Impaired hypoxic response in senescent mouse brain

Tamer Rabie*, Reiner Kunze, Hugo H. Marti

Institute of Physiology and Pathophysiology, University of Heidelberg, D-69120 Heidelberg, Germany

*Present address and corresponding author:

Tamer Rabie, PhD
School of Life Sciences
University of Hertfordshire
College Lane
AL10 9AB Hatfield
United Kingdom
Phone: +44 1707 28 5096
Fax: +44 1707 28 5046
Email: t.rabie@herts.ac.uk

Abstract

Tissue hypoxia leads to activation of endogenous adaptive responses that involve a family of prolyl hydroxylase domain proteins (PHD1-3) with oxygen sensing properties, hypoxia inducible transcription factors (HIFs), and cytoprotective HIF target genes such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF). The hypoxic induction of these genes is regulated by oxygen-dependent hydroxylation of HIFα subunits by PHDs, which signals their proteasomal degradation. In this study, mice of different age were exposed to hypoxia or subjected to cerebral ischemia after hypoxic pre-conditioning. We found an impaired hypoxic response in the brain, characterized by elevated levels and impaired downregulation of PHD1. Furthermore, an attenuated hypoxic activation of VEGF and EPO, as well as of other HIF-target genes such glucose transporter-1 and carbonic anhydrase 9 was found in senescent brain. Finally, we observed a loss of the protective effect of hypoxic pre-conditioning on subsequent cerebral ischemia with increasing age. Thus, the impaired hypoxic adaptation, resulting in compromised hypoxic activation of neuroprotective factors, could contribute to neurodegenerative processes with increasing age, and might have implications for treating age-related disorders.

Keywords: aging; hypoxia; HIF; PHD; VEGF; erythropoietin; neuroprotection

Abbreviations: CA-IX, carbonic anhydrase 9; EPO, erythropoietin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter-1; HIF, hypoxia-inducible factor; MCAO, middle cerebral artery occlusion; PHD, prolyl hydroxylase domain protein; RPS12, ribosomal protein S12; TBP, TATA binding protein; VEGF, vascular endothelial growth factor

1. **Introduction**

Perturbation of cellular oxygen availability leads to the activation of endogenous adaptive mechanisms. Upon hypoxia/ischemia, several cytoprotective factors such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) are induced (Goldberg et al. 1994). In addition, many other genes are activated during hypoxia, among them glucose transporter-1 (GLUT1) and carbonic anhydrase 9 (CA-IX). Both genes are frequently used as markers for a hypoxic response (Stiehl et al. 2006). The expression of these factors is under the influence of hypoxia inducible transcription factors (HIFs) (Wang et al. 1995). HIFs are heterodimers composed of an α and a β subunit. Three isoforms of the α subunit exist, 1α, 2α, and 3α (Gu et al. 1998; Tian et al. 1997; Wang et al. 1995). Under normoxic conditions, the α subunit is hydroxylated on specific proline residues leading to the binding of the von Hippel-Lindau (pVHL) protein E3 ubiquitin ligase and its subsequent proteasomal degradation (Ivan et al. 2001; Jaakkola et al. 2001). This prolyl hydroxylation is mediated by a family of proteins called prolyl hydroxylase domain proteins (PHDs), which require molecular oxygen for the hydroxylation reaction (Bruick et al. 2001; Epstein et al. 2001). Under hypoxic conditions, where oxygen is limited, PHD is inactive, no prolyl hydroxylation occurs, and thus HIF-α is stabilized leading to transcription of HIF target genes (Kaelin, Jr. et al. 2008). Three PHD family members have been identified (PHD1-3) (Bruick et al. 2001; Epstein et al. 2001) which are differentially expressed. While PHD2 is ubiquitously expressed, PHD1 is expressed mainly in the testis, and PHD3 mainly in the heart (Willam et al. 2006). Interestingly, whereas PHD1 is constitutively expressed, PHD2 and PHD3 expression is induced by hypoxia itself, indicative of a negative feedback mechanism (Willam et al. 2006). The PHDs also have different substrate specificity. *In vitro* studies suggest PHD2 preference towards HIF-1α, while PHD3 seems to be more active towards HIF-2α (Appelhoff et al. 2004).

The HIF-1α abundance in different organs is not only dependent on the PHD isoform present, but also shows organ specific variations. Although hypoxia-induced HIF-1α

stabilization/activation occurs in all organs, the degree of organ-specific stabilization varies *in vivo*. In the kidney, peak levels of HIF-1α are reached after 1 h of systemic hypoxia and return to basal levels 4 h thereafter. In contrast, peak HIF-1α levels in the brain are reached only after 5 h and remain elevated for at least 12 h (Stroka et al. 2001).

Evidence exists that the endogenous adaptive response to hypoxia is impaired during the aging process (Rivard et al. 1999; Rohrbach et al. 2005). For instance, HIF-1α protein has been shown to be reduced under hypoxia in smooth muscle cells isolated from old rabbits as compared to young animals (Rivard et al. 2000). Moreover, DNA binding activity of HIF-1 is impaired in senescent mice exposed to hypoxia (Frenkel-Denkberg et al. 1999). It has been suggested that reduced HIF levels in aged organs could be the result of increased PHD expression, as the expression of PHD3 was increased in the heart of old mice and humans (Rohrbach et al. 2005). As many neuroprotective factors such as VEGF and EPO are direct HIF target genes, an impaired hypoxic response in the senescent brain might facilitate neurodegenerative processes. We therefore aimed to gain more insight into the effect of aging on the HIF system and thus on the hypoxic adaptive response in the elderly, and subjected young and old mice to hypoxia for varying durations and measured the levels of the major molecular players involved in the hypoxic response.

2. Materials and methods

2.1 Animals

All experiments were performed using male C57BL/6 mice (3 to 21 months of age). Animals were maintained at the animal facility of the University of Heidelberg. All animal procedures were approved by the animal welfare committee.

Hypoxia was induced by substituting nitrogen for oxygen using a Digamix 5SA 18/3A pump (Woesthoff, Bochum, Germany) as described (Marti et al. 1998). Mice (n = 3-4 per group)

breathed 6% oxygen for 2, 12 or 48 h, or were kept at 20% oxygen. After hypoxic exposure, brains were removed for total RNA isolation and nuclear protein extract preparation.

For hypoxic pre-conditioning and middle cerebral artery occlusion (MCAO), 16 animals were exposed to 6% O_2 for 4 h or kept at 20%. 72 h later, animals underwent filament-induced MCAO using a 7-0 silicon rubber-coated monofilament (Doccol Cooperation Redlands, USA). This is a modification of a previously published hypoxic pre-conditioning model (Prass et al. 2003). Mice were anaesthetized by a mixture containing 4% halothane, 70% N_2O, and remainder O_2, and were maintained by reducing the halothane concentration to 1.0-1.5%. Body temperature was maintained at 37 °C using a temperature controlled heating pad. MCAO was performed as described previously (Hata et al. 2000). After 90 min occlusion, reperfusion was allowed for 24 h. Subsequently, brains were removed and frozen. From each brain, 24 coronal cryosections (10 µm thick each; 0.4 mm apart) were prepared and silver-stained. Healthy tissue appears dark while infarcted tissue appears light (silver-deficient). The total infarct volume was calculated as the summation of the total infarct area of each section multiplied by the distance between each section. The area of each hemisphere was measured by ImageJ software. To calculate the infarct area of each section the following equation was applied: I = (SD+CT-IT), where I = infarct area in mm^2, SD = silver-deficient area in mm^2, CT = total area of the contralateral hemisphere in mm^2, IT = total area of the ipsilateral hemisphere in mm^2. Thus, the total infarct volume in mm^3 = $\sum$I*0.4, where 0.4 = the distance between each section in mm. This equation was used to correct for the increase in volume of the ipsilateral hemisphere due to swelling (Lin et al. 1993).

2.2 Real-time RT-PCR

After 2, 12 or 48 h of hypoxic exposure, animals were sacrificed and organs isolated and snap frozen in liquid nitrogen. Total RNA was isolated using the TRI reagent (Invitrogen, Darmstadt, Germany) according to manufacturer`s instructions. For digestion of residual DNA, 10 µg of total RNA was incubated in a 25 µl reaction mix containing 1x DNase-buffer,

40 U RNasin and 1 U DNase (Promega, Mannheim, Germany) for 30 min at 37 °C. Reverse transcription (1 µg RNA) was carried out with Access RT-PCR Kit (Promega). Real-time RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) corresponding to manufacturer`s instructions. The housekeeping genes ribosomal protein S12 (RPS12) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as controls. The following primers (MWG Biotech, Ebersberg, Germany) were used: CA-IX sense GACAGTTCATCTGGGGAGGA, CA-IX antisense AAGGGACTCCTCCATCCTGT, EPO sense CCACCCTGCTGCTTTTACTC, EPO antisense CTCAGTCTGGGACCTTCTGC, GAPDH sense TCACCATCTTCCAGGAGCG, GAPDH antisense CTGCTTCACCACCTTCTTGA, GLUT1 sense GCTGTGCTTATGGGCTTCTC, GLUT1 antisense AGAGGCCACAAGTCTGCATT, PHD1 sense GCTAGGCTGAGGGAGGAAGT, PHD1 antisense CCCCAAGTTGTCCTTGA, PHD2 sense TCCATCTGGACACGAAACAA, PHD2 antisense TTCCCCGAATTCAAAACTTG, PHD3 sense GCTATCCAGGAAATGGGACA, PHD3 antisense TGGCGTCCCAATTCTTATTC, RPS12 sense GAAGCTGCCAAAGCCTTAGA, RPS12 antisense AACTGCAACCAACCACCTTC, VEGF sense GTACCTCCATGCCAAGT, VEGF antisense ACTCCAGGGCTTCATCGTTA.

2.3 Nuclear protein extract preparation

One brain hemisphere was homogenized in 750 µl of buffer A containing 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, 1% Nonidet P40, 2 mM Na_3VO_4 (all from Sigma-Aldrich, Steinheim, Germany). After 15 min incubation at 4 °C, samples were centrifuged at 850 g for 10 min. Pellets were resuspended in 1 ml of buffer A, incubated on ice for 15 min, then centrifuged at 14,000 g for 1 min. Pellets were resuspended in buffer C containing 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, 2 mM Na_3VO_4 and incubated on ice for 30 min with gentle shaking. After a final centrifugation at 14,000 g for 10 min, supernatants (nuclear protein extract) were snap-frozen in liquid nitrogen.

2.4 Immunoblotting

Nuclear proteins (25 µg) were run on a SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. The TATA binding protein (TBP) was used as loading control. The membrane was incubated with primary antibodies against HIF-1α (1:500; NB100-449, Novus Biologicals, Cambridge, UK), PHD2 (1:1000; NB100-2219, Novus Biologicals), PHD3 (1:1000; NB100-303, Novus Biologicals) or TBP (1:1000; ab818, Abcam, Cambridge, UK). All antibodies were diluted in 5% non-fat dry milk. After rinsing in tris-buffered saline, blots were incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; 31460, Thermo Fisher Scientific, Bonn, Germany). ECL Western blot detection reagents (GE Healthcare, Freiburg, Germany) were used for protein detection.

2.5 Data evaluation and statistical analysis

The influence of age on expression of the target genes under basal conditions was analyzed by relating the values from old mice (15, 18 and 21 mo, respectively) to young mice (3 mo) under normoxic conditions. With the exception of PHD1 (Fig.2) no age-dependent changes in basal gene expression were determined. One-way ANOVA combined with Bonferroni post-test was applied to determine statistical significance. The effect of hypoxia on gene expression was first evaluated separately for 3, 15, 18 and 21 months old mice by comparing the values from mice exposed to 2, 12 and 48 h hypoxia, respectively, to these from mice kept under normoxia. Then, all data calculated for old mice (15, 18 and 21 mo) were averaged and statistical significance for hypoxia-induced alterations in gene expression of young (3 mo) and old (15-21 mo) mice, respectively, was defined with one-way ANOVA followed by Bonferroni post-test. The strength of hypoxic response was compared between young (3 mo) and old (15-21 mo) mice, and was statistically evaluated by using two-way ANOVA combined with Bonferroni post-test.

Results

3.1 Relative PHD expression in the brain.

As tissue-specific PHD expression has been reported, we first determined the expression level (relative to GAPDH) for the different PHD forms in the adult brain. PHD2 was the most abundant form, followed by PHD1, whereas PHD3 was 2-3 times weaker expressed, as compared to PHD1, and PHD2, respectively (Fig.1). Next, we characterized age-dependent PHD expression in the brain, as increased PHD3 expression had been shown for the aged heart (Rohrbach et al. 2005). We found no changes in expression levels for PHD2 and PHD3, neither in brain nor heart, when comparing young mice (3 mo) with 15, 18 or 21 months old animals (data not shown). However, PHD1 expression significantly increased 1.6-1.7-fold in 15-21 mo old mice as compared to young mice (Fig.2), whereas no change for PHD1 was found in the heart of the same mice (data not shown).

3.2 Hypoxic induction of PHDs in brain.

To test the effect of aging on the hypoxic response, mice of various ages (3, 15, 18, and 21 months) were subjected to hypoxia (6% O_2) for 2 and 12 h, young animals in addition to 48 h, and PHD mRNA levels were measured by quantitative PCR. Surprisingly, PHD1 expression in the brain was significantly downregulated (by 50%) after 2 h of hypoxia, but tended to recover after longer hypoxic exposure (Fig.3). Older mice (18, 21 mo) only showed a non significant 25% reduction, suggesting that hypoxic responsiveness of PHD1 expression decreases with age (Fig.3). For PHD2, a slight but significant increase was seen after prolonged hypoxia (12 and 48 h) in young animals, which was not detectable in older mice (Fig.3). PHD3 expression in the brain showed neither hypoxic responsiveness nor age-dependent changes (Fig.3). On the protein level, however, neither PHD2 nor PHD3 levels significantly changed in the brain after hypoxic exposure or during aging (Fig.4). Unfortunately, no PHD1 antibody suitable for mouse protein detection in Western blots was available to us.

To test whether hypoxia-induced HIF-1α stabilization was affected by aging, nuclear protein extracts from brain of hypoxic mice were prepared. In agreement with the subtle changes in PHD expression in senescent mice, no major difference for HIF-1α expression was detected. However, HIF-1α protein levels tended to be reduced in older animals after 2 h hypoxia as compared to young mice (Fig.4).

3.3 Impaired HIF-target gene expression in senescent mouse brain.

The total increase in PHD levels (mainly PHD1), and reduced hypoxic responsiveness (PHD1, PHD2, HIF-1α) in senescent brain, prompted us to analyze expression levels of known HIF targets and hypoxia-responsive factors important for neuroprotection. The hypoxia response of VEGF and EPO was clearly reduced in senescent mice. Whereas VEGF induction was almost 5-fold in young animals, it was less than 3-fold in older animals. Even more dramatic differences were seen for EPO. Hypoxic exposure of young animals induced EPO expression in the brain 25-35-fold, whereas induction level was less than 10-fold in older animals (Fig.5). For both neuroprotective factors we found a clear declining hypoxic response with increasing age. To verify the diminished hypoxic response in senescent brain tissue, we quantified expression levels of two further HIF-target genes, CA-IX and GLUT1. We found a significant transient 2-fold induction of CA-IX (after 12 h of hypoxia) and a sustained 2.5-fold increase in GLUT1 expression in young animals, which was clearly reduced in older mice, thus confirming the blunted hypoxic responsiveness. The dysfunctional hypoxic response was also found in the heart, as induction of VEGF mRNA expression was totally absent in old animals (data not shown).

3.4 Hypoxic pre-conditioning fails in aged mice.

All these results suggested that the hypoxic response in the brain of old mice is blunted. To test whether this impaired hypoxic adaptation has functional consequences, we analyzed the effect of hypoxic pre-conditioning on infarct size after cerebral ischemia. It had been previously shown that subjecting animals to a short period of hypoxia has a protective effect

against a subsequent strong ischemic insult (Bergeron et al. 2000; Miller et al. 2001). Young (3 months) and old (18 months) mice were subjected to 4 h hypoxia (6% O_2), followed by cerebral ischemia induced by occluding the middle cerebral artery 72 h later. As shown in figure 6, hypoxic pre-conditioning significantly reduced the cerebral infarct size by 20% in young mice. Strikingly, this protective effect was not observed in old mice, demonstrating that the impaired hypoxic response in the brain of aged mice has functional consequences.

4. Discussion

The results of this study demonstrate that the hypoxic adaptive response is altered in the brain of old mice. PHD1 levels were higher in old mice, and hypoxic upregulation of PHD2 as well as downregulation of PHD1 expression attenuated in senescent brain. Although this did not result in clear alterations of hypoxia-induced HIF-1α levels, the hypoxic activation of the neuroprotective HIF-target genes VEGF and EPO was significantly attenuated with increasing age. Furthermore, other HIF target genes, such as CA-IX and GLUT1 also showed attenuated hypoxic inducibility with increasing age. Consequently, this study also demonstrated a loss of the protective effect of hypoxic pre-conditioning on a subsequent ischemic insult in senescent brain.

Adaptation to reduced oxygen supply (hypoxia) is essential for normal cell and organ function. Therefore, any impairment of this adaptive mechanism is likely to contribute to pathological changes and disease. Hypoxia is thought to significantly contribute to the etiology of several age-related neurodegenerative diseases, most notably Alzheimer's disease (Sun et al. 2006). Our results suggest that the impaired adaptive response to hypoxia in the elderly results in attenuated activation of neuroprotective factors and thus might contribute to the progress of neurodegenerative diseases.

4.1 PHD expression in the brain

We could show that in the brain of adult mice PHD2 is the most abundant PHD protein, followed by PHD1, while PHD3 was much weaker expressed in comparison to both PHD1 and PHD2. Thus, the distribution of different PHDs appears to be organ specific, as PHD3 and PHD1 were reported to be the predominant PHD forms in heart and testis, respectively (Willam et al. 2006). Under hypoxia we found downregulation of PHD1 mRNA expression in the adult brain. While hypoxic activation of PHD2 and PHD3 has been described before (Willam et al. 2006), our results suggest for the first time that also PHD1 is responsive to changes in inspired oxygen concentration. The underlying mechanisms need to be defined. It has recently been shown that absence of PHD1 improves hypoxia tolerance by shifting glucose metabolism towards the anaerobic pathway (Aragones et al. 2008). Thus, the observed downregulation of PHD1 mRNA expression by hypoxia in the brain seems to be part of the adaptive response towards hypoxia, enabling the brain to cope with reduced oxygen levels and to improve anaerobic energy generation.

Several *in vitro* studies demonstrated a strong hypoxia-dependent upregulation of PHD2 and PHD3 expression (Metzen et al. 2005; Pescador et al. 2005). However, *in vivo*, we only observed a slight increase in PHD2 expression on mRNA but not protein level, while PHD3 expression in the adult brain was not altered under hypoxic conditions at all. Measurement of partial oxygen pressure in normal cerebral cortex of cats revealed mean values of around 20 mmHg (Whalen et al. 1970; Padnick et al. 1999), thus corresponding to an oxygen concentration of about 2.5-3%. One can assume, that exposure to 6% oxygen (inspired air) results in a further reduction of tissue oxygenation, although no clear data exist. Thus, the degree of tissue hypoxia is difficult to predict. Tissue oxygenation in living animals is a very complex process, depending on ventilation and organ perfusion, enabling a certain degree of adaptation which is not possible in cell culture systems. Indeed, available data concerning PHD expression in hypoxic tissues are conflicting. While in the brain of mice exposed to 7.5% O_2 for up to 72 h no significant changes in PHD2 and PHD3 expression were found (Stiehl et al. 2006), expression of PHD2 and PHD3 even decreased in the brain of rats exposed to 8% O_2 for 8 h (Willam et al. 2006). On the other hand, functional anemia (by

exposure to carbon monoxide) and thus reduction of the oxygen carrying capacity of the blood, increased expression of PHDs 1.5-2.2-fold (Stiehl et al. 2006), suggesting that animals can better compensate for inspiratory hypoxia than for reduced oxygen carrying capacity. Thus, the regulation of hypoxia-induced PHD genes appears to be more subtle under physiological inspiratory hypoxia than under *in vitro* conditions (Stiehl et al. 2006). However, prolonged exposure to hypoxia has been recently shown to increase mRNA and protein expression of both PHD2 and PHD3 in the brain of rats (Ndubuizu et al. 2009). The discrepancy to our results might be due to extended hypoxic exposure time, severity of hypoxia, or species differences. While we used mice for a short exposure (2 and 12 h) to 6% O_2, Ndubuizu et al. used rats which were exposed for 24 h to 10% followed by additional 48 h exposure to 8% O_2.

4.2 Age-dependent changes in PHD expression in the brain

If we consider hypoxic downregulation of PHD1 as an adaptive protective mechanism in the brain, then our findings that the hypoxic inhibition of PHD1 in old mice was attenuated support the hypothesis - in concert with increased PHD1 levels in older animals - that this adaptive response is lost in the brain of older mice indicative of reduced hypoxia tolerance.

While PHD1 expression in the brain increased with age, we did not find any age-related changes for PHD2 or PHD3 neither in the brain, nor in the heart (data not shown). This is in contrast to previous publications, where PHD3 expression was increased in heart (Rohrbach et al. 2005), liver, skeletal muscle (Rohrbach et al. 2008), and brain (Ndubuizu et al. 2009) of senescent tissue isolated from mice, rats and humans. The difference could be due to different experimental settings. Nevertheless, the common feature of our and the previous results is the elevated level of PHD expression in senescent tissue, either as PHD1 in brain, or as PHD3 in liver, skeletal muscle and heart.

The hypoxic responsiveness of PHD expression in our *in vivo* model was rather small, even in the young animals; however we could detect an attenuated PHD1 downregulation and

blunted PHD2 upregulation after hypoxic exposure in the brain of old animals supporting the idea of defective hypoxia responsiveness.

In line with the small changes in PHD expression in old mice, HIF-1α protein levels showed no major changes with increasing age in the brain, while decreased HIF-1α stabilization upon hypoxia was correlated with increasing age in the lung (Hwang et al. 2007), and the heart (Rohrbach et al. 2005), and similar findings were reported for smooth muscle cells obtained from old rabbits in comparison with young animals (Rivard et al. 2000). Ndubuizu et al. have recently reported a decrease in HIF-1α stabilization in the brain of old rats exposed for 24 h to 10%, followed by 48 h to 8% O_2 (Ndubuizu et al. 2009). This discrepancy might be due to the longer exposure period to milder hypoxia and/or species-specific effects.

4.3 Hypoxia-induced gene expression is impaired in old mice

Although hypoxia-induced stabilization of HIF-1α appeared to be normal in the brain of old mice, we observed a clear impairment of hypoxic induction of the HIF-1 target genes VEGF, EPO, CA-IX and GLUT1. This is in line with recent findings showing impaired hypoxic induction of various HIF target genes in the brain of old rats (Ndubuizu et al. 2009). Impaired transcriptional activity in senescent brain tissue might not necessarily be dependent on reduced amounts of transcription factor present, but could be the result of decreased DNA binding activity. Indeed, a decreased HIF-1α/DNA binding activity in brain, liver, kidney, and lung tissue of senescent mice has been reported (Frenkel-Denkberg et al. 1999).

The impaired hypoxic response, characterized by reduced inducibility of VEGF and EPO, had functional consequences, as hypoxic pre-conditioning did not protect old mice against the subsequent ischemic insult. The protective pre-conditioning effect on ischemic damage has been attributed to the upregulation of EPO (Prass et al. 2003). The impaired hypoxic induction of EPO and VEGF in the brain of old mice could thus be responsible for the lack of protection after hypoxic pre-conditioning. It is tempting to speculate that the adaptation to hypoxic (and ischemic) stress in the brain results in upregulation of EPO and VEGF which in turn protects neurons from hypoxic damage. Indeed, several reports demonstrate that EPO

and VEGF can exert their neuroprotective function via activation of common survival pathways such as PI3 kinase/Akt and ERK-1/-2 (Kilic et al. 2005; Kilic et al. 2006; Rabie et al. 2008; Zacchigna et al. 2008). In addition, recent data suggest that VEGF can protect neuronal cells from damage by depression of synaptic transmission (McCloskey et al. 2005), involving inactivation of glutamate receptors of the AMPA type (Bogaert et al. 2010).

GLUT1 and CA-IX may support the adaptive response of the brain during hypoxia. While GLUT1 facilitates glucose transport from blood into brain parenchyma trough the blood-brain barrier, transmembrane CA-IX regulates cellular pH by catalyzing the reversible conversion of carbon dioxide to bicarbonate and H^+ (Boado et al. 2002; van den Beucken et al. 2009). The lack of oxygen caused by hypoxia leads to a switch from aerobic to anaerobic glucose metabolism, characterized by increased production of lactic acid and a lowered intracellular pH (Swietach et al. 2007). Thus, increased levels of GLUT1 could satisfy the elevated glucose requirement for anaerobic glycolysis, and increased CA-IX levels may account for the stabilization of the intracellular pH under hypoxia. Impaired induction of GLUT1 and CA-IX, as observed in the brain of old animals, may thus have functional relevance for the protective effect of hypoxic pre-conditioning on ischemic damage, as efficient ATP production might be impaired.

Taken together, our results show that the hypoxic response in senescent brain is impaired and suggest that the protective effect, elicited by hypoxia-induced VEGF and EPO, as well as other hypoxia-inducible genes, is attenuated, and could contribute to progressive neurodegeneration with increasing age.

Figure legends

Figure 1: Expression of PHD1-3 on mRNA level in the brain of young mice. Brains were isolated from mice (3 months old), RNA was prepared and RT-PCR was performed. *N* = 4 (per group).

Figure 2: PHD1 mRNA expression in the brain is increased in an age-dependent manner. Brains were isolated from mice (3 to 21 months old), RNA was prepared and real-time RT-PCR was performed. Significant differences determined by Bonferroni post-test are indicated with ** (p<0.01). *N* = 3 (per group).

Figure 3: Hypoxia decreases PHD1 and increases PHD2 mRNA expression in the brain of young mice. Mice (3 to 21 months old) were subjected to hypoxia (6% O_2) for 2, 12 or 48 h. Then, brains were isolated, RNA was prepared and real-time RT-PCR was performed. Significant differences determined by Bonferroni post-test are indicated with * (p<0.05) or ** (p<0.01). *N* = 3-4 (per group).

Figure 4: Effect of hypoxia on HIF-1α stabilization and protein expression of PHD2 and PHD3 in the brain. Mice (3 to 21 months old) were subjected to hypoxia (6% O_2) for 2 or 12 h. Then, brains were isolated, nuclear proteins were prepared and Western blot analysis was performed. *N* = 3-4 (per group).

Figure 5: Hypoxia-induced expression of HIF-1α target genes is impaired in the brain of old mice. Mice (3 to 21 months old) were subjected to hypoxia (6% O_2) for 2, 12 or 48 h. Then, brains were isolated, RNA was prepared and real-time RT-PCR was performed. Significant differences determined by Bonferroni post-test are indicated with * (p<0.05), ** (p<0.01) or *** (p<0.001). *N* = 3-4 (per group)

Figure 6: Hypoxic pre-conditioning reduces the infarct volume upon focal cerebral ischemia in young but not in old mice. Mice (3 and 18 months old) were subjected to hypoxia (6% O_2) for 4 h or kept at 20% O_2. 72 h later, focal cerebral ischemia was induced for 90 min followed by 24 h of reperfusion. Then, brains were removed and the infarct volume was determined. Statistical significance was determined by using one-way ANOVA combined with Bonferroni post-test. Significant differences determined by Bonferroni post-test are indicated with * (p<0.05). N = 3-4 (per group).

References

Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., Gleadle, J. M., 2004. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem 279, 38458-38465.

Aprelikova, O., Chandramouli, G. V., Wood, M., Vasselli, J. R., Riss, J., Maranchie, J. K., Linehan, W. M., Barrett, J. C., 2004. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem 92, 491-501.

Aragones, J., Schneider, M., van Geyte, K., Fraisl, P., Dresselaers, T., Mazzone, M., Dirkx, R., Zacchigna, S., Lemieux, H., Jeoung, N. H., Lambrechts, D., Bishop, T., Lafuste, P., Diez-Juan, A., Harten, S. K., Van Noten, P., De Bock, K., Willam, C., Tjwa, M., Grosfeld, A., Navet, R., Moons, L., Vandendriessche, T., Deroose, C., Wijeyekoon, B., Nuyts, J., Jordan, B., Silasi-Mansat, R., Lupu, F., Dewerchin, M., Pugh, C., Salmon, P., Mortelmans, L., Gallez, B., Gorus, F., Buyse, J., Sluse, F., Harris, R. A., Gnaiger, E., Hespel, P., Van Hecke, P., Schuit, F., Van Veldhoven, P., Ratcliffe, P., Baes, M., Maxwell, P., Carmeliet, P., 2008. Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. Nat Genet 40, 170-180.

Bergeron, M., Gidday, J. M., Yu, A. Y., Semenza, G. L., Ferriero, D. M., Sharp, F. R., 2000. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol 48, 285-296.

Boado, R. J. and Pardridge, W. M., 2002. Glucose deprivation and hypoxia increase the expression of the GLUT1 glucose transporter via a specific cis-acting regulatory element. J Neurochem 80, 552-554.

Bogaert, E., Van Damme, P., Poesen, K., Dhondt, J., Hersmus, N., Kiraly, D., Scheveneels, W., Robberecht, W., Van Den, B. L., 2010. VEGF protects motor neurons against excitotoxicity by upregulation of GluR2. Neurobiol Aging 31, 2185-2191.

Bruick, R. K. and McKnight, S. L., 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294, 1337-1340.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., Ratcliffe, P. J., 2001. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107, 43-54.

Frenkel-Denkberg, G., Gershon, D., Levy, A. P., 1999. The function of hypoxia-inducible factor 1 (HIF-1) is impaired in senescent mice. FEBS Lett 462, 341-344.

Goldberg, M. A. and Schneider, T. J., 1994. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J Biol Chem 269, 4355-4359.

Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L., Bradfield, C. A., 1998. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr 7, 205-213.

Hata, R., Maeda, K., Hermann, D., Mies, G., Hossmann, K. A., 2000. Evolution of brain infarction after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab 20, 937-946.

Hwang, I. S., Fung, M. L., Liong, E. C., Tipoe, G. L., Tang, F., 2007. Age-related changes in adrenomedullin expression and hypoxia-inducible factor-1 activity in the rat lung and their responses to hypoxia. J Gerontol A Biol Sci Med Sci 62, 41-49.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., Kaelin, W. G., Jr., 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292, 464-468.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292, 468-472.

Kaelin, W. G., Jr. and Ratcliffe, P. J., 2008. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell 30, 393-402.

Kilic, E., Kilic, U., Soliz, J., Bassetti, C. L., Gassmann, M., Hermann, D. M., 2005. Brain-derived erythropoietin protects from focal cerebral ischemia by dual activation of ERK-1/-2 and Akt pathways. FASEB J 19, 2026-2028.

Kilic, E., Kilic, Ü., Wang, Y., Bassetti, C. L., Marti, H. H., Hermann, D. M., 2006. The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF's neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. FASEB J 20, 1185-1187.

Lin, T. N., He, Y. Y., Wu, G., Khan, M., Hsu, C. Y., 1993. Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24, 117-121.

Marti, H. H. and Risau, W., 1998. Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. Proc Natl Acad Sci U S A 95, 15809-15814.

McCloskey, D. P., Croll, S. D., Scharfman, H. E., 2005. Depression of synaptic transmission by vascular endothelial growth factor in adult rat hippocampus and evidence for increased efficacy after chronic seizures. J Neurosci 25, 8889-8897.

Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W., Acker, H., Fandrey, J., 2003. Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. J Cell Sci 116, 1319-1326.

Metzen, E., Stiehl, D. P., Doege, K., Marxsen, J. H., Hellwig-Burgel, T., Jelkmann, W., 2005. Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J 387, 711-717.

Miller, B. A., Perez, R. S., Shah, A. R., Gonzales, E. R., Park, T. S., Gidday, J. M., 2001. Cerebral protection by hypoxic preconditioning in a murine model of focal ischemia-reperfusion. Neuroreport 12, 1663-1669.

Ndubuizu, O. I., Chavez, J. C., LaManna, J. C., 2009. Increased Prolyl 4-Hydroxylase (PHD) Expression and Differential Regulation of Hypoxia Inducible Factors (HIFs) in the Aged Rat Brain. Am J Physiol Regul Integr Comp Physiol 297, 158-165.

Padnick, L. B., Linsenmeier, R. A., Goldstick, T. K., 1999. Oxygenation of the cat primary visual cortex. J Appl Physiol 86, 1490-1496.

Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landazuri, M. O., Del Peso, L., 2005. Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene. Biochem J 390, 189-197.

Prass, K., Scharff, A., Ruscher, K., Lowl, D., Muselmann, C., Victorov, I., Kapinya, K., Dirnagl, U., Meisel, A., 2003. Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. Stroke 34, 1981-1986.

Rabie, T. and Marti, H. H., 2008. Brain protection by erythropoietin: a manifold task. Physiology 23, 263-274.

Rivard, A., Berthou-Soulie, L., Principe, N., Kearney, M., Curry, C., Branellec, D., Semenza, G. L., Isner, J. M., 2000. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 275, 29643-29647.

Rivard, A., Fabre, J. E., Silver, M., Chen, D., Murohara, T., Kearney, M., Magner, M., Asahara, T., Isner, J. M., 1999. Age-dependent impairment of angiogenesis. Circulation 99, 111-120.

Rohrbach, S., Simm, A., Pregla, R., Franke, C., Katschinski, D. M., 2005. Age-dependent increase of prolyl-4-hydroxylase domain (PHD) 3 expression in human and mouse heart. Biogerontology 6, 165-171.

Rohrbach, S., Teichert, S., Niemann, B., Franke, C., Katschinski, D. M., 2008. Caloric restriction counteracts age-dependent changes in prolyl-4-hydroxylase domain (PHD) 3 expression. Biogerontology 9, 169-176.

Stiehl, D. P., Wirthner, R., Köditz, J., Spielmann, P., Camenisch, G., Wenger, R. H., 2006. Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. J Biol Chem 281, 23482-23491.

Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Neil, D. A., Bauer, C., Gassmann, M., Candinas, D., 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J 15, 2445-2453.

Sun, X., He, G., Qing, H., Zhou, W., Dobie, F., Cai, F., Staufenbiel, M., Huang, L. E., Song, W., 2006. Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. Proc Natl Acad Sci U S A 103, 18727-18732.

Swietach, P., Vaughan-Jones, R. D., Harris, A. L., 2007. Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev 26, 299-310.

Tian, H., McKnight, S. L., Russell, D. W., 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 11, 72-82.

van den Beucken, T., Koritzinsky, M., Niessen, H., Dubois, L., Savelkouls, K., Mujcic, H., Jutten, B., Kopacek, J., Pastorekova, S., van der Kogel, A. J., Lambin, P., Voncken, W., Rouschop, K. M., Wouters, B. G., 2009. Hypoxia-induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response. J Biol Chem 284, 24204-24212.

Wang, G. L. and Semenza, G. L., 1995. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270, 1230-1237.

Whalen, W. J., Ganfield, R., Nair, P., 1970. Effects of breathing O_2 or O_2 + CO_2 and the injection of neurohumors on the PO_2 of cat cerebral cortex. Stroke 1, 194-200.

Willam, C., Maxwell, P. H., Nichols, L., Lygate, C., Tian, Y. M., Bernhardt, W., Wiesener, M., Ratcliffe, P. J., Eckardt, K. U., Pugh, C. W., 2006. HIF prolyl hydroxylases in the rat; organ distribution and changes in expression following hypoxia and coronary artery ligation. J Mol Cell Cardiol 41, 68-77.

Zacchigna, S., Lambrechts, D., Carmeliet, P., 2008. Neurovascular signalling defects in neurodegeneration. Nature Rev Neurosci 9, 169-181.

Figure 1

Click here to download high resolution image

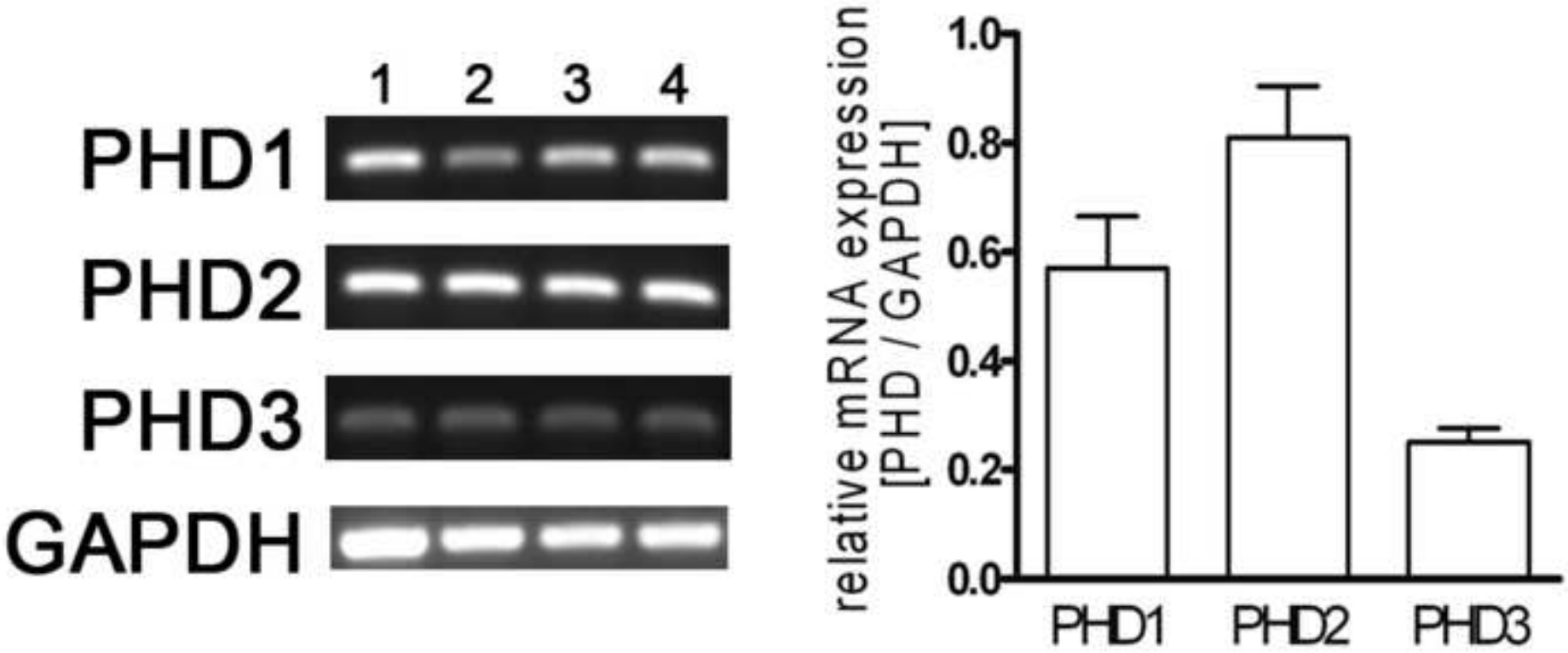

Figure 2

Click here to download high resolution image

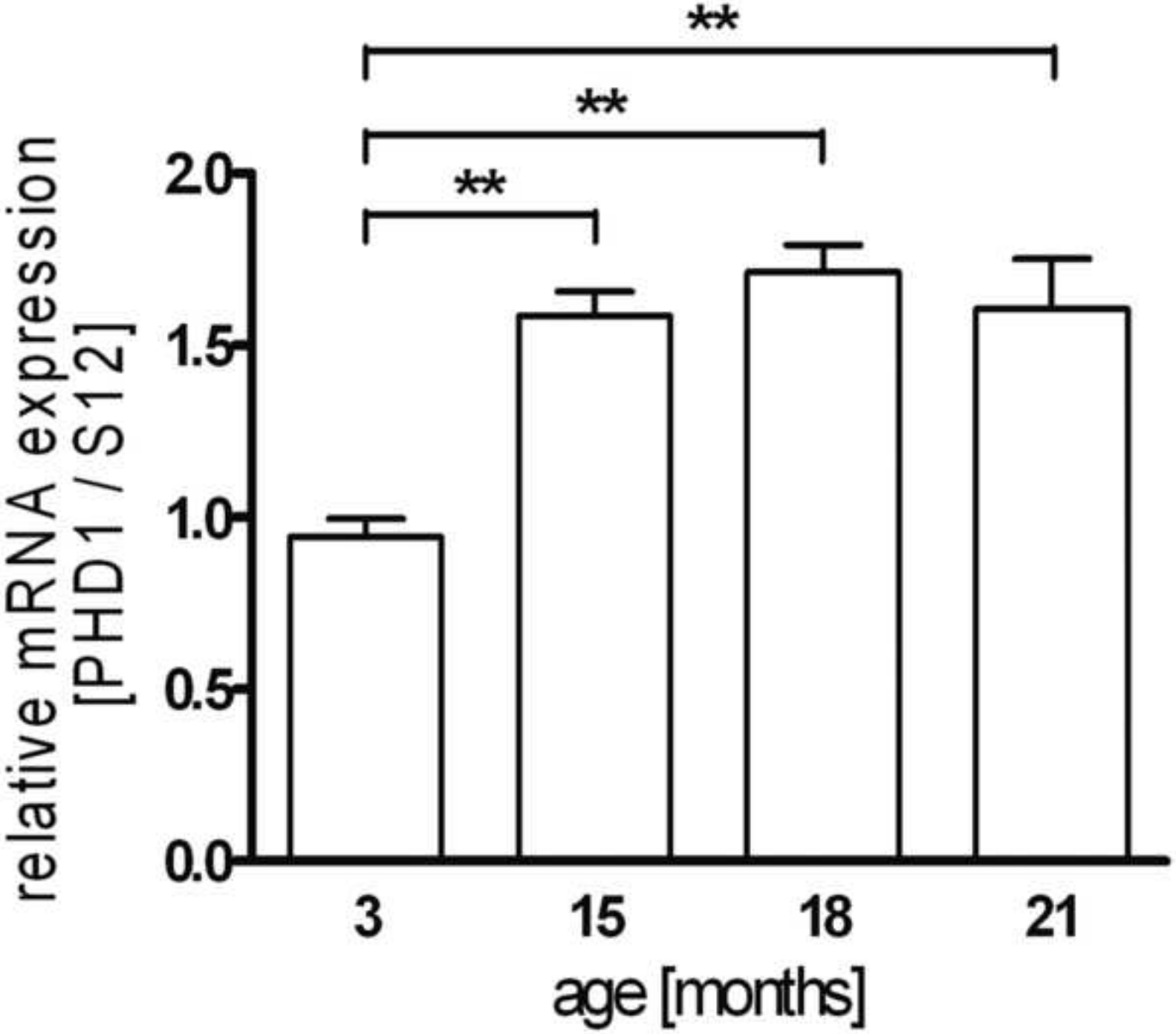

Figure 3

Click here to download high resolution image

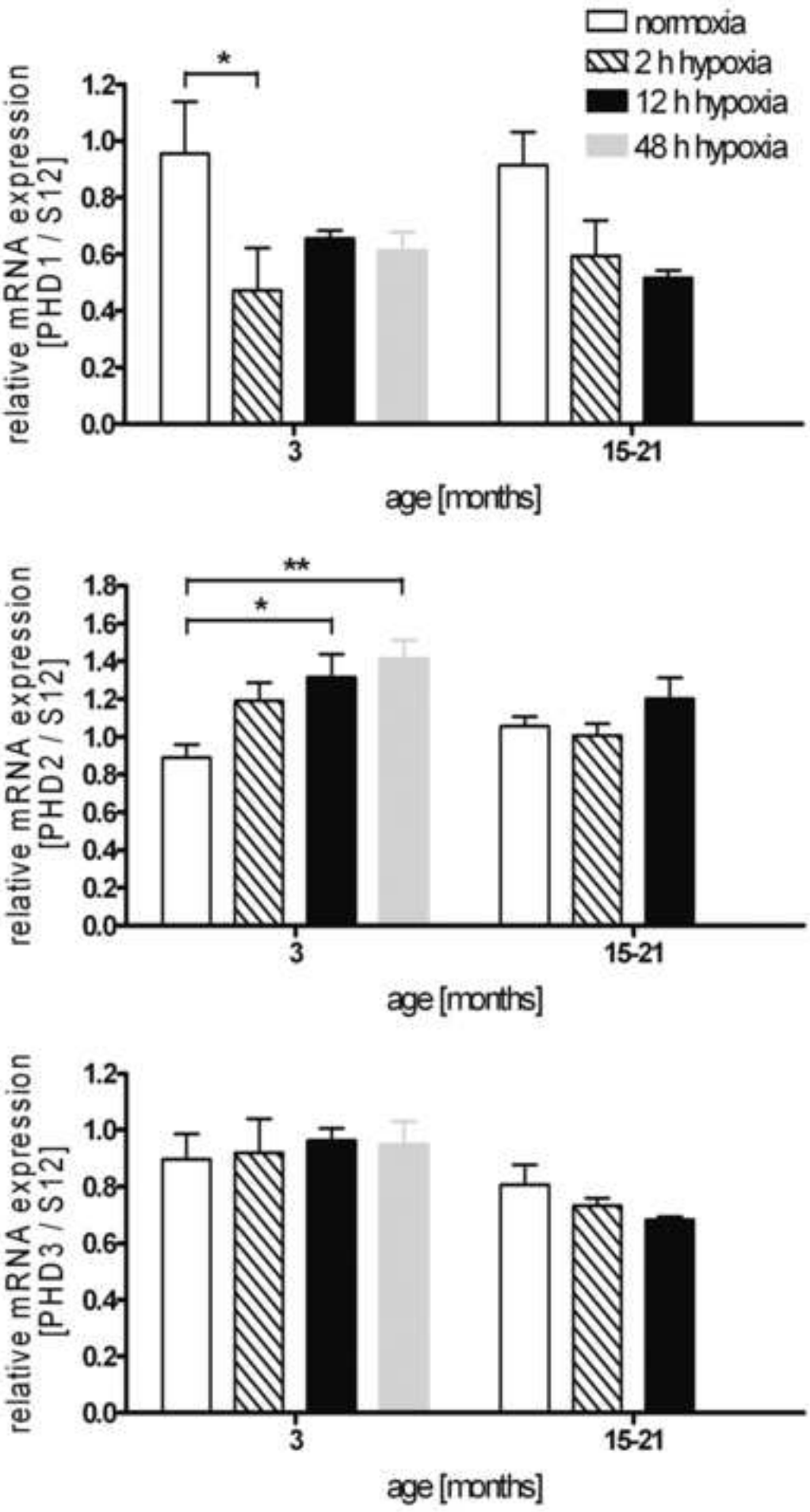

Figure 4

Click here to download high resolution image

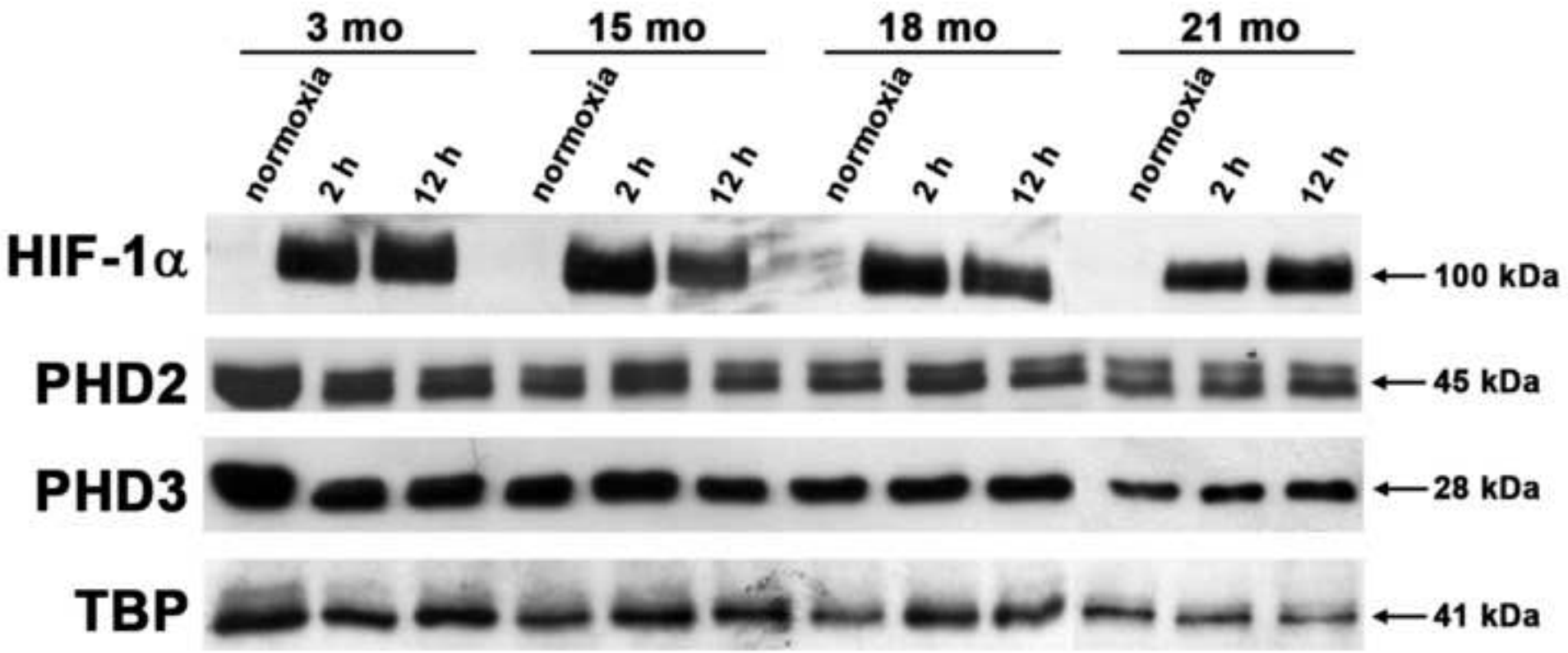

Figure 5

Click here to download high resolution image

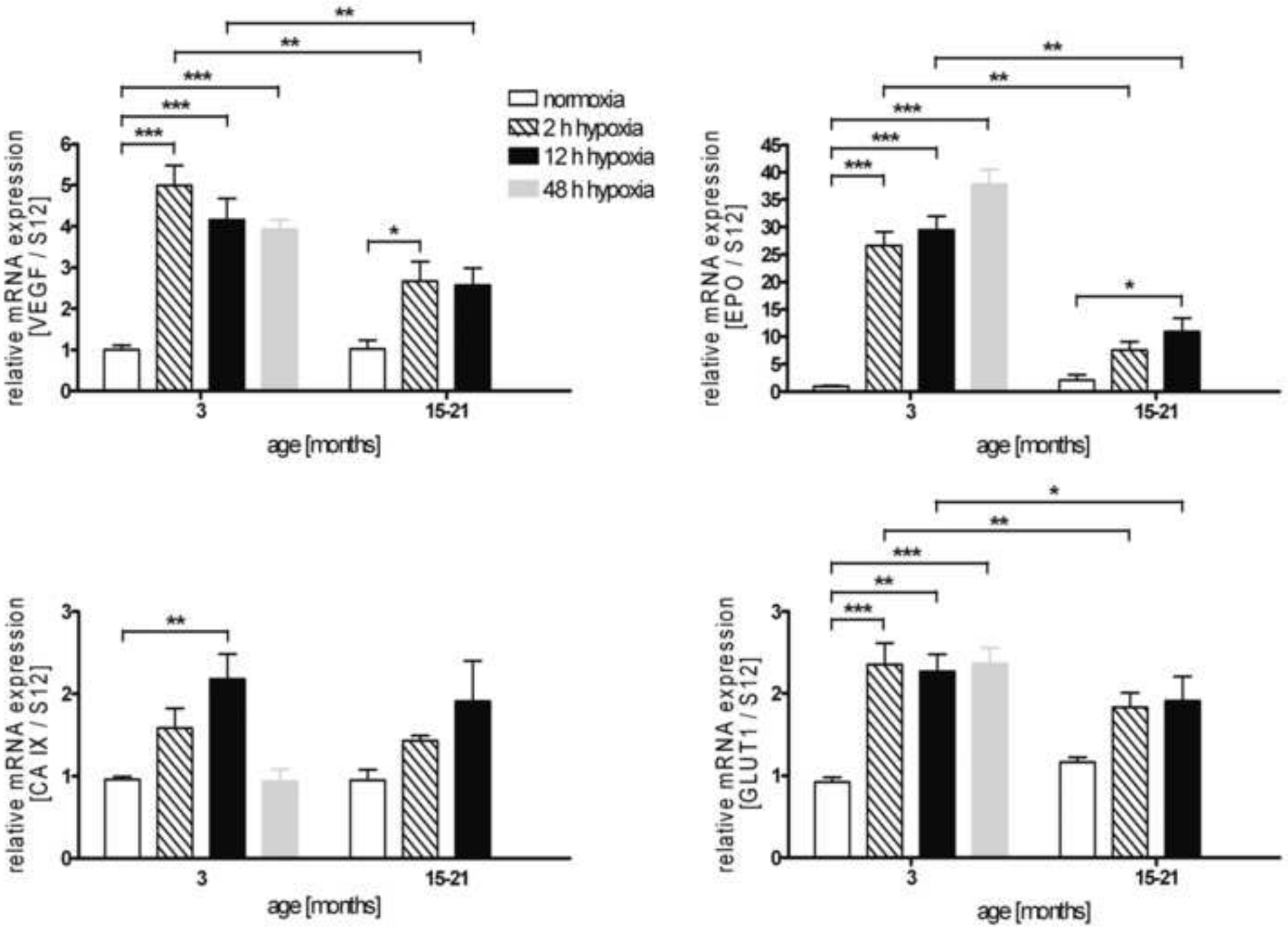

Figure 6

Click here to download high resolution image