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RESEARCH ARTICLE

HIF1α and physiological responses to hypoxia are correlated in mice but not in rats

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ABSTRACT

We previously reported that rats and mice that have been raised for more than 30 generations in La Paz, Bolivia (3600 m), display divergent physiological responses to high altitude, including improved respiratory and metabolic control in mice. In the present study, we asked whether these traits would also be present in response to hypoxia at sea level. To answer this question, we exposed rats (Sprague Dawley) and mice (FVB) to normoxia (21% O_2) or hypoxia (15 and 12% O_2) for 6 h and measured ventilation and metabolic rate (whole-body plethysmography), and expression of the transcription factor HIF-1α (ELISA and mass spectrometry) and other proteins whose expression are regulated by hypoxia (glucose transporter 1, pyruvate dehydrogenase kinase 1 and angiopoietin 2; mass spectrometry) in the brainstem. In response to hypoxia, compared with rats, mice had higher minute ventilation, lower metabolic rate and higher expression of HIF-1α in the brainstem. In mice, the expression level of HIF-1α was positively correlated with ventilation and negatively correlated with metabolic rate. In rats, the concentration of brainstem cytosolic protein decreased by 38% at 12% O_2, while expression of the glucose transporter 1 increased. We conclude that mice and rats raised at sea level have divergent physiological and molecular responses to hypoxia, supporting the hypothesis that mice have innate traits that favor adaptation to altitude.

KEY WORDS: Respiratory control, Metabolic rate, Hypoxia, Protein expression, Brain

INTRODUCTION

The house mouse (*Mus musculus*) is not a native high-altitude (HA) species, but natural populations have been able to establish viable colonies at altitudes up to 4000 m, as reported in South America (Storz et al., 2007). However, some reports show that rats (*Rattus rattus* or *Rattus norvegicus*) are more difficult to observe at a similar altitude, particularly in Bolivia (Anderson, 1997; Innes, 2005). In Madagascar, though, some populations of *R. rattus* are found at 2400 m above sea level (SL) (Brouat et al., 2013) – a mid-range altitude to which it might be easier to adapt or acclimatize. We previously compared the physiological adjustments to HA in laboratory Sprague Dawley (SD) rats and FVB mice that have been bred and raised at 3600 m above SL (La Paz, Bolivia) for more than 20 years and over 30 generations. Compared with rats, mice had lower hematocrit and hemoglobin levels, reduced right ventricular hypertrophy (a sign of reduced pulmonary hypertension), increased alveolar surface area and lung volume, higher tidal volume and metabolic rate (Jochmans-Lemoine et al., 2015). Along with the data obtained at HA, we also reported values obtained at SL under normoxic conditions showing that rats and mice had similar lung volume, total alveolar surface area and metabolic rate (Jochmans-Lemoine et al., 2015). Therefore, the different traits observed at HA required several generations to appear, might be developmentally regulated, or could indicate that mice have innate predispositions to withstand hypoxic exposure that are absent in rats.

The response to acute hypoxia at SL leads to a rapid (within a few seconds) elevation of the minute ventilation, and if the exposure is prolonged for more than a few minutes, metabolic rate typically declines. Mice and rats are commonly used for studies of respiratory control, and while we are not aware of systematic comparisons between the two species, both have been found to show robust respiratory responses mediated by peripheral chemoreceptors upon exposure to acute hypoxia (Izumizaki et al., 2004; Marcouiller et al., 2014; Morgan et al., 2016; Roux et al., 2000; Soliz et al., 2005). In contrast, long-term exposure to hypoxia (days to weeks) induces a long-term plasticity of the respiratory control system (ventilatory acclimatization to hypoxia), in which hypoxia-inducible factor 1 (HIF-1) plays a key role (Kline et al., 2002; Powell and Fu, 2008; Prabhakar and Semenza, 2012). HIF-1 is a heterodimer composed of one alpha and one beta subunit; under normoxia, the HIF-1α subunit is degraded by the proteasome, but under hypoxia it is stabilized, translocated to the nucleus and dimerized with the HIF-1β subunit to regulate the expression of its target genes (Prabhakar and Semenza, 2012). In the brain of mice, the HIF-1α response has been readily observed in western blots: it gradually increases during the first hours of hypoxic exposure, and reaches a peak after 5 to 6 h (Stroka et al., 2001). By contrast, after 6 h of exposure to hypoxia, HIF-1α expression in rats is limited to a small group of brainstem neurons involved in the cardiorespiratory responses to hypoxia, including the nucleus tractus solitarius (NTS; the central projection site of carotid body chemoafferents; Pascual et al., 2001). This different pattern of response clearly suggests that there are some differences in the magnitude of the expression and effect of HIF-1α between rats and mice. Besides its role in the plasticity of the respiratory control system under hypoxia, HIF-1 has also profound effects on metabolic pathways, by slowing down oxygen (O_2) consumption in mitochondria and favoring glycolytic anaerobic pathways for ATP synthesis (Ebert et al., 1995; Papandreou et al., 2006; Schönenberger and Kovacs, 2015; Wheaton and Chandel, 2011). These responses help maintain high cellular O_2 pressure under hypoxia, and are therefore important traits for survival under conditions of limited O_2 levels.

Because SD rats and FVB mice had different respiratory and metabolic responses at HA (Jochmans-Lemoine et al., 2015), we tested the hypothesis that at SL, SD rats and FVB mice have different ventilatory and metabolic responses to hypoxia which

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List of symbols and abbreviations

Angpt2	angiopoietin 2
f_R	respiratory frequency
Glut1	glucose transporter 1
HA	high altitude
HIF	hypoxia-inducible factor
MRM	multiple reaction monitoring
mTOR	mammalian target of rapamycin
NTS	nucleus tractus solitarius
PDGF	platelet-derived growth factor
Pdk1	pyruvate dehydrogenase kinase 1
SL	sea level
TNF	tumor necrosis factor
$\dot{V}_{CO_2}$	carbon dioxide production rate
$\dot{V}_{CO_2}/\dot{V}_{O_2}$	respiratory exchange ratio
$\dot{V}_E$	minute ventilation
$\dot{V}_{O_2}$	oxygen consumption rate
V_T	tidal volume

could be releated to a different expression of HIF-1α in the brainstem, the main region harboring respiratory neurons. To answer these questions, the animals were exposed for 6 h to normoxia (21% O_2) or two levels of hypoxia (15 and 12% O_2). We choose 6 h of exposure to hypoxia because previous studies suggested clear differences between the expression of HIF-1α in the brain of rats and mice (see above). We recorded ventilation and metabolic rate, and assessed HIF-1α expression in the brainstem with an ELISA on nuclear protein homogenates. We also used cytosolic protein extracts and mass spectrometry to assess the expression level of proteins involved in the metabolic and angiogenic responses to hypoxia including glucose transporter 1 (Glut1; ensures transport of glucose into cells), pyruvate dehydrogenase kinase 1 (Pdk1; inhibits pyruvate dehydrogenase to limit mitochondrial O_2 consumption) and angiopoietin 2 (Angpt2; involved in vascular remodeling).

MATERIALS AND METHODS

Animals and experimental groups

We used 17 adult SD male rats and 21 adult FVB male mice (2–3 months old) ordered from Charles-River, St Constant, Quebec, Canada. Before the experiments, all the animals were housed for at least 7 days under standard conditions, with access to food and water *ad libitum*, and exposed to a 12 h:12 h light:dark cycle. All protocols have been reviewed and approved by the local committee of animal care and use and are in concordance with the guidelines of the Canadian Council of Animal Care. Adult rats and mice were divided into three groups: a control group was exposed for 6 h to room air conditions (six rats and six mice), and two other groups were exposed for 6 h to hypoxia at 15% (five rats and seven mice) and 12% O_2 (six rats and eight mice).

Exposure to hypoxia

On the day of the experiment, the animals were placed in a whole-body plethysmograph chamber for adult mice or rats (Emka Technologies, Paris, France) flushed constantly with fresh room air. The airflow through the chamber was maintained at 200 ml min^{-1} for the mice and 1.5 l min^{-1} for the rats. The plethysmograph chamber was equilibrated with the desired gas mixture, and the animal was immediately exposed to the desired O_2 level when entering the chamber. After 6 h, the animals were deeply anesthetized with 2–3% of isoflurane flushed inside the plethysmograph chamber, and mixed with the corresponding normoxia or hypoxia gas mixture. Rectal temperature was measured immediately as the animal fell unconscious, then the animals were killed by decapitation.

The brain was removed from the skull and laid on a pre-chilled surface, and then the brainstem (pons+medulla oblongata) was separated from the midbrain at the most rostral part of the cerebellum and from the spinal cord a few millimeters below the caudal part of the cerebellum. The cerebellum was removed, and the brainstem was immediately frozen on dry ice and kept at −80°C.

During the exposure to hypoxia or normoxia, the outflowing air was directed towards gas analyzers for measurements of water pressure (RH-300, Sable Systems, Las Vegas, NV, USA), and O_2 and carbon dioxide (CO_2) percentage (S3A and CD3A analyzers, AEI Technologies; previously calibrated with a certified gas tank), the airflow through the chamber was recorded by a mass flowmeter (TSI series 4140 mass flowmeter; TSI, Shoreview, MN, USA). All signals (from the plethysmograph, gas analyzers and flowmeter) were directed toward a computer for storage and analysis using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Ventilatory and metabolic variables were obtained towards the end of the sixth hour of exposure by selecting periods of 30–60 s preceded at least by 10 min of stable breathing pattern. During these periods, the animals were in a curled-up or ball position, suggesting they were sleeping (van Betteray et al., 1991), despite the effect of hypoxia to reduce sleep time (Pappenheimer, 1977). Tidal volume (V_T), respiratory frequency (f_R), minute ventilation ($\dot{V}_E$; reported at body conditions, BTPS), O_2 consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$; both reported at ambient conditions not saturated in H_2O, ATP) were calculated as previously described (Marcouiller et al., 2014) using standard equations.

Mass-corrected data using allometric scaling

As previously described (Jochmans-Lemoine et al., 2015) we used allometric scaling to compare physiological and morphological variables between mice and rats. Each specific variable A is reported relative to body mass (M, in g) to the exponent b (A/M^b), and we used the scaling variables calculated by Stahl (1967), which are: b=0.8 for minute ventilation, b=1.04 for tidal volume, b=−0.25 for respiratory frequency and b=0.76 for O_2 consumption and CO_2 production. The data corrected for the allometric scaling variables are referred to as 'mass-corrected' (presented in figures) whereas data normalized to body mass (/100 g) are referred to as 'mass-specific' (cf. Tables 1 and 2).

Extraction of the cytosolic and nuclear proteins

We used the nuclear protein extraction kit (item no. 10009277) from Cayman Chemical (Ann Arbor, MI, USA). The brainstem was weighed and put into a pre-chilled vial containing an ice-cold hypotonic buffer (3 ml g^{-1}) supplemented with dithiothreitol and Nonidet P-40. The sample was homogenized on ice, and then centrifuged at 300 ***g*** for 10 min at 4°C to separate the first part of the cytosolic fraction (supernatant). The pellet obtained after centrifugation was gently re-suspended in an additional volume of the hypotonic buffer to complete the lysis, followed by 15 min additional incubation on ice. After incubation, Nonidet P-40 was added to the tubes, which were then centrifuged at 14,000 ***g*** for 30 s at 4°C to separate the second part of the cytosolic fraction. The pellet was then re-suspended in ice-cold extraction buffer. Each vial was vortexed for 15 s at the highest setting and then shaken gently on ice for 30 min (with a brief vortex spin after 15 min). The sample was then centrifuged at 14,000 ***g*** for 10 min at 4°C and the supernatant was collected as the nuclear fraction. The protein concentration in

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Table 1. Comparison between mass-specific ventilatory variables in normoxia (21% O_2) and in response to sustained hypoxia (15 and 12% O_2; 6 h each) in adult male rats and mice

	O_2 level (%)	Rats	Mice
n per group	21	6	6
	15	5	7
	12	6	8
f_R (beats min^{-1})	21	103±3	136±9
	15	134±6$^{\circ\circ}$	217±6$^{\circ\circ\circ\circ}$
	12	162±6$^{\circ\circ\circ\circ}$	269±7$^{\circ\circ\circ\circ}$
V_T (ml 100 g^{-1})	21	0.48±0.05	0.62±0.03
	15	0.43±0.03	0.61±0.06
	12	0.63±0.06	0.89±0.11$^{\circ\circ}$
$\dot{V}_E$ (ml min^{-1} 100 g^{-1})	21	49.5±4.3	86.2±8.3
	15	57.7±6.6	131±14°
	12	101±9.7°	236±29$^{\circ\circ\circ\circ}$

f_R, respiratory frequency; V_T, tidal volume; $\dot{V}_E$, minute ventilation. Species differences are indicated by gray shading (at least P<0.01). $^{\circ}P$<0.05, $^{\circ\circ}P$<0.01 and $^{\circ\circ\circ\circ}P$<0.0001: significant difference versus 21% O_2.

the nuclear and cytosolic fractions was immediately measured with a standard colorimetric assay, then each fraction was aliquoted and stored at −80°C until used for further assays.

The presence of nuclear proteins in the nuclear fraction was verified by western blot using a standard protocol with an antibody for the TATA binding protein TBP (nuclear loading control antibody; Abcam ab51841). TATA binding protein TBP is a general transcription factor that binds to TATA boxes in the promoter region of approximately one-quarter of the genes. Briefly, 30 µg of protein from the brainstem exctracts of mice and rats exposed to 21% O_2 were denaturated, loaded and separated on a 15% agarose gel. After tranfer to nitrocellulose membrane, incubation with primary and secondary antibodies and revelation, we found a two to three times stronger signal in the nuclear fractions compared with the cytosolic fractions, as expected from the Abcam datasheet.

ELISA assay for HIF-1α in the nuclear protein fraction

We used the HIF-1α transcription factor assay kit (item no. 10006910, Cayman Chemical) on the nuclear protein fractions. Each well of the ELISA plate is pre-coated with a specific double-stranded DNA (dsDNA) sequence containing the HIF-1α response element (5′-ACGTG-3′, coated by the manufacturer). A known volume of complete assay buffer, a competitor to the dsDNA, positive control and nuclear fraction of each sample were added in the appropriate wells. The ELISA plate was incubated at 4°C overnight. After incubation, all the wells were washed five times and the HIF-1α antibody was added to each well (except two blanks) followed by 1 h incubation at room temperature. After incubation, the wells were washed properly and the secondary antibody was added, followed by incubation for 1 h at room temperature, the wells were washed again, and the developing solution was added, followed by incubation for 30 min. The stop solution was added in each well and the absorbance was measured at 450 nm. The optical density was corrected by the concentration of protein into each well, and normalized for normoxic values.

Table 2. Comparison between mass-specific metabolic variables in normoxia (21% O_2) and in response to sustained hypoxia (15 and 12% O_2; 6 h each) in adult male rats and mice

	O_2 level (%)	Rats	Mice
$\dot{V}_{O_2}$ (ml min^{-1} 100 g^{-1})	21	3.38±0.07	7.03±0.58
	15	1.90±0.30°	3.86±0.61$^{\circ\circ\circ\circ}$
	12	1.93±0.08°	2.56±0.15$^{\circ\circ\circ\circ}$
$\dot{V}_{CO_2}$ (ml min^{-1} 100 g^{-1})	21	1.76±0.09	3.31±0.25
	15	1.49±0.06	2.81±0.39
	12	1.33±0.08	1.49±0.09$^{\circ\circ\circ\circ}$
$\dot{V}_{CO_2}/\dot{V}_{O_2}$	21	0.52±0.02	0.50±0.04
	15	0.89±0.18$^{\circ\circ}$	0.54±0.09
	12	0.69±0.04	0.56±0.02
Rectal temperature (°C)	21% O_2	36.8±0.2	35.4±0.3
	15% O_2	36.3±0.3	35.6±0.3
	12% O_2	35.6±0.3$^{\circ\circ\circ}$	34.6±0.1°

$\dot{V}_{O_2}$, oxygen consumption rate; $\dot{V}_{CO_2}$, carbon dioxide production rate; $\dot{V}_{CO_2}/\dot{V}_{O_2}$, respiratory exchange ratio. Species differences are indicated by gray shading (at least P<0.01). $^{\circ}P$<0.05, $^{\circ\circ}P$<0.01, $^{\circ\circ\circ}P$<0.001 and $^{\circ\circ\circ\circ}P$<0.0001: significant difference versus 21% O_2.

Mass spectrometry assays

Our colleagues from the proteomic platform of the Centre Hospitalier Universitaire de Quebec have performed all mass spectrometry analyses. By incorporating stable isotope labeled peptides to the samples, multiple reaction monitoring (MRM) mode was used to detect and quantify the expression of HIF-1α (that gave results only in rats), Glut1 (rats and mice), PDK1 (rats and mice) and Angpt2 (only in mice) in normoxia and in response to 6 h of hypoxic exposure at 12% O_2 in nuclear or cytosolic fractions. We initially tried several other proteins (including HIF-2α, Epo, VEGF and HIF-1α in mice to be used as a control for the ELISA, and Angpt 2 in rats), and preliminary experiments were encouraging, but unfortunately, specific mass spectrometry signals were not detectable in the final analysis. Mass spectrometry in the MRM mode is now considered as a reliable tool to assess protein levels in biological samples, and it is not necessary to validate the results with conventional tools such as western blots (Aebersold et al., 2013).

Peptide selection and synthesis

To select tryptic peptides that are the most suitable for sensitive and selective protein detection, target proteins were separately digested *in silico* using the open source Skyline v2.5 program (MacCoss Lab, Seattle, WA, USA) to create a list of potential tryptic peptides with length ranging from 5 to 25 aa. Non-unique peptides were eliminated. Theoretical MRM transitions were generated by including y-ions from +2 and +3 charge state precursor with mass above 300 Da and below 1500 Da. A pool of samples was constituted and analyzed by MRM to monitor the list of transitions generated by the Skyline software. Two peptides per protein were selected based on peak shape and intensity. However, for some proteins, according to those criteria, only one peptide could be selected.

The corresponding crude peptides containing labeled [$^{13}C_6$,$^{15}N_2$]Lys and [$^{13}C_6$, $^{15}N_4$]Arg were synthesized (Pierce Protein Biotechnology, ThermoFisher Scientific). They were diluted in 0.1% formic acid and mixed with variable concentrations ranging from 25 fmol µl^{-1} to 5 pmol µl^{-1}. The concentration was adjusted in order to generate signal equivalent to the endogenous signal.

In-solution digestion of proteins extracts

A volume equivalent to 50 µg of proteins per sample was centrifuged using an Amicon Ultra Spin column, 3 kDa (EMD Millipore), with three successive washes using 50 mmol l^{-1} ammonium bicarbonate to remove salts and detergents. Samples were dried in a Speedvac, and then diluted in denaturation buffer (ammonium bicarbonate: 50 mmol l^{-1}, pH 8; sodium deoxycholate:

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1%; 30 µl final volume), heated to 95°C for 5 min. Disulfide bonds were reduced with 1 µg dithiothreitol (30 min at 37°C) and alkylated with 5 µg iodoacetamide (30 min at 37°C in the dark). Finally, protein digestion was performed with trypsin (1 µg, Sequencing Grade Modified Trypsin, Promega) at 37°C overnight. Digestion was stopped and sodium deoxycholate was removed by acidification (350 µl 5% formic acid). Mixtures were left for 10 min at room temperature and centrifuged at 16,000 ***g*** for 5 min. The supernatant was desalted on a Hydrophilic-Lipophilic-Balance column (Waters). Digested peptides were eluted with a solution of 70% acetonitrile (ACN)/0.1% formic acid and dried in a SpeedVac.

MRM-MS analysis

Dried digested samples were reconstituted with the diluted standard solution. Four micrograms of digested proteins in 10 µl were analyzed on a 6500QTRAP™ hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a nanoLC 400 cHiPLCnanoflex controlled by Analyst 1.6™ (Sciex, Concord, ON, Canada) and with a nanospray ionization source. Mass spectrometry analyses were conducted in positive ion mode with an ionspray voltage of 2500 V. Peptides were desalted on a 200 µm×6 mm chip trap column packed with ChromXP C18, 3 µm (Eksigent), at 2 µl min^{-1} of solvent A (formic acid, 0.1%). The peptides were then eluted at a flow rate of 1 µl min^{-1} on a 200 µm×15 cm chip column packed with ChromXP C18, 3 µm (Eksigent), on a 30 min linear gradient from 5 to 40% of solvent B (ACN with formic acid, 0.1%) and a 10 min linear gradient from 40 to 95% of solvent B. The nebulizer gas was set at 20 (Gas 1), curtain gas at 35 and heater at 100°C. The MRM analysis was conducted in schedule mode with MRM detection windows of 240 s.

MRM-MS analyses were performed using the three most intense transitions for each of the target peptides. The two MRM transitions that gave the highest area counts were subsequently used for the quantitation, with the other transition used as qualifier transition to confirm peptide retention times and the fragment ion ratios. The quantification was done with MultiQuant 2.1 (Sciex) and was based on the relative areas of the synthetic internal standards and endogenous peptides. A blank solvent injection was run between biological samples to prevent sample carryover and the samples were injected in random order. Samples were analyzed in duplicate. Samples containing 5 fmol of digested BSA were injected periodically in order to confirm the stability of the system. For each protein, the relative peak areas were all normalized to the mean normoxic values and expressed in arbitrary units (a.u.) as a ratio of expression (peak value/mean peak value in normoxia).

Statistical analysis

We used GraphPad Prism 6.0c for all analyses. For the variables measured at different levels of O_2 percentage, we first performed two-way ANOVAs with species and O_2 percentage (hypoxia) as grouping variables. When significant effects of species or hypoxia, or a significant interaction between species and hypoxia, appeared, a *post hoc* analysis was performed (Fisher's LSD) to assess species differences at different O_2 levels, or the effects of O_2 level in each species. Correlations were computed using standard equations for linear regressions in Prism. The effects of hypoxia for the level of protein expression assessed by mass spectrometry were tested with unpaired *t*-tests.

All values are reported as means±s.e.m., and the significant *P*-value was set at 0.05. *P*-values are reported in the figures with the

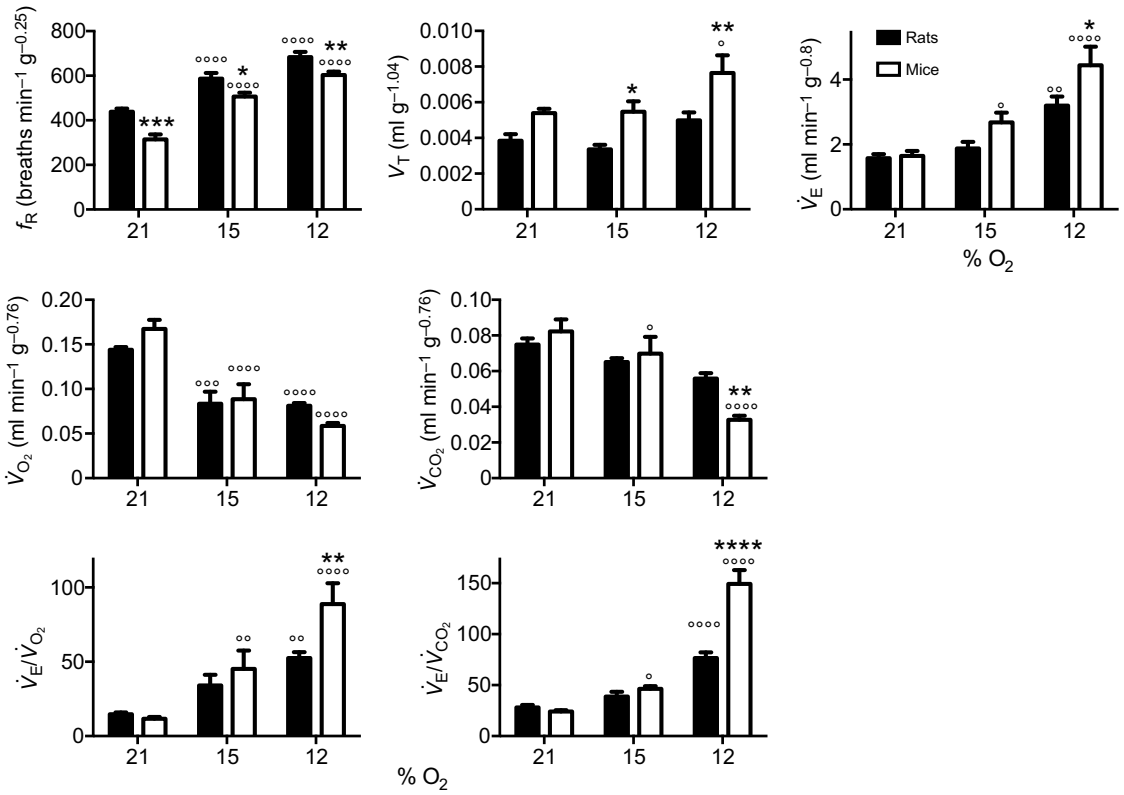

Fig. 1. Mass-corrected ventilatory and metabolic variables in normoxia (21% O_2) and sustained hypoxia (15 and 12% O_2 for 6 h) in rats and mice. Respiratory frequency (f_R; breaths min^{-1} $g^{-0.25}$), tidal volume (V_T; ml $g^{-1.04}$), minute ventilation ($\dot{V}_E$; ml min^{-1} $g^{-0.8}$), O_2 consumption ($\dot{V}_{O_2}$; ml min^{-1} $g^{-0.76}$), CO_2 production rate ($\dot{V}_{CO_2}$; ml min^{-1} $g^{-0.76}$), and ventilatory exchange ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) and CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$) in rats and mice. Data are means+s.e.m. (**P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 for mice versus rats; °*P*<0.05, °°*P*<0.01 and °°°°*P*<0.0001 for 15% versus 21% O_2 or 12% versus 15% O_2).

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following general pattern: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

RESULTS

Mice have higher respiratory response to sustained hypoxia than rats

All mass-specific values are presented in Table 1 for respiratory and metabolic parameters. f_R (breaths min^{-1}) was higher in mice than in rats, and increased more under hypoxic exposure in mice compared with rats (species×hypoxia, P<0.0001). Compared with the values in normoxia, under 12% O_2, f_R had increased by 100±16% in mice and by 59±10% in rats. Similarly, $\dot{V}_E$ increased by 209±37% in mice and by 111±22% in rats, with a significant interaction between hypoxia and species (P=0.016). V_T increased by 52±21% in mice and by 35±16% in rats, and while there was no significant species×hypoxia interaction (P=0.6), there was a significant effect of species (P=0.002), and the *post hoc* ANOVA showed a higher V_T in mice versus rats at 12% O_2.

Mass-corrected values are presented in Fig. 1. When mass-specific corrections were applied, there was no significant species×hypoxia interaction for f_R (P=0.5), V_T (P=0.7) or $\dot{V}_E$ (P=0.2). However, there were significant effects of species for these variables (P<0.0001, P=0.0002 and P=0.016, respectively). The *post hoc* analysis showed that hypoxic exposure significantly increased V_T in mice, but not in rats, and at 15% and 12% O_2, mice had a higher mass-corrected V_T than rats. Mass-corrected $\dot{V}_E$ increased in rats and mice in response to hypoxia, but mass-corrected $\dot{V}_E$ was higher at 15% compared with normoxia only in mice, and at 12% O_2 mice had higher mass-corrected $\dot{V}_E$ than rats.

Mice have a larger reduction of metabolic rate than rats, leading to higher $\dot{V}_E/\dot{V}_{O_2}$ and $\dot{V}_E/\dot{V}_{CO_2}$ in hypoxia

Mass-specific values for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (ml min^{-1} 100 g^{-1}) were higher in mice than in rats (species, both P<0.0001; Table 2). For both values, there were significant species×hypoxia interactions (P=0.002 for $\dot{V}_{O_2}$; P=0.007 for $\dot{V}_{CO_2}$), and the *post hoc* analysis showed higher $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in mice under normoxia and at 15% O_2, but not at 12% O_2. Mice had a stronger decrease of $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ compared with rats: $\dot{V}_{O_2}$ declined by 61±5% in mice at 12% O_2 compared with normoxia, and by 43±2% in rats, and $\dot{V}_{CO_2}$ declined by 52±5% in mice and by 24±5% in rats. The respiratory exchange ratio ($\dot{V}_{CO_2}/\dot{V}_{O_2}$) in normoxia was similar in rats and mice, but at 15% O_2 it was higher in rats compared with mice.

Mass-corrected values are presented in Fig. 1. There was no significant effect of species for mass-corrected $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ but there was a significant effect of hypoxia for both values (P<0.0001) and for mass-corrected $\dot{V}_{CO_2}$ a significant interaction between hypoxia and species (P=0.01). The *post hoc* analysis showed that mass-corrected $\dot{V}_{O_2}$ decreased in rats and mice at 15% O_2 compared with normoxia, and decreased further at 12% O_2 compared with 15% O_2 only in mice. At 12% O_2, mass-corrected $\dot{V}_{CO_2}$ was lower in mice compared with rats (Fig. 1). Ventilatory exchange ratio for O_2 ($\dot{V}_E/\dot{V}_{O_2}$) and CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$; both calculated using mass-specific values, corrected to 100 g) were consequently higher in mice compared with rats at 12% O_2.

Finally, rectal temperature measured at the end of the exposure was lower in mice than in rats (species, P<0.0001), and decreased in hypoxia (P=0.0004) without significant interaction between species and hypoxia (P=0.46; Table 2).

To further emphasize the relationship between ventilation and metabolism in response to sustained hypoxia in rats and mice, we plotted the mass-specific $\dot{V}_E$ against $\dot{V}_{O_2}$ (Fig. 2, left) or $\dot{V}_{CO_2}$ (Fig. 2, right). Below 15% O_2, rats are no longer able to decrease their metabolic rate and only slightly increase their ventilation, whereas mice decrease their metabolic rate between 15 and 12% O_2 exposure and further increase their ventilation.

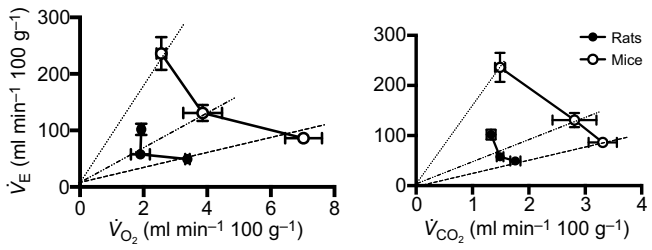

Fig. 2. Correlation between $\dot{V}_E$ and $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ showing the effect of hypoxia in rats and mice. The dashed, dot-dashed and dotted lines represent the values of $\dot{V}_E/\dot{V}_{O_2}$ or $\dot{V}_E/\dot{V}_{CO_2}$ reported for mice and rats at levels of 21%, 15% and 12% O_2, respectively, from bottom to top.

HIF-1α protein level in the brainstem increased in response to hypoxia in mice but not in rats, and is correlated with the ventilatory response

Western blot controls for nuclear protein are presented in Fig. 3A,B. We used an antibody for the TATA binding protein TBP (see

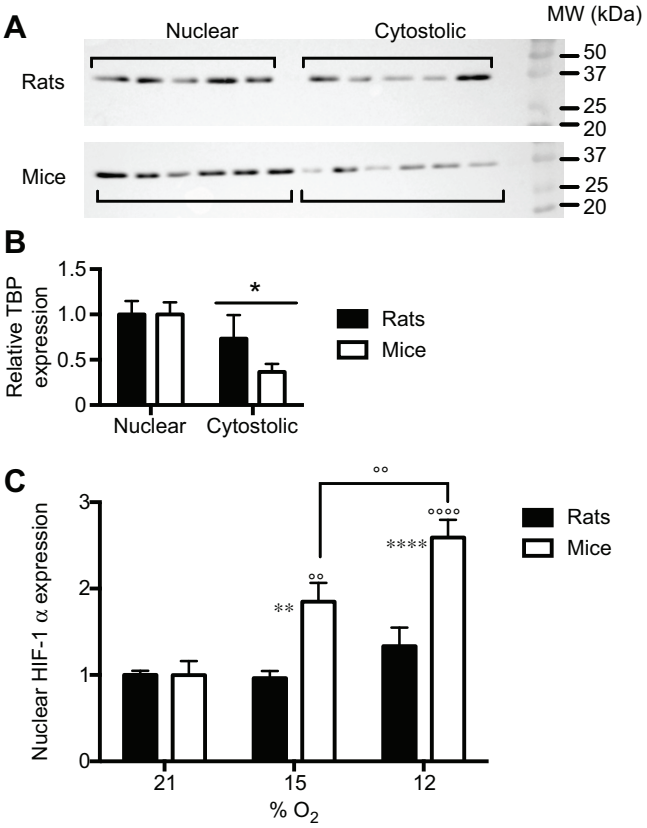

Fig. 3. Control of nuclear extraction with the TATA binding protein TBP, and expression level of HIF-1α in the brainstem in normoxia (21% O_2) and sustained hypoxia (15 and 12% O_2 for 6 h). (A) Representative immublots of rats and mice from the normoxic group with 30 µg of protein from the nuclear or cytosolic fraction loaded into each well. MW, molecular weight. (B) Cytosolic expression level normalized to nuclear expression level. *P<0.05 for the nuclear versus cysotoslic fraction (ANOVA). (C) Relative expression of HIF-1α normalized to the expression in normoxia. **P<0.01 and ****P<0.0001 for mice versus rats; °°P<0.01 and °°°°P<0.0001 for 15% versus baseline (21% O_2) or 12% versus 15% O_2. All values in B and C are means+s.e.m.

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Table 3. Concentrations of protein (μg mg^{-1} tissue) in the nuclear fraction from brainstem samples

O_2 level (%)	Rats	Mice
21	7.82±0.51	20.1±1.1
15	9.69±0.63	19.9±1.1
12	6.76±0.85	20.7±1.6

Materials and methods). As expected with this antibody, we found a two to three times stronger signal in the nuclear fractions compared with the cytosolic fractions (Fig. 3B).

The concentration of protein in the nuclear fractions obtained from the brainstem are presented in Table 3, and the relative protein level of HIF-1α in Fig. 3C. HIF-1α protein increased in mice but not in rats (hypoxia×species, P=0.004). At 12% O_2, the HIF-1α level had increased by 2.6-fold in mice (versus 21%, P<0.0001), but was not significantly increased in rats (1.3-fold; versus 21%, P=0.2). Even at a modest hypoxia level, in mice, HIF-1α was significantly increased compared with normoxia (at 15% O_2, 1.8-fold, P=0.002), and the HIF-1α level was significantly higher at 12% O_2 versus 15% O_2.

Interestingly, in mice, there were significant positive correlations between the relative protein level of HIF-1α in brainstem nuclear extracts and the mass-corrected value of $\dot{V}_E$ or $\dot{V}_E/\dot{V}_{CO_2}$ across all groups (Fig. 4, upper panels), and negative correlations between HIF-1α level and $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$ (Fig. 4, lower panels). These correlations were absent in rats.

Cytosolic protein level decreased in hypoxia in rats, and Glut1 expression level increased in the brainstem of rats and mice

In rats, the concentrations of protein in the cytosolic fractions obtained from the brainstem had declined by 38% at 12% O_2 compared with normoxic values (P=0.0004), while it remained at the same level in mice (hypoxia×species, P=0.039; Table 4). In rats, the protein level of Glut1, Pdk1 and HIF-1α in the cytosolic fraction increased in response to hypoxia when expressed relative to the protein content, while in mice the cytosolic level of Glut1 and Angpt2 increased in response to hypoxia (Fig. 5). However, in rats, if the absolute expression level is reported, then only Glut1 increased, and HIF-1α expression in the cytosolic fraction was only marginally increased (24% higher versus normoxia, P=0.051), while Pdk1 remained at a stable level of expression.

DISCUSSION

This study shows that under hypoxic exposure sustained for 6 h, compared with SD rats, FVB mice had a higher ventilatory response to hypoxia, a greater metabolic decline and a higher induction of HIF-1α expression. In FVB mice, the expression level of HIF-1α was positively correlated with minute ventilation, and negatively correlated with metabolic rate. This is in line with studies showing the role of HIF-1α for the respiratory plasticity (Kline et al., 2002; Powell and Fu, 2008; Prabhakar and Semenza, 2012) and control of metabolic pathways (Papandreou et al., 2006; Schönenberger and Kovacs, 2015; Wheaton and Chandel, 2011) under hypoxia. In SD rats, the concentration of cytosolic proteins in the brainstem declined during the exposure to 12% O_2. Similar responses have been obtained *in vitro* using rats neuronal cells and reflected a decline of protein synthesis (Hernández-Jiménez et al., 2012; Véga et al., 2006). Interestingly, in FVB mice, cytosolic protein content remained stable in hypoxia, while the expression of HIF-1α increased, suggesting that expression of HIF-1α under hypoxia is a way to ensure adequate ATP synthesis and maintain protein synthesis. We conclude that FVB mice and SD rats have different responses to short-term hypoxia. These differences could represent a set of innate traits present in mice but not in rats, and can provide key advantages for mice under hypoxic conditions.

Methodological considerations

Most protocols of hypoxic exposure use either shorter (a few minutes) or longer (days to weeks) exposure duration to investigate mechanisms of acute responses to hypoxia, or the long-term process of ventilatory acclimatization to hypoxia (Powell et al., 1998). We chose 6 h of exposure, because it has been shown that in mice, this corresponds to the peak expression for HIF-1α in the brain (Stroka et al., 2001), and as expected we found a marked upregulation of

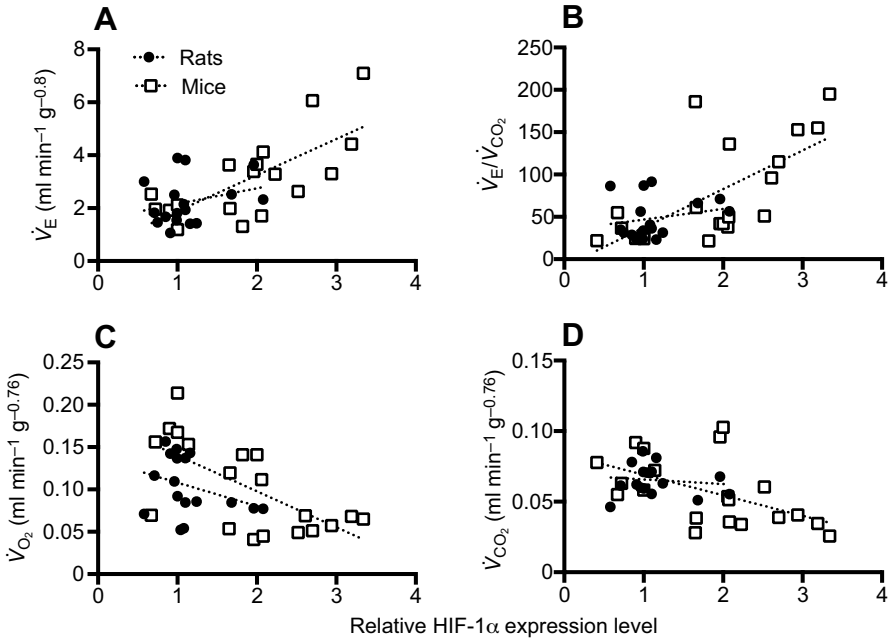

Fig. 4. Correlation between the expression level of HIF-1α in the brainstem and $\dot{V}_E$, $\dot{V}_E/\dot{V}_{CO_2}$, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ in rats and mice. Correlation between the relative HIF-1α expression level and (A) mass-corrected $\dot{V}_E$ (ml min^{-1} g$^{-0.8}$; rats, r^2=0.08, P=0.3; mice, r^2=0.51, P=0.0008), (B) $\dot{V}_E/\dot{V}_{CO_2}$ (rats, r^2=0.05, P=0.4; mice, r^2=0.45, P=0.001), (C) mass-corrected $\dot{V}_{O_2}$ (ml min^{-1} g$^{-0.76}$; rats, r^2=0.10, P=0.2; mice, r^2=0.44, P=0.002) and (D) mass-corrected $\dot{V}_{CO_2}$ (ml min^{-1} g$^{-0.76}$; rats, r^2=0.01, P=0.6; mice, r^2=0.28, P=0.016) in rats and mice. Data are individual values. Dashed lines are correlation slopes for rats and mice.

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Table 4. Concentrations of protein (μg mg^{-1} tissue) in the cytosolic fraction from brainstem samples

O_2 level (%)	Rats	Mice
21	50.9±4.6	23.6±2.3
15	44.3±6.7	23.2±2.0
12	31.4±2.2 °°°	21.8±2.0

°°°P<0.001: significant difference versus 21% O_2.

HIF-1α in the brainstem of mice. In rats, previous studies have shown (by immunohistochemistry) that exposure to hypoxia for 6 h (10% O_2) induces the expression of HIF-1α in a restricted pool of neurons in cardiorespiratory areas of the brainstem (Pascual et al., 2001). It remains possible that ELISA does not allow the measurement of these changes in rats, and we cannot speculate on HIF-1α expression level in restricted areas of the brainstem such as the NTS that receive and integrate signals from the peripheral chemoreceptors (Pascual et al., 2001). By contrast, the clear increased expression of HIF-1α in mice might reflect a widespread response, contrasting with the restricted response in rats observed by Pascual et al. (2001).

Values of $\dot{V}_{CO_2}/\dot{V}_{O_2}$ recorded in normoxia (0.5–0.6) were lower than the expected lowest value (0.7). The most likely explanation is that inflowing water pressure level was not recorded during the measurements and we could not take into account its diluting effect on O_2 concentration. This leads to an over-estimation of $\dot{V}_{O_2}$, reducing $\dot{V}_{CO_2}/\dot{V}_{O_2}$.

HIF-1α expression is correlated with respiratory and metabolic responses in mice

The α subunit of the HIF-1 transcription factor is tightly regulated by O_2 level and plays an important role in the neural circuits controlling breathing. Heterozygous knockout mice for HIF-1α have deficient peripheral chemoreceptors responses to acute hypoxia and the process of ventilatory acclimatization to hypoxia is not possible in these animals (Kline et al., 2002). A similar effect is reported in mice with a specific deletion of the HIF-1α gene in the central nervous system (Bavis et al., 2007), while the induction of HIF-1α expression with desferrioxamine (an iron chelator) in rats enhances ventilatory response to hypoxia (Nguyen et al., 2007). The plasticity occurring in the central nervous system in response to sustained hypoxia involves activation of the glutamatergic signaling pathway in the NTS (Pamenter et al., 2014; Reid and Powell, 2005), and a recent study pointed out that HIF coordinates the transcriptional activation of multiple genes encoding glutamate transporters and receptors in response to hypoxia in cancer cells (Hu et al., 2014); therefore, this might be a way by which HIF-1 induces higher ventilation under hypoxia in mice. HIF-1 is also a key element for metabolic control by promoting glycolysis, and by suppressing the activity of the mitochondrial Krebs cycle and

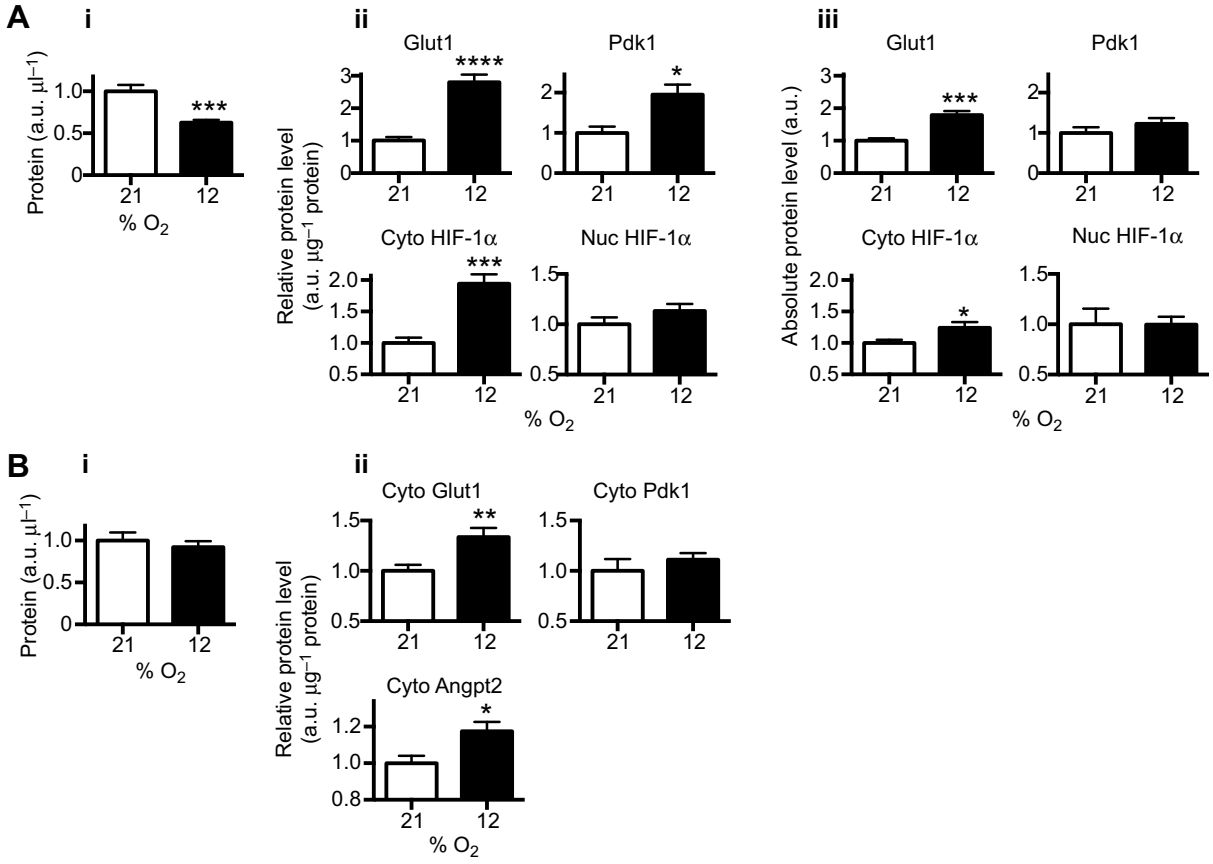

Fig. 5. Protein concentrations in relative (a.u. μg^{-1} protein) or absolute (a.u.) levels in the nuclear and cytosolic fraction in rats and mice. (A) Protein level in the brainstem of rats: (i) protein concentration in the cytosolic fraction (a.u. μl^{-1}), (ii) relative protein level (a.u. μg^{-1} protein) in the cytosolic fraction of glucose transporter 1 (Glut1), pyruvate deshydrogenase kinase isoform 1 (Pdk1) and hypoxia-inducible factor 1 subunit alpha (HIF-1α) and in the nuclear fraction of HIF-1α, and (iii) absolute protein level (a.u.) in the cytosolic fraction of Glut1, Pdk1 and HIF-1α and in the nuclear fraction of HIF-1α. (B) Protein expression in the brainstem of mice: (i) protein concentration in the cytosolic fraction (a.u. μl^{-1}) and (ii) relative protein level (a.u. μg^{-1} protein) in the cytosolic fraction of Glut1, Pdk1 and angiopoietin 2 (Angpt2). Data are means+s.e.m.

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oxidative phosphorylation (Papandreou et al., 2006; Schönenberger and Kovacs, 2015). After 6 h of hypoxic exposure, HIF-1α expression level in the brainstem of mice was positively correlated with $\dot{V}_E$, and negatively correlated with $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, suggesting a causal link between these responses and HIF-1α expression under these conditions.

We also observed an increased expression of Angpt2 in the brainstem of mice exposed to 12% O_2 for 6 h. Angpt2 is a pro-angiogenic factor, whose expression in the brain is enhanced by HIF-1α in hypoxia (Simon et al., 2008), and it contributes to vascular remodeling (Benderro and LaManna, 2014).

The fact that the respiratory and metabolic responses in mice are correlated with the expression level of HIF-1α suggests a potential role for long-term acclimatization to hypoxia in mice, and other species displaying such responses. Supporting this view, it is interesting that in the plateau pika, a lagomorph that is considered adapted to HA, the expression level of HIF-1α in cell nuclei of the lung, liver, spleen and kidney is higher compared with SL mice, and HIF-1α expression augmented with the increase in the altitude range of the animal (Li et al., 2009). These results are in concordance with what we observed in the brainstem of mice after 6 h of sustained hypoxia at 15 and 12% O_2.

Lack of physiological responses linked to reduced protein synthesis and a HIF-independent switch to glycolytic metabolism in rats?

By contrast with mice, rats displayed reduced respiratory and metabolic responses to hypoxia, and the expression level of HIF-1α in the nuclear fraction of the protein extracts was not increased in response to hypoxia. Strikingly, we also observed a decreased in the cytosolic protein content. One might argue that it would be necessary to normalize protein concentration to dry tissue mass to avoid potential confounding effects of brain edema induced by hypoxia. However, brain edema increases brain water content by less than 10% in rats (Paczynski et al., 2000; Yu et al., 2016), thus unlikely accounting for a 40% decrease of cytosolic protein concentration. In PC-12 cells that have been differentiated by nerve growth factor (used as a model of sympathetic neurons from rats), exposure to hypoxia for 4 h inhibits protein synthesis by approximately 25%. This response is mediated by an inhibition of the cytosolic protein complex that regulates the translation of mRNAs to proteins (Hernández-Jiménez et al., 2012). Two mechanisms explained this reduction of protein synthesis. One of them involves a HIF-1-dependent pathway, through inhibition of mTOR activity and dephosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). The other mechanism does not depend on HIF-1, and involves the translocation of the eukaryotic translation initiation factor 4E (eIF4E) from the cytosol to the nucleus (Hernández-Jiménez et al., 2012). This is also in concordance with a previous study that reported a decreased protein content after 1 day of hypoxia in cultured astrocytes (Véga et al., 2006). Moreover, it is noteworthy that this response is generally considered to limit cellular energy requirements. Because this was only observed in SD rats, one might argue that FVB mice develop physiological responses to hypoxia that provide enough O_2 to maintain cellular energy requirements and thus avoid the decreased protein synthesis.

In parallel with the decreased protein content, there was also an increased expression of the glucose transporter Glut1 in rats, and to a lesser extend also in mice. Glut1 is particularly abundant in astrocytes and endothelial cells in the brain, and is essential for the transport of glucose into cells, which is a limiting step of glycolytic activity (Duelli and Kuschinsky, 2001). It is well established that hypoxic response in the brain includes increased glycolytic activity and anaerobic metabolism, and that this response ensures sufficient ATP production in face of the reduced O_2 availability. In cultured astrocytes, Glut1 expression doubles after 1 day of hypoxia, but is reduced after 3 weeks of exposure (Véga et al., 2006), and glycolytic capacity doubles after 8 h of hypoxia (Marrif and Juurlink, 1999). It is notable that while we observed a sustained elevation of Glut1 expression in rats, there was no measureable increase of HIF-1α. Contrastingly, in mice, HIF-1α increased, but the effect of hypoxia on the expression of Glut1 was less important than in rats. While it is largely recognized that hypoxia induces Glut1 expression through binding of HIF-1α on the *GLUT1* gene promoter (Ebert et al., 1995), other regulatory pathways are involved in the regulation of Glut1 protein level by post-transcriptional regulation of its mRNA (Qi and Pekala, 1999). For example, hypoglycemia (Bruckner et al., 1999), TNFα (Boado and

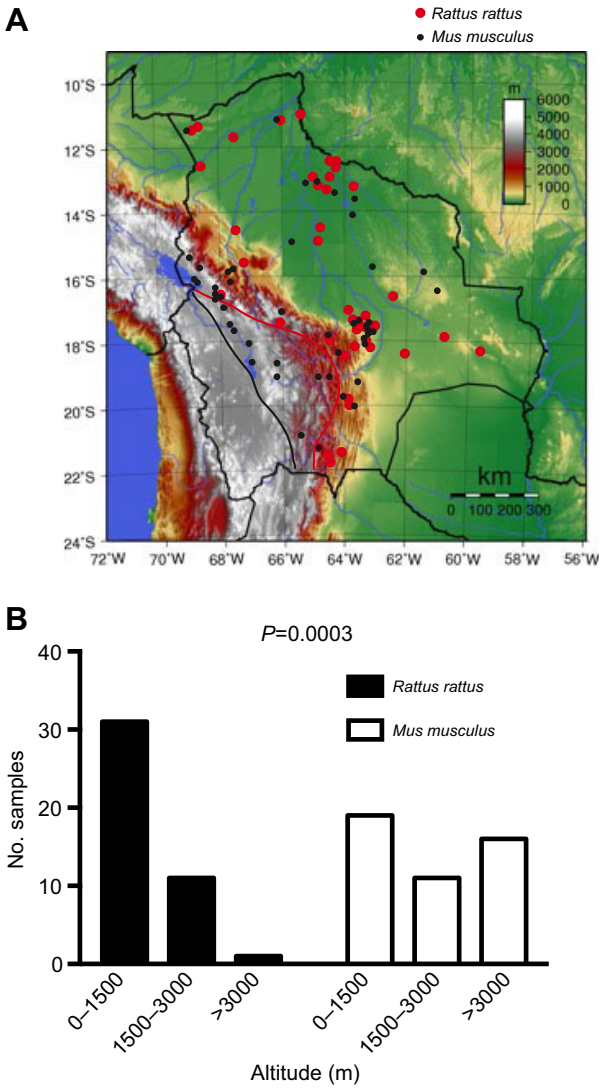

Fig. 6. Altitudinal distribution of rats and mice in Bolivia. (A) Samples of *Rattus rattus* (red circles) and *Mus musculus* (black circles) reported by Anderson (1997), superposed on a geographical map of Bolivia showing elevation range. (B) Number of samples for altitude ranges (0–1500, 1500–3000 and >3000 m above sea level) for *R. rattus* and *M. musculus* based on A. *P*-value reported for a chi-square test showing significant differences in sample numbers across altitude ranges.

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Pardridge, 1993) and PDGF (Rollins et al., 1988) are all able to induce Glut1 expression. Glut1 expression is also tightly associated with brain glycolytic activity (Duelli and Kuschinsky, 2001); therefore, metabolic signaling pathway(s) likely regulate Glut1 expression. In our previous studies, we showed that the respiratory exchange ratio in rats raised at high altitude was close to 1 (a sign of glycolytic metabolism), while it was lower in mice (Jochmans-Lemoine et al., 2015). Interestingly, in the present study, the respiratory exchange ratio increased in response to hypoxia in rats (although this was only significant at 15% O_2 compared with normoxia). Taken together, these results likely underlie a general response strategy in rats that rely on glycolytic pathways when exposed to hypoxia, while species considered as being adapted to HA can rely on other sources of metabolic fuels (Cheviron et al., 2012; Jochmans-Lemoine et al., 2015). Finally, we did not observed specific increase of Pdk1 in rats or in mice, despite its well-known induction by HIF-1α, and its role to inhibit pyruvate dehydrogenase and limit the conversion of pyruvate to acetyl-CoA, the entry point of the Krebs cycle (Prabhakar and Semenza, 2012). The fact that this is mainly a mitochondrial enzyme probably limited our ability to measure changes of expression induced by hypoxia.

Mice in hypoxia: a model of 'pre-adapation' to altitude?

We conclude from this study that adult SD rats and FVB mice living at SL have divergent ventilatory and metabolic responses to 6 h of sustained hypoxia. Overall, these results seem to indicate that responses to hypoxia are more efficient in FVB mice than in SD rats. This is line with our previous findings obtained in the HA colonies of FVB mice and SD rats (Jochmans-Lemoine et al., 2015), in which we concluded that the specific physiological traits observed in laboratory mice at HA could explain why mice are commonly found at HA. By contrast, and as also presented in the Introduction, it is less common to find rats at HA. To better visualize the differences between the two species, we used the data published by Anderson (1997) for the repartitioning of *M. musculus* and *R. rattus* in Bolivia, superposed with a physical map, and we counted the number of samples observed for specific altitude zones (Fig. 6). It is striking to observe the difference between the two species, particularly in regions that are above the altitude of 3000 m. In his book, Anderson reports only one specimen of rats from the city of La Paz, but he also noted that based on an 'external source', 'the species does not reproduce at the elevation of La Paz and the Altiplano', and that it was possible 'that it came from some lower elevation in the department of La Paz and not from the city itself'. If we generalize our findings and the observation of Anderson, it is thus tempting to speculate that a form of 'pre-adaptation' to hypoxia is present in mice, but not in rats. We acknowledge that this hypothesis remains mostly speculative, but it is in line with the proposition made by Monge and Leon-Velarde (1991), stating that 'pre-adaptation' is a 'capacity to invade a new ecological niche as a consequence of previously acquired appropriate structural and/or functional changes'. Finally, it seems useful to note that current phylogeography data indicate that the area in which *M. musculus* originaly evolved and differentiated includes a region encompassing the countries of Iran, Afghanistan and Pakistan. Thus, Bonhomme and Searle (2012) stated that during their phase of initial expansion from this regions, mice should have crossed moutain barriers (Bonhomme and Searle, 2012). It is thus tempting to speculate that through its evolution in this region, *M. musculus* has gained, and retained, specific traits of resitance to low oxygen levels. Similar processes occured in human Andean natives over a time frame of 10,000 to 15,000 years (Bigham et al., 2013), or roughly 500–1000 generations of HA residents, that can be achieved in a much shorther time frame in mice.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.J., J.S. and A.J.-L. developed the concepts and designed the experiments. A.J.-L., M.S. and V.J. performed the experiments. A.J.-L., M.S. and V.J. analyzed the data and prepared the figures. V.J., J.S. and A.J.-L. prepared or edited the manuscript prior to submission.

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