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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 ε=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 horseradish 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 λ=505 nm and the