.78 ± 0.17 | < 0.0001 |

[a] $P < 0.05$ vs. 30%$O_2$.
[b] $P < 0.05$ vs. 21%$O_2$ (ANOVA and Tukey post test).

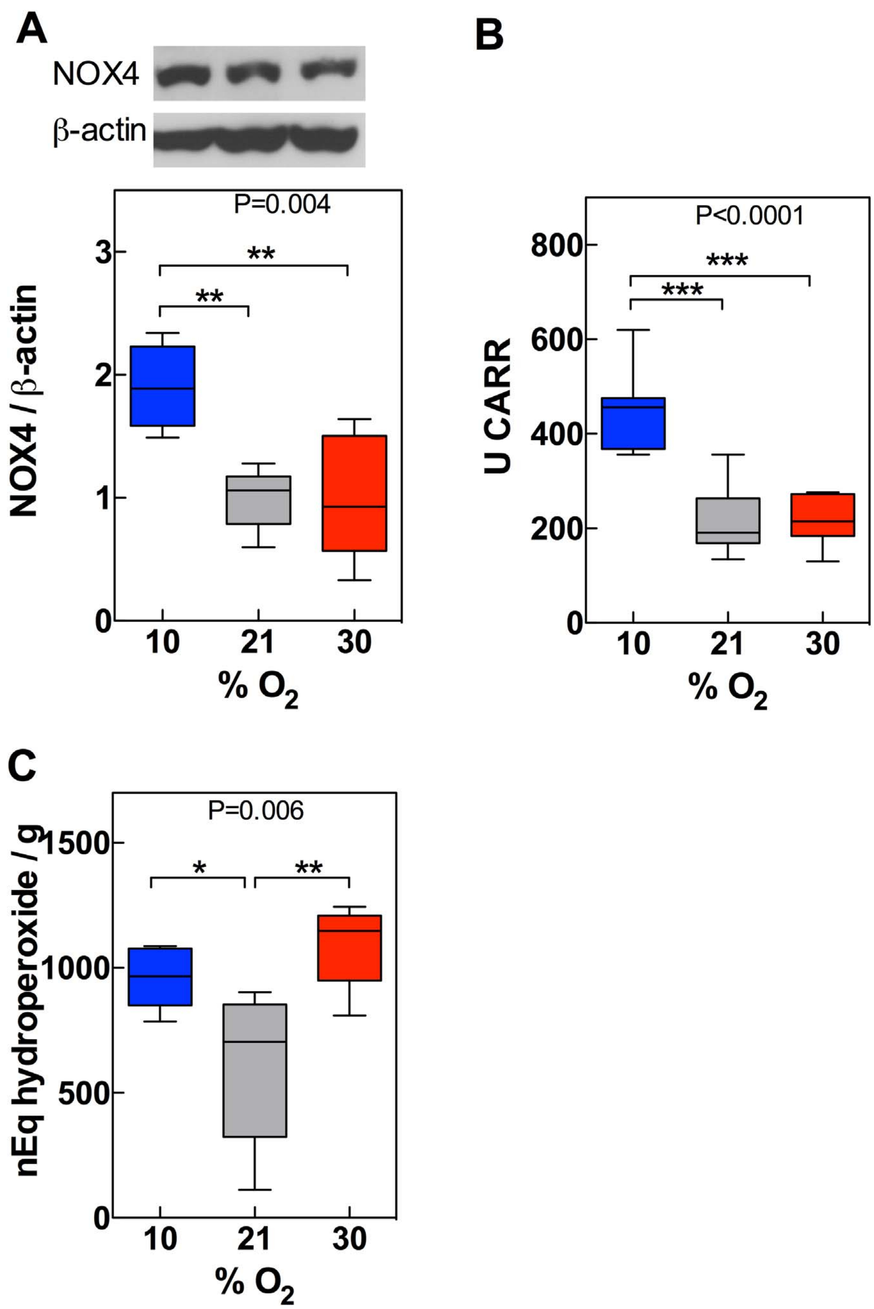


**Fig. 1.** Redox imbalance at the end of the exposure to hypoxia (10%$O_2$), normoxia (21%$O_2$) and hyperoxia (30%$O_2$) for 28 days (n=7, 6 and 6, respectively). Panel A. Expression level of NADPH oxidase subunit 4 (NOX4). Panel B. D-ROMs test to estimate the pro-oxidant capacity of plasma samples towards a chromogenic indicator, expressed in U CARR units. Panel C. Lipotiss test to estimate the hydroperoxide level in cerebral tissue. Data are expressed as box plots indicating the 25th percentile, the median and the 75th percentile, with whiskers indicating the max and min values. The inset reports the ANOVA test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Tukey multiple comparison post-test).

concentration of lipoperoxides was calculated and expressed as nanoequivalent hydroperoxydes/g tissue.

### 2.6. Statistics

Data are expressed as box plots indicating the 25th percentile, the

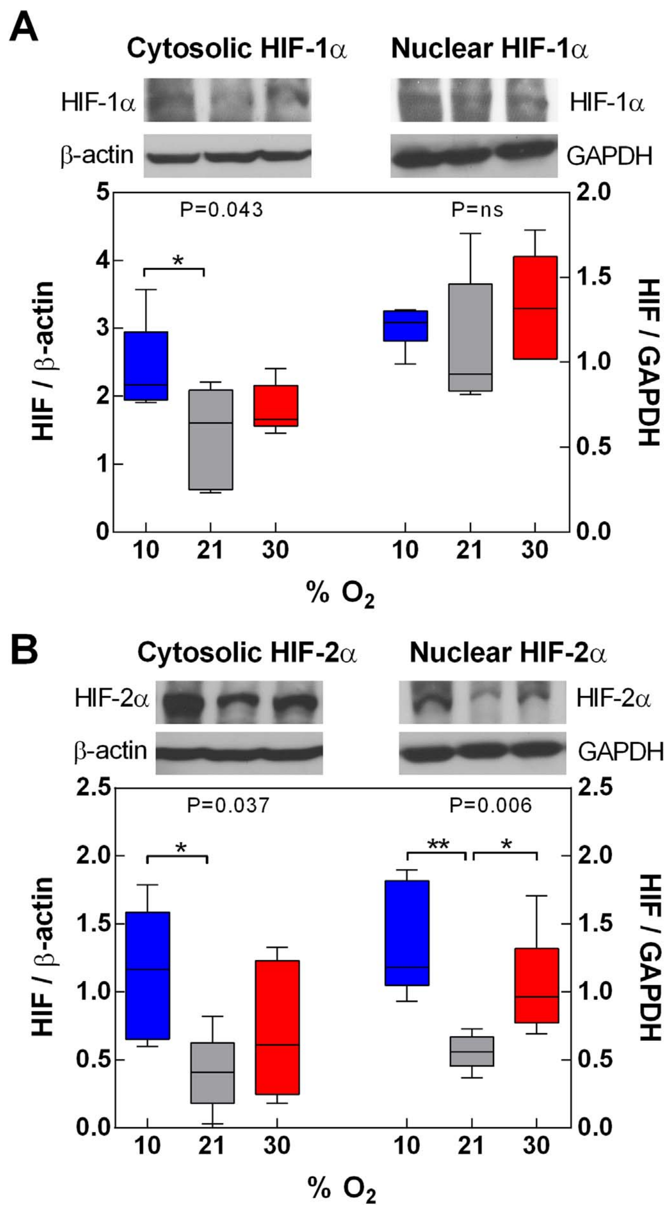


**Fig. 2.** Hypoxia signaling. Panel A. Expression level of cytosolic (left) and nuclear (right) hypoxia-inducible factor (HIF)−1α. Panel B. Expression level of cytosolic (left) and nuclear (right) HIF-2α. Data expressed as described in Fig. 1.

median and the 75th percentile, with whiskers indicating the max and min values. To assess the significance of the between-treatment differences, we performed One-way ANOVA followed by the Tukey multiple comparison post-test if $P < 0.05$. Statistics was performed using Prism (GraphPad Software, Inc.), with the significance level set at $P=0.05$ (two-tailed).

## 3. Results

### 3.1. Systemic changes

All mice survived the various treatments. The body weight decreased slightly in 10%$O_2$ vs. a modest and a marked increase in 21% $O_2$ and 30%$O_2$, respectively. Blood [Hb] was higher and lower, respectively, in 10%$O_2$ and 30%$O_2$ than in 21%$O_2$ (Table 1).

### 3.2. Pro-oxidant mechanisms

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit 4 (NOX4) is one of the proteins responsible for the ROS production in cerebral tissue [14]. The expression level of NOX4 in 10%$O_2$ was twice that in 21%$O_2$ (Fig. 1A), indicating greater pro-oxidant potential in hypoxia. The NOX4 expression level was the same in 30%$O_2$ and 21%$O_2$, indicating loss of $O_2$-dependence in hyperoxia.

The D-ROM (reactive $O_2$ metabolites) test addresses the level of oxidant species in plasma [15]. The value found in 21%$O_2$, which reflects the situation typical of healthy humans [15], doubled in 10%$O_2$ (P=0.001), showing that sustained mild hypoxia markedly impaired the systemic redox balance (Fig. 1B). However, 30%$O_2$ did not affect the systemic pro-oxidant pool with respect to 21%$O_2$.

To assess how ROS affects cerebral tissue integrity [16], we measured the hydroperoxide level in brain tissue. It appeared that 21%$O_2$ displayed the lowest hydroperoxide level, and that both 10%$O_2$ and 30%$O_2$ had higher level of hydroperoxides (Fig. 1C).

### 3.3. Hypoxia signaling

Fig. 2 reports the results related to the hypoxia-inducible factors (HIFs) as measured by Western blot in both the cytosolic and the nuclear fractions. The expression level of HIF-1α increased in 10%$O_2$ in the cytosolic fraction (P=0.05), but not in the nuclear fraction (P=NS) (Fig. 2A). By contrast, the expression level of HIF-2α in 10%$O_2$ was markedly increased with respect to that in 21%$O_2$ in both the cytosolic (P=0.05) and nuclear (P=0.01) fractions (Fig. 2B). Remarkably, the expression of HIF-2α in the nuclear fraction, where the transcription factor is active was higher in 30%$O_2$ with respect to 21%$O_2$ (P=0.05).

### 3.4. Protective mechanisms

To assess the response of brain tissue to the redox imbalance, we measured the expression level of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein, a transcription factor activated within the molecular mechanisms aimed at compensating the redox imbalance [17]. Fig. 3A shows that both the nuclear and cytosolic expression levels of Nrf2 remained unchanged in either treatment.

Protein kinase B (Akt) is part of a relevant defense mechanism often recruited by cells under stress [18]. The expression of total Akt remained unchanged in the three groups (not shown), but the phosphorylated form at $Ser^{473}$ (*p*-Akt), which plays a critical role in controlling survival and apoptosis [19], showed a monotonic decrease from 10%$O_2$ to 30%$O_2$ (P=0.0004, linear regression test), thereby displaying an $O_2$-dependent pattern inversely related to %$O_2$ (Fig. 3B).

The erythropoietin (EPO)/EPO receptor (EPO-R) axis represents another defense mechanism particularly active in cerebral tissue [20–22]. The tissue level of EPO remained unchanged (P=ns) in the three situations (Fig. 3C), as confirmed by semi-quantitative confocal microscopy analysis (Fig. 1S, supplementary data). However, the expression level of EPO-R displayed a %$O_2$-related decrease (P=0.038), consistent with the decrease of p-Akt shown above (Fig. 3C).

### 3.5. Angiogenic potential

To assess the angiogenic potential, we measured some of the proteins related to tissue vascularization. No appreciable changes were observed in the expression of the vascular endothelial growth factor (VEGF) (Fig. 4A). However, VEGF receptor (VEGFR-2, also known as KDR/Flk-1), the hematopoietic progenitor cell antigen CD34, and the platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31), increased only in 10%$O_2$ and remained constant in 30%$O_2$ compared to 21%$O_2$ (Fig. 4B–D).

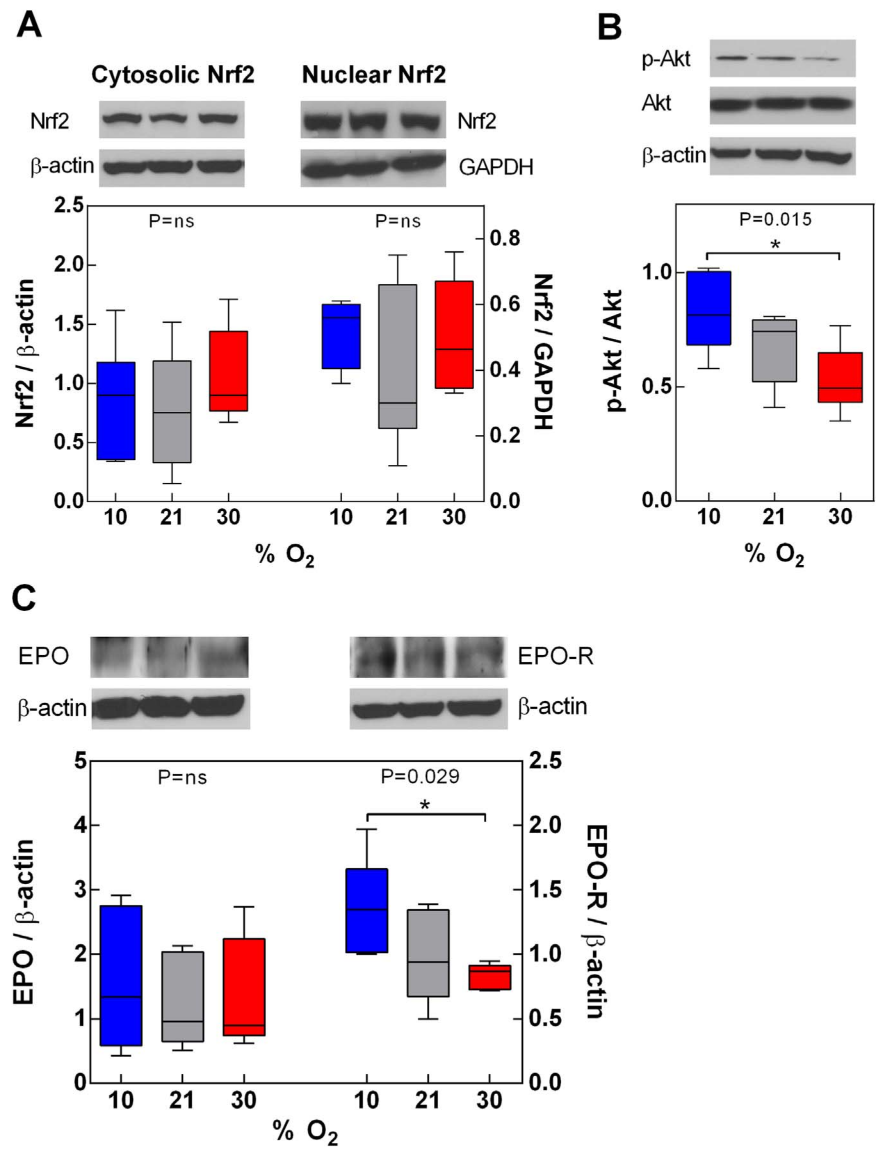


**Fig. 3.** Protective mechanisms. Panel A. Expression level of cytosolic (left) and nuclear (right) nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Panel B. Ratio between the expression levels of total protein kinase B (Akt) and the phospho-Akt-Ser[473] (*p*-Akt). Data expressed as described in Fig. 1. Panel C**.** Tissue expression level of erythropoietin (EPO, left) and of the EPO receptor (EPO-R, right).

### *3.6. Apoptosis*

The triple labeling procedure described in Material and Methods enabled distinguishing neuronal and non-neuronal apoptosis (Fig. 5A). Apoptotic neurons (NeuN+ cells) accounted for 8.2 ± 1.8% of all neuronal nuclei in 21%$O_2$ (Fig. 5B). In 10%$O_2$, the count rose to 25.6 ± 2.4% (P=0.001 vs 21%$O_2$), indicating a pro-apoptotic effect of hypoxia. In 30%$O_2$, the count was 20.4 ± 1.9% (P=0.01 vs 21%$O_2$), indicating a pro-apoptotic effect of hyperoxia similar to that led by hypoxia. By contrast, the degree of apoptosis attributable to non-neuronal cells remained essentially unchanged in the three groups.

## **4. Discussion**

In this study, we examined the cerebral tissue after 28-d normobaric hypoxia or hyperoxia, with normoxia as control. Confounding phenomena related to reoxygenation or deoxygenation events were excluded especially in the sacrifice phase to avoid uncontrolled ROS bursts and rapid stabilization/destabilization of HIFs and downstream proteins [23]. The gas chamber, where mice dwelled for the entire study duration, and the compensation chamber, where mice were handled and sacrificed, are designed to minimize the %$O_2$ fluctuations to < 1%$O_2$ throughout, leaving %$O_2$ in breathed air as the only independent variable. The %$O_2$ selected for hypoxia, comparable to 5000 m altitude, induces sub-lethal metabolic and signaling changes in cerebral tissue [24–26]. The %$O_2$ selected for hyperoxia mimics a situation common in pulmonary patients who breath with the aid of portable $O_2$ concentrators [27]. Regular spacing of %$O_2$ in an almost linear progression (10–21-30) enables detecting linear correlations with %$O_2$. Whereas a few of them were linearly related with %$O_2$, for most the $O_2$-dependence was lost. As a final outcome, both hypoxia and hyperoxia worsened neuron apoptosis, yet through different mechanisms.

Among the mechanisms underlying the redox imbalance in hypoxic cerebral tissue, the mitochondrial complex I may be relevant [28], but NOX4, which nearly doubled from 21%$O_2$ to 10%$O_2$, is likely a pivotal factor because it responds directly to hypoxia [29] and stands out as the main enzyme family that is dedicated to forming ROS [30], thereby constituting a major ROS source [14,31]. On the other hand, Nrf2, one of the genes that are altered in rat brains after 12 h high altitude exposure [32], may represent a major mechanism that regulates antioxidant defenses [17]. Yet, we did not observe changes in Nrf2,

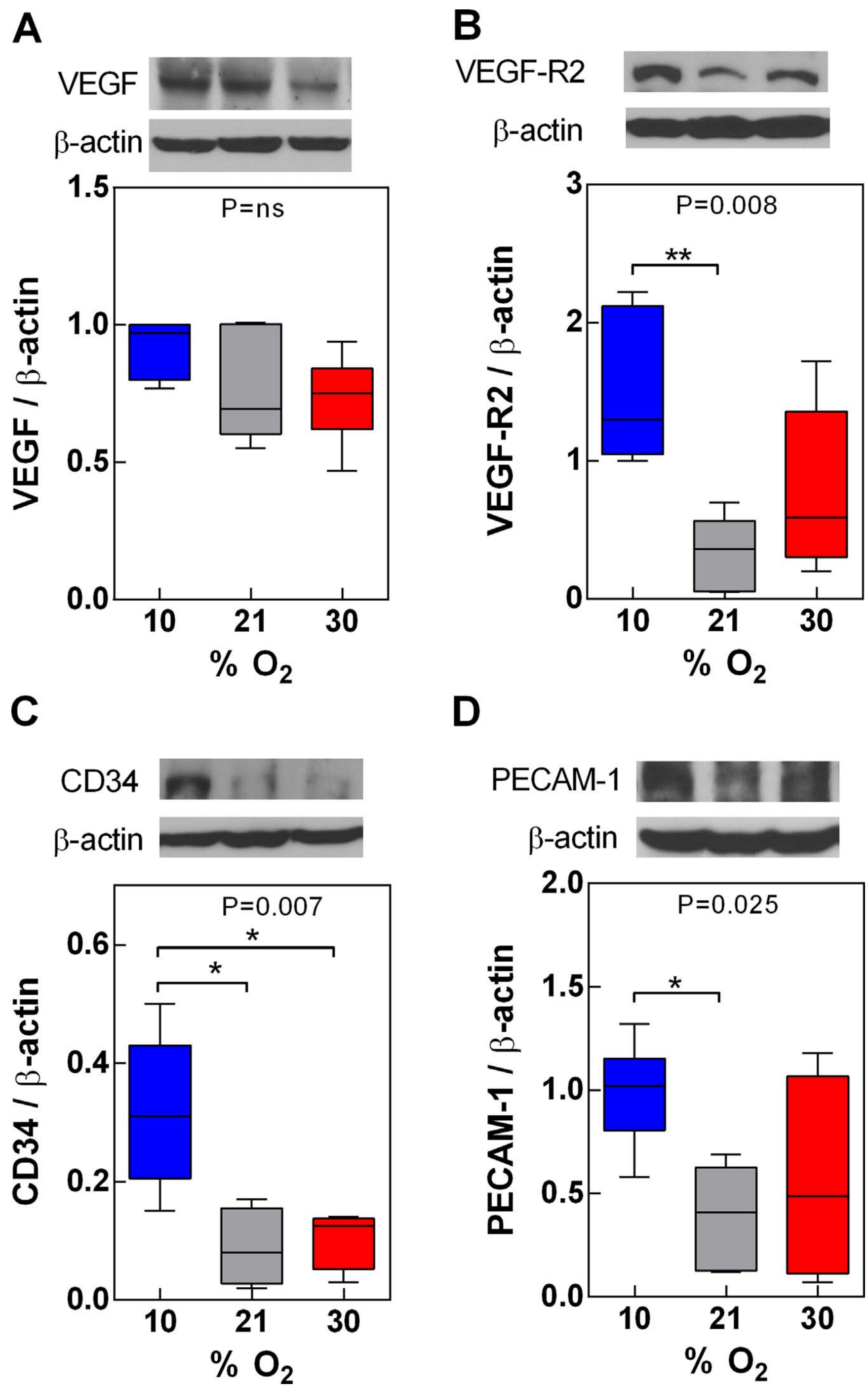


**Fig. 4.** Markers of vascularization. Panel A. Expression level of tissue vascular endothelial growth factor (VEGF). Panel B. Expression level of tissue VEGF receptor 2. Panel C. Expression level of hematopoietic progenitor cell antigen (CD34). Panel D. Expression level of platelet endothelial cell adhesion molecule (PECAM-1). Data expressed as described in Fig. 1.

possibly because Nrf2 might undergo normalization after sustained exposure to altered %$O_2$. By contrast, Akt, a serine/threonine-specific protein kinase that plays a key role in neuroprotection by inhibiting apoptosis [33,34], may represent a protective mechanism even in chronic situations, possibly overriding that potentially elicited by Nrf2. Despite unchanged total Akt expression, $Ser^{473}$ phosphorylation increased in 10%$O_2$ and decreased in 30%$O_2$ with respect to normoxia, indicating that the Akt mechanism becomes progressively more depressed with increasing %$O_2$. Collectively, it appears that mild hypoxia increases both ROS production and defense mechanisms, but the latter is clearly insufficient with respect to the first, thereby worsening the redox imbalance.

In hyperoxia, the major ROS source (NOX4) was not up-regulated, but high %$O_2$ is expected to augment non-enzymatic ROS production [35]. This is the main reason for which life-saving $O_2$ therapies are sometimes considered noxious. In some studies, it appeared that normobaric hyperoxia does not induce appreciable redox imbalance, at least if applied for a short duration [1], and another study in a rat model of subdural hematoma failed to document a clear increase in free radicals with 100% $O_2$ hyperoxia [36]. In qualitative agreement with the above studies, despite possible interferences with serum components [37], the D-ROM test shows that the systemic redox imbalance

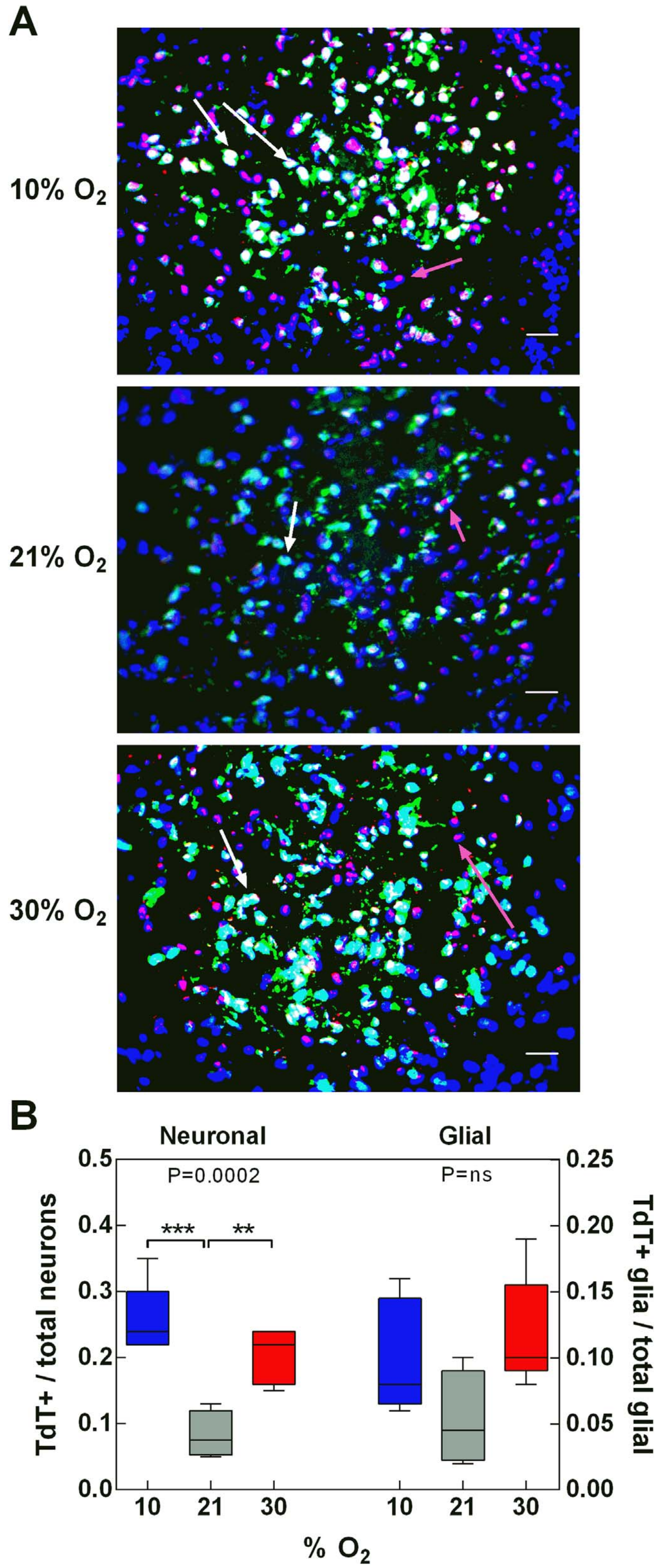


**Fig. 5.** Brain damage. Panel A. Representative immunofluorescence pictures obtained in cerebral tissue samples from hypoxia (10%$O_2$), normoxia (21%$O_2$) and hyperoxia (30% $O_2$) for 28 days. The green (neurons), red (TdT positive) and blue (nuclei) channels are displayed. The white arrows identify spots associated with apoptosis in neurons (green +red+blue), while purple arrows identify spots associated with apoptosis in non-neuronal cells (red+blue). The bar represents 50 µm. Panel B**.** Percent of neuronal (left) and glial (right) cells that are positive for TdT as counted in 5 sections from each specimen. Data expressed as described in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was not appreciable in 30%$O_2$. It is relevant to note that the d-ROMs test is designed to measure the level of all the hydroperoxides generated from the oxidation of biological molecules, and that the contribution of chloramines and ceruloplasmin can't be ruled out. Although unspecific, this test enables evaluating collectively the effects driven by an array of oxidizing agents thereby providing a global assessment of the redox imbalance in serum and reinforcing the potential clinical significance of the present approach.

Accurate comparison of the above findings with literature is difficult due to profound differences in the $O_2$ level, the duration of the exposure and the target organ or system. The observations gathered in the present investigation converge in attributing a marginal role for the systemic redox imbalance in sustained exposure to mild 30%$O_2$ hyperoxia, but the situation in cerebral tissue is different. The Lipotiss test shows almost the same degree of cerebral tissue redox imbalance in 10%$O_2$ and 30%$O_2$. Likewise, neuronal apoptosis was elevated in either situation. Remarkably, the Nrf2 antioxidant response was similar, yet Akt phosphorylation followed an $O_2$-dependent fashion. These findings converge into the schematic representation proposed in the graphical abstract, whereby ROS production was higher in hypoxia than in hyperoxia, but the anti-oxidant defenses were also proportionally greater in hypoxia than in hyperoxia. The final outcome is similar tissue damage, because it does not strictly depend on ROS production per se, but rather on the imbalance between ROS production and anti-oxidant defenses.

To get an insight into the underlying mechanisms, we tested the roles of the $O_2$-dependent transcription factors. Although HIF-1α is the most established member of the HIF family, stabilized by different extents in various organs [24], HIF-2α (endothelial Pas domain protein 1) is today recognized as a major player in hypoxia adaptation [38–40]. Perhaps, whereas HIF-1α is more active during short, intense hypoxia, HIF-2α may become preponderant during prolonged, mild hypoxia [41]. As $O_2$ destabilizes both isoforms by activating prolyl hydroxylases, HIF-2α accumulation in hyperoxia seems paradoxical, but similar patterns were observed in prostate tumors [42], brain tissue [5], hepatocytes and liver hemopoietic cells in newborn rats [43], as well as in embryonic myocardium [44]. The molecular mechanisms underlying HIF-1α activation independently of hydroxylation are being elucidated [45]. Remarkably, the redox imbalance in the sympathetic nervous system is caused by the disruption of the balance between HIF-1α-dependent pro-oxidant and HIF-2α-dependent antioxidant enzymes [46]. Irrespectively of the cause, HIFs up-regulation is expected to trigger an array of downstream events through up-regulation of specific proteins. For example, Akt activation is both a consequence [47] and a trigger of HIF's signaling [48]. HIF-1α downregulation after 28-d mild hypoxia is not surprising because it quickly normalizes in vivo after 21 h hypoxia [49]. The observed increase in cytosolic HIF-1α and HIF-2α in 10% $O_2$ might be secondary to increased mitochondrial ROS that are released to the cytosol and inhibit prolyl hydroxylases, which stabilize HIF's [50].

EPO, a cytokine produced in large part by kidneys, is the main regulator of erythropoiesis, but in the central nervous system it also exerts an important protective role mediated by EPO-R on neuron membrane [20,51–53]. This mechanism is particularly relevant in hypoxia [26] and has been proposed to contribute to endogenous neuroprotection during carotid endarterectomy [54]. The mechanism whereby EPO exerts its neuroprotective action might involve activation of the anti-apoptotic STAT3 pathway [21,51], increase of the antioxidant enzyme expression and reduction of ROS production [55,56]. Despite the reported up-regulation of EPO expression in astrocytes by HIF-2α [57], we were unable to observe elevated EPO as that observed in rats exposed to 50%$O_2$ for 3 weeks [5]. By contrast, we found linear dependency on %$O_2$ in the expression of EPO-R. These findings, together with the confocal microscopy observation that EPO appears to be expressed mostly in non-neuronal cells (Fig. 1S, supplementary data), indicate that mild hypoxia improves the recruitment of the EPO/

EPO-R signaling neuroprotective pathway, whereas mild hyperoxia has the opposite effect.

Brain may react to hypoxia by preserving $O_2$ and nutrients supply through angiogenic changes downstream HIFs up-regulation [58]. Although we did not observe VEGF up-regulation, other components of vascularization as VEGF-R2, CD34 and PECAM-1 were markedly increased by hypoxia, but were not affected by hyperoxia. This is in agreement with reports showing that chronic hypoxia doubles capillary density [59], but is in contrast with the decreased capillary density observed in mice exposed to 50%$O_2$ for 3 weeks [5]. Although differences in experimental conditions may account for this discrepancy, it remains to be clarified whether changes in the angiogenic potential turn out to be physiologically relevant in terms of cerebral blood flow.

Sustained exposure to hypoxia is well known to induce brain damage [25] and cognitive impairment [60–64]. Remarkably, the processes associated to cell death and cognitive impairment are beginning to be appreciated in hyperoxia too [65,66]. If the concept that the redox imbalance is the expression of the equilibrium between ROS formation and the anti-oxidant defense is valid, the case of hypoxia vs. hyperoxia may represent a noticeable paradigm that needs further investigation to understand the molecular mechanisms of HIFs and their differential effects on cerebral blood flow, $O_2$ delivery, NO-driven modulation [67] and cerebral metabolic rate auto-regulation [68].

## 5. Conclusions

We assessed whether the molecular pathways of cerebral tissue adaptation to different %$O_2$ in breathed air depend on %$O_2$ only or if other factors are involved. We show that, whereas HIFs display a biphasic pattern of adaptation to altered %$O_2$, only the p-Akt response was directly linked to %$O_2$. By contrast, neuron apoptosis worsened both in hypoxia and hyperoxia compared to normoxia. Thus, mild hyperoxia represents a condition as challenging as mild hypoxia with respect to redox imbalance and brain damage. This suggests that, despite profound fluctuations in Earth atmosphere %$O_2$ in the past geological eras [69], terrestrial mammals are now adapted to live in an atmosphere containing 20.96%$O_2$, and that any deviation from this value, irrespectively if higher or lower, might trigger the occurrence of a potentially lethal situation. This may have important clinical implications for the 800,000 individuals that need supplemental oxygen at a cost of 1.8 billion dollars/year in the US [70].

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.10.018.

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