Regulation of ventilatory sensitivity and carotid body proliferation in hypoxia by the PHD2/HIF-2 pathway

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Key points

- Sustained hypoxic exposure increases ventilatory sensitivity to hypoxia as part of physiological acclimatisation.
- Oxygen-sensitive signals are transduced in animal cells by post-translational hydroxylation of transcription factors termed hypoxia-inducible factors (HIFs).
- Mice heterozygous for the principal ‘oxygen-sensing’ HIF hydroxylase PHD2 (prolyl hydroxylase domain 2) show enhanced ventilatory sensitivity to hypoxia.
- To analyse the underlying mechanisms, functional (hypoxic ventilatory responses, HVRs) and anatomical (cellular proliferation within carotid bodies) responses were studied in genetic models of inducible and constitutive inactivation of PHD2 and its principal hydroxylation substrates, HIF-1α and HIF-2α.
- Inducible PHD2 inactivation enhanced HVR, similar to constitutive inactivation; both responses were almost entirely compensated for by specific inactivation of HIF-2α.
- Inducible inactivation of HIF-2α, but not HIF-1α, strikingly reduced ventilatory acclimatisation to hypoxia and associated carotid body cell proliferation.
- These findings demonstrate a key role for PHD2 and HIF-2α in ventilatory control and carotid body biology.

Abstract Ventilatory sensitivity to hypoxia increases in response to continued hypoxic exposure as part of acute acclimatisation. Although this process is incompletely understood, insights have been gained through studies of the hypoxia-inducible factor (HIF) hydroxylase system. Genetic studies implicate these pathways widely in the integrated physiology of hypoxia, through effects on developmental or adaptive processes. In keeping with this, mice that are heterozygous for the principal HIF prolyl hydroxylase, PHD2, show enhanced ventilatory sensitivity to hypoxia and carotid body hyperplasia. Here we have sought to understand this process better through comparative analysis of inducible and constitutive inactivation of PHD2 and its principal targets HIF-1α and HIF-2α. We demonstrate that general inducible inactivation of PHD2 in tamoxifen-treated Phd2⁺/⁻;Rosa26⁺/⁻CreERT2 mice, like constitutive, heterozygous PHD2 deficiency, enhances hypoxic ventilatory responses (HVRs: 7.2 ± 0.6 vs. 4.4 ± 0.4 ml min⁻¹ g⁻¹ in controls, \( P < 0.01 \)). The ventilatory phenotypes associated with both inducible and constitutive inactivation of PHD2 were strongly compensated for by concomitant inactivation of HIF-2α, but not HIF-1α. Furthermore, inducible inactivation of HIF-2α strikingly impaired ventilatory acclimatisation to...
chronic hypoxia (HVRs: 4.1 ± 0.5 vs. 8.6 ± 0.5 ml min⁻¹ g⁻¹ in controls, P < 0.0001), as well as carotid body cell proliferation (400 ± 81 vs. 2630 ± 390 bromodeoxyuridine-positive cells mm⁻² in controls, P < 0.0001). The findings demonstrate the importance of the PHD2/HIF-2α enzyme–substrate couple in modulating ventilatory sensitivity to hypoxia.

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**Abbreviations**  BrdU, bromodeoxyuridine; CB, carotid body; CH, chronic hypoxia; HIF, hypoxia-inducible factor; HVR, hypoxic ventilatory response; PHD, prolyl hydroxylase domain; TH, tyrosine hydroxylase; VAH, ventilatory acclimatisation to hypoxia; VHL, von Hippel–Lindau protein.

**Introduction**

The control of breathing is a fundamental component of oxygen homeostasis, and ventilation increases within seconds in response to hypoxaemia. This acute hypoxic ventilatory response (HVR) defends against hypoxia, but is limited by hyperventilation-induced hypocapnia. With exposure to more prolonged hypoxia, a further progressive increase in ventilation and arterial oxygenation develops over a period of hours to days in spite of the hypocapnia. This secondary response, often referred to as ventilatory acclimatisation to hypoxia (VAH; reviewed by Robbins, 2007), is associated with an increase in chemoreceptor sensitivity and, whilst usually associated with adaptation to altitude, is also important in diseases associated with chronic hypoxaemia. Despite intense study in many species, the mechanisms underlying chemoreceptor acclimatisation remain largely unknown. An understanding of this process could, however, represent an important target for therapeutic control of chemoreceptor activity.

Molecular insights into the regulation of gene expression by the hypoxia-inducible factor (HIF) system have generated new opportunities for the understanding of such physiological responses to hypoxia (reviewed by Kaelin & Ratcliffe, 2008; Prabhakar & Semenza, 2012; Ratcliffe, 2013). HIF is an α/β heterodimeric transcription factor whose α subunits are regulated by oxygen levels through post-translational hydroxylation of specific amino acid residues. The most important of these is the prolyl hydroxylation of residues that promote association of HIF-α proteins with von Hippel–Lindau protein (pVHL) ubiquitin ligase and their subsequent proteasomal degradation. HIF prolyl hydroxylation is catalysed by the prolyl hydroxylase domain (PHD) enzymes, a series of closely related enzymes belonging to the 2-oxoglutarate-dependent dioxygenase family. A fall in oxygen availability impairs prolyl hydroxylation allowing HIF-α proteins to escape destruction and form the transcriptional complex.

The HIF hydroxylase system is conserved throughout the animal kingdom, consisting of a single PHD and HIF-α in the simplest animal *Trichoplax*, but undergoing whole genome duplications and deletions during vertebrate evolution, resulting in multiple copies of both HIF-α (of which 1 and 2 are the best characterised) and PHD (1, 2 and 3) in mammals (Loenarz et al. 2010). Although PHD2 is the most abundant enzyme and the main regulator of HIF-1α (Berra et al. 2003; Appelhoff et al. 2004), all PHD enzymes are able to regulate both HIF-1α and HIF-2α, with PHD1 and 3 having relatively greater action on HIF-2α in some cell lines. Transcriptional profiling of HIF-1α and HIF-2α at the cellular level indicates that they activate distinct, although partially overlapping, sets of target genes (Mole et al. 2009; Schödel et al. 2012), generating interest in understanding the existence and nature of their differentiated physiological functions (Keith et al. 2012; Prabhakar & Semenza, 2012). Interestingly, despite HIF-1α being the principal target of PHD2 in cell lines, studies in intact animals have identified a specific role for the PHD2/HIF-2α couple in the regulation of erythropoiesis (Arsenault et al. 2013; Franke et al. 2013), a major component of acclimatisation to altitude.

The PHD/HIF system has also been shown to play an important part in ventilatory acclimatisation (Kline et al. 2002; Peng et al. 2006, 2011; Bishop et al. 2013; Yuan et al. 2013). Studies to date have investigated ventilatory control in mice with heterozygous loss of Phd2, Hif-1α and Hif-2α, which are viable and have no major phenotype in normoxia (Yu et al. 1999; Brusselmans et al. 2003; Aragones et al. 2008; Mazzone et al. 2009) – unlike their homozygous counterparts which are embryonic lethal (Carmeliet et al. 1998; Iyer et al. 1998; Kotch et al. 1999; Peng et al. 2000; Compernolle et al. 2002, 2003; Takeda et al. 2006) or, in the case of Hif-2α−/− mice, develop gross abnormalities even if they survive embryonic development (Compernolle et al. 2002; Scortegagna et al. 2003). For instance, Phd2+/− mice show enhanced HVR similar to that observed after chronic exposure to hypoxia and overgrowth of the carotid body (CB) (Bishop et al. 2013). However, the PHD/HIF system also has profound effects on development, including that of sympathoadrenal and neuroendocrine systems that are relevant to ventilatory
control (Tian et al. 1998; Compernolle et al. 2003; Bishop et al. 2008; Pan et al. 2012; Kenchegowda et al. 2014). The findings in constitutively heterozygous Phd2+/− mice therefore raise important questions as to the extent to which these effects are developmental as opposed to a reflection of adaptive effects of hypoxia on the activity of PHD2, and which targets (HIF-α proteins or other proposed PHD2 substrates, e.g. Takahashi et al. 2011) mediate the observed effects.

To address these questions we have performed comparative studies of multiple genetic models that reflect both inducible (acute) inactivation of PHD2 and its principal HIF-α targets: HIF-1α and HIF-2α, and constitutive (chronic) heterozygous inactivation of PHD2 and HIF-1/2α. Our findings reveal that acute inducible inactivation of PHD2 increases ventilatory sensitivity to hypoxia and that these effects are strongly dependent on the integrity of HIF-2α. HIF-2α was also critical for both the enhanced HVR, and the cellular proliferation within the CB, which were observed in response to a 7-day exposure to chronic hypoxia. These effects were reflected in the association of HIF-2α heterozygosity with reduced ventilatory sensitivity, both in the context of PHD2 heterozygosity and after chronic hypoxia. Together our findings implicate the PHD2/HIF-2 couple as the dominant mediator of enhanced HVR during ventilatory acclimatisation to hypoxia, at least under the conditions of our experiments.

Methods

Animals

All animal procedures were compliant with the UK Home Office Animals (Scientific Procedures) Act 1986 and Local Ethical Review Procedures (University of Oxford Medical Sciences Division Ethical Review Committee). The authors understand the ethical principles of The Journal of Physiology and all work was conducted in compliance with stated standards (Grundy, 2015). Male mice, approximately 3 months old and from the same litter, were used for all comparisons, unless stated otherwise. Phd2f/f, Hif-1αf/f and Hif-2αf/f (where f denotes the floxed allele) conditional knockout and Rosa26CreERT2 mice have all been described previously and were obtained from these sources (Vooijs et al. 2001; Cramer et al. 2003; Higgins et al. 2004; Gruber et al. 2007; Mazzone et al. 2009). Each line had been backcrossed with C57BL/6 for at least five generations (Adam et al. 2011) and was intercrossed to generate littermates of appropriate genotypes. Phd2f/f, Hif-1αf/f and Hif-2αf/f mice are as described previously (Carmeliet et al. 1998; Brusselmans et al. 2003; Mazzone et al. 2009); these lines were intercrossed to generate mice that were maintained on a mixed Swiss/129/SvEv/C57BL/6 genetic background. Genotype was determined by PCR (details on request). Mice were housed in individually ventilated cages with free access to food and water.

Tamoxifen administration

Tamoxifen (prepared to 20 mg ml−1 in corn oil; Sigma, St Louis, MO, USA) was administered by oral gavage to ~6-week-old mice at a dose of 2 mg day−1 for five consecutive days as described by Arsenault et al. (2013). Recombination was assessed using DNA isolated from ear biopsies obtained 10 days after the first dose of tamoxifen (PCR primers used to test for recombination are as described by Adam et al. 2011). Ventilation measurements were also taken (and chronic hypoxia treatment started) at this time point, i.e. 10 days after the first tamoxifen dose, to allow for assessment of ventilatory responses prior to the onset of other steady-state, long-term effects of PHD2/HIF inactivation such as polycythaemia.

Plethysmography

Tidal volume and respiratory rate were measured in awake, unrestrained mice using individual whole body plethysmographs (600 ml volume; Model PLY4211, Buxco, DSI, St. Paul, MI, USA). Minute ventilation was calculated from tidal volume and respiratory rate. Ventilatory parameters were derived using FinePointe software (Buxco) and adjusted to body weight as measured immediately prior to plethysmography. Premixed gas was delivered to each chamber as described previously (Bishop et al. 2013). The acute hypoxic stimuli consisted of 10% oxygen, balance nitrogen or 10% oxygen, 3% carbon dioxide, balance nitrogen. The HVR to each stimulus was defined as the difference between minute ventilation (or tidal volume or respiratory rate; see Table 1) during the 1 min prior to the onset of hypoxia and the first 1 min of stable hypoxia (i.e. excluding the first 30 s of hypoxia).

Chronic hypoxic exposure of mice and bromodeoxyuridine labelling

Mice were housed in a normobaric chamber containing 10% oxygen for 7 days with controlled temperature, humidity and carbon dioxide levels, and free access to food and water, as described previously (Bishop et al. 2013) and removed from this chamber only for plethysmography testing at 48 h. All mice were 20–35 g body weight at the start of the procedure. Mice were given 50 mg kg−1 bromodeoxyuridine (BrdU, Sigma) by intraperitoneal injection immediately prior to the start of hypoxia exposure (day 0) followed by supplementation of drinking water with 1 mg ml−1 BrdU until termination of the experiment.
as described previously (Pardal et al. 2007; Bishop et al. 2013).

**Blood measurements**

Blood was taken from the tail vein of mice using heparinised capillary tubes, and haematocrits were measured using a haematocrit centrifuge (model C-MH30, Unico, Dayton, NJ, USA).

**Immunohistochemistry**

Mice were killed by overdose of isoflurane and exsanguination followed by immediate dissection of CBs and fixation in 4% paraformaldehyde/PBS overnight prior to transfer into 70% ethanol and processing. Paraffin-embedded tissue samples were sectioned to 4 μm thickness and immunostained with a polyclonal anti-tyrosine hydroxylase (TH) antibody (Novus Biologicals, Abingdon, UK) as previously described (Bishop et al. 2013), or with an anti-BrdU antibody according to the manufacturer’s instructions (Becton Dickinson, Oxford, UK). To quantify BrdU-positive cells, the density of positively stained cells was estimated using Image J software; adjacent sections were immunostained for TH to delineate the CB. Stereological analyses of TH-positive cell density, TH-positive cell number and CB volume were performed using Image J software on every fourth section throughout CBs harvested from mice 8–9 weeks after tamoxifen treatment.

**Statistical analysis**

Data are shown as mean ± SEM. Statistical analyses were performed using unpaired Student’s t tests. For repeated measures, data were analysed by ANOVA, followed by Tukey’s test or t tests with Holm–Sidak correction for multiple comparisons, as appropriate. P values < 0.05 were considered significant.

### Table 1. Acute hypoxic ventilatory responses after conditional Phd2 inactivation in adult mice

| Acute hypoxic stimulus | Phd2<sup>ff</sup> | Phd2<sup>ff</sup>;CreER | P value |
|------------------------|------------------|-------------------------|---------|
| Minute ventilation (ml min<sup>-1</sup> g<sup>-1</sup>) | 10% O<sub>2</sub>/3% CO<sub>2</sub> | 4.44 ± 0.44 | 7.19 ± 0.60 | 0.003 |
|                        | 10% O<sub>2</sub>  | 1.52 ± 0.40 | 2.18 ± 0.46 | 0.286 |
| Tidal volume (ml g<sup>-1</sup>) | 10% O<sub>2</sub>/3% CO<sub>2</sub> | 9.11 ± 0.53 | 15.14 ± 1.10 | 0.001 |
|                        | 10% O<sub>2</sub>  | 2.83 ± 0.40 | 4.84 ± 0.51 | 0.009 |
| Respiratory rate (breaths min<sup>-1</sup>) | 10% O<sub>2</sub>/3% CO<sub>2</sub> | 98.9 ± 42.8 | 97.53 ± 18.6 | 0.975 |
|                        | 10% O<sub>2</sub>  | 47.0 ± 34.7 | 49.1 ± 27.3 | 0.961 |

Minute ventilation, tidal volume and respiratory rate in response to the indicated acute hypoxic stimulus, following tamoxifen-induced recombination. Mean ± SEM; n = 6 littermate pairs; P < 0.05 highlighted in bold type.

### Results

**Enhanced ventilatory responses after conditional PHD2 inactivation in adult mice**

To better define the role of PHD2 in regulating ventilatory acclimatisation we first examined the effect of inducible inactivation of PHD2 on the HVR using Phd2<sup>ff</sup>; Rosa26<sup>+</sup>/CreERT2 (hereafter referred to as Phd2<sup>ff</sup>;CreER) mice. Administration of tamoxifen at ~6 weeks of age causes general inactivation of PHD2 by Cre recombinase-mediated excision of sequences encoding the PHD2 catalytic domain. Ventilation was analysed in conscious, unrestrained Phd2<sup>ff</sup>;CreER mice by whole animal plethysmography before, during and after a 5 min acute hypoxic stimulus both before and after (measured 10 days after the first dose) tamoxifen treatment. Responses were compared with those of littermate control Phd2<sup>ff</sup> mice lacking the Rosa26<sup>CreERT2</sup> allele (Fig. 1; Table 1).

In control Phd2<sup>ff</sup> animals prior to tamoxifen treatment, acute exposure to hypoxia (10% oxygen) elicited an immediate but poorly sustained HVR (Fig. 1A). The response to unregulated breathing of a hypoxic atmosphere decreases as hyperventilation induces respiratory alkalosis, which in turn depresses ventilation. This can be offset by adding a small concentration of carbon dioxide to the hypoxic atmosphere used for acute stimulation. Thus, control mice exposed to 10% oxygen alone (Fig. 1A) have a greater HVR than when exposed to 7% oxygen (Ishiguro et al. 2006), exhibited a more sustained HVR (Fig. 1D) than when exposed to 10% oxygen alone (Fig. 1A). Treatment of the control Phd2<sup>ff</sup> mice with tamoxifen did not alter HVR with either 10% oxygen or 10% oxygen with 3% carbon dioxide (Fig. 1B vs. A, E vs. D).

In Phd2<sup>ff</sup>;CreER mice, acute exposure to hypoxia (either 10% oxygen or 10% oxygen with 3% carbon dioxide) elicited HVRs of similar magnitude to those
of littermate control Phd2<sup>f/f</sup> mice when tested prior to tamoxifen treatment (Fig. 1A, D). Ten days after the first dose of tamoxifen genomic recombination was efficient (data not shown) and mice appeared healthy, without visible abnormalities. Ventilatory responses to 10% oxygen and 10% oxygen with 3% carbon dioxide were again tested. Large increases in HVR were observed in Phd2<sup>f/f</sup>;CreER mice compared to their Phd2<sup>f/f</sup> littermate controls. Responses were particularly clearly observed in the sustained HVR observed in response to breathing 10% oxygen with 3% carbon dioxide (Fig. 1E; Table 1). These were similar in magnitude and character to those observed after 7 days of pre-exposure to hypoxia (Bishop et al. 2013, and see below) and primarily involved an increase in tidal

![Figure 1. Acute hypoxic ventilatory responses before and after conditional Phd2 inactivation in adult mice.](image)

Minute ventilation before, during and after an acute hypoxic exposure to 10% oxygen (A–C, closed bars), or 10% oxygen with added 3% carbon dioxide (D–F, open bars) in littermate controlled Phd2<sup>f/f</sup> versus Phd2<sup>f/f</sup>;CreER mice. Animals studied before (A and D) and 10 days after (B and E) starting tamoxifen treatment. Hypoxic ventilatory responses (HVRs) calculated from B and E are shown in C and F, respectively. Mean ± SEM, n = 6 littermate pairs; **P < 0.01.
volume (Table 1). Furthermore, this was accompanied by a small but significant increase in basal minute ventilation (in 21% oxygen) in Phd2\(^{+/−}\);CreER mice compared to Phd2\(^{+/−}\) controls (4.09 ± 0.3 vs. 3.10 ± 0.3 ml min\(^{−1}\) g\(^{−1}\), \(P = 0.03\)). These findings reveal that induced, general loss of PHD2 enhances the acute ventilatory response to hypoxia, and over a comparable period of time to ventilatory acclimatisation to hypoxia, consistent with the hypothesis that hypoxic inhibition of PHD2 plays a key mechanistic role in this process.

**Combined inactivation of PHD2 and HIF-\(\alpha\) isoforms**

As PHD2 regulates both HIF-1\(\alpha\) and HIF-2\(\alpha\) and has been reported to hydroxylate many non-HIF substrates, we next analysed whether the PHD2-dependent ventilatory phenotypes are mediated through HIF regulation and whether they are specifically dependent on a particular HIF-\(\alpha\) isoform.

In the first instance, we tested this by combining acute general inactivation of PHD2 with that of either HIF-1\(\alpha\) or HIF-2\(\alpha\) by breeding Phd2\(^{+/−}\);Hif-1\(\alpha\)^}\(^{−/−}\);CreER or Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\);CreER mice and comparing ventilatory responses to those of Phd2\(^{+/−}\);CreER control mice, after treatment with tamoxifen. HVR was measured in response to both 10% oxygen and 10% oxygen with 3% carbon dioxide. Prior to tamoxifen treatment, similar HVR values were obtained in all mice, regardless of genotype (data not shown). As previously, ventilatory responses were re-measured 10 days after the first dose of tamoxifen, at which point efficient genomic recombination had taken place (data not shown), but other systemic responses such as changes in haematocrit had not developed. Effects were again more clearly observed on the sustained HVR observed with 10% oxygen and 3% carbon dioxide. HVRs to this stimulus were significantly reduced in Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\);CreER mice but not Phd2\(^{+/−}\);Hif-1\(\alpha\)^}\(^{−/−}\);CreER mice when compared to Phd2\(^{+/−}\);CreER control mice (Fig. 2A–C, Table 2). There were no significant effects on basal ventilation in either case, despite a trend towards reduced ventilation in Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\);CreER mice. The results indicate that the acute ventilatory effects of PHD2 inactivation can be largely reversed through concomitant acute inactivation of HIF-2\(\alpha\) but not HIF-1\(\alpha\).

After longer periods of PHD2 inactivation these interventions have been reported to result in major changes in haematopoiesis. To enable comparison of our work with those reports, we measured haematocrits 6 weeks following tamoxifen treatment. In line with previous reports (Takeda et al. 2007, 2008; Minamishima et al. 2008), Phd2\(^{+/−}\);CreER mice developed severe polycythaemia (Table 6A) and hepatosplenomegaly (data not shown). Concomitant inactivation of HIF-2\(\alpha\) (tamoxifen-treated Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\);CreER mice), but not HIF-1\(\alpha\) (tamoxifen-treated Phd2\(^{+/−}\);Hif-1\(\alpha\)^}\(^{−/−}\);CreER mice), ablated the development of polycythaemia (Table 6A). These findings are thus in line with previous findings using similar but not identical mouse lines (Arsenault et al. 2013; Franke et al. 2013), and confirm the dominant role of HIF-2\(\alpha\) in regulating erythropoiesis.

Ventilation measurements in these mice were made prior to the onset of the polycythaemia when mice appeared healthy, at the earliest time point following tamoxifen treatment when recombination could be confirmed. Nevertheless, these models may be confounded by, for example, changes in iron status that precede the onset of polycythaemia. To confirm the findings on the HIF-\(\alpha\) isoform dependence of PHD2-dependent increases in HVR in an independent genetic model, we analysed the HIF-\(\alpha\) dependence of the ventilatory effects observed in Phd2\(^{+/−}\) mice (Bishop et al. 2013) using double heterozygotes (Phd2\(^{+/−}\);Hif-1\(\alpha\)^}\(^{−/−}\)/Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\)/animals). These mice developed normally and were essentially indistinguishable from wild-type mice under normoxic conditions; haematocrits were normal in Phd2\(^{+/−}\) mice, with Phd2\(^{+/−}\);Hif-1\(\alpha\)^}\(^{−/−}\)/Hif-2\(\alpha\)^}\(^{−/−}\)/mice being only very mildly polycythaemic/anaemic, respectively, when compared to Phd2\(^{+/−}\) mice (Table 6A).

The required intercrosses generated mice on a mixed genetic background rather than the C57BL/6 background on which we had previously observed enhanced HVR in Phd2\(^{+/−}\) animals (Bishop et al. 2013), and HVR has been reported to vary with genetic background (Han et al. 2002; Balbir et al. 2007). For those reasons, we first confirmed enhanced HVRs in Phd2\(^{+/−}\)/versus wild-type littermate mice (data not shown). The effect of heterozygous inactivation of HIF-1\(\alpha\) or HIF-2\(\alpha\) on the Phd2\(^{+/−}\)/ventilatory responses was tested by directly comparing HVRs in Phd2\(^{+/−}\)/mice with littersmates that were also heterozygous for the different HIF-\(\alpha\) isoforms. HVRs were significantly reduced in Phd2\(^{+/−}\);Hif-2\(\alpha\)^}\(^{−/−}\)/versus Phd2\(^{+/−}\)/mice, but not in the equivalent HIF-1\(\alpha\)/comparison (Fig. 2D–F, Table 3). The findings are therefore consistent with those following tamoxifen-inducible general inactivation. Furthermore, the results demonstrate that although ventilatory control and erythropoiesis are both strongly regulated by PHD2 and HIF-2\(\alpha\), the two processes can be uncoupled from each other, with changes in HVR occurring independently of erythropoietic stimulation, in the different settings.

**A major role for HIF-2\(\alpha\) in ventilatory acclimatisation to hypoxia**

Given that our findings implicate HIF-2\(\alpha\) in mediating increased HVR in the different models of PHD2 inactivation, we proceeded to test the role of the different...
HIF-α isoforms in enhanced HVR following pre-exposure to hypoxia. This was done by measuring HVR in mice after inactivation of HIF-1α or HIF-2α using both the inducible and the constitutive systems, i.e. comparison of tamoxifen-treated Hif-1α or Hif-2α;CreER versus Hif-1α or Hif-2α;CreER control mice and comparison of heterozygous Hif-1α or Hif-2α versus wild-type mice (Figs 3 and 4). Animals were pre-exposed to chronic hypoxia (10% oxygen in a normobaric chamber for 7 days), starting 10 days after the first tamoxifen dose in the case of the conditional system. HVR was measured before and after 48 h and 7 days at 10% oxygen.

Progressive respiratory acclimatisation was observed in control (i.e. wild-type or tamoxifen-treated Hif-1α;CreER and Hif-2α;CreER) mice, with a significant increase in HVR after 48 h at 10% oxygen, which was further enhanced after 7 days at 10% oxygen (Figs 3 and 4; Tables 4 and 5).

![Graphs showing HVR responses](image-url)
Neither acute inactivation of HIF-1α in tamoxifen-treated Hif-1α+/−;CreER mice (compared to control, tamoxifen-treated Hif-1α+/+) nor constitutive loss of HIF-1α in Hif-1α−/− mice (compared to wild-type littermates) significantly altered HVR measured over 48 h to 7 days at 10% oxygen (Fig. 3; Tables 4A and 5A), although there was a trend towards decreased HVRs in Hif-1α+/−;CreER and Hif-1α−/− mice after 48 h of hypoxia (Tables 4A and 5A). In contrast, acute inactivation of HIF-2α (tamoxifen-treated Hif-2α+/−;CreER mice) and constitutive inactivation of HIF-2α in Hif-2α+/− mice significantly reduced the increases in HVR after 48 h or 7 days of hypoxia (Fig. 4; Tables 4B and 5B). Interestingly, effects on HVR at baseline (i.e. prior to chronic hypoxia pretreatment) were also observed in Hif-2α+/−;CreER mice (Fig. 4A; Table 4B) without any significant effect on basal ventilation in normoxia; a similar trend towards a reduction in HVRs was noted in normoxic Hif-2α+/− mice but this did not reach statistical significance (Fig. 4E; Table 5B). Together these results demonstrate that, regardless of the genetic model, HIF-2α plays a major role in ventilatory acclimatisation to hypoxia (and contributes to maintenance of basal HVR), consistent with dependence of the enhanced HVR on HIF-2α in PHD2 deficient animals.

Although both Hif-2α+/− and tamoxifen-treated Hif-2α+/−;CreER mice had reduced ventilatory acclimatisation to hypoxia, they differed in their erythropoietic status. Erythropoiesis was unaffected by loss of HIF-2α or HIF-1α in Hif-2α+/− or Hif-1α+/− mice (Table 6B); likewise, it was unaffected in tamoxifen-treated Hif-1α+/−;CreER mice (Table 6B). In contrast, hypoxia-induced erythropoiesis was abrogated in tamoxifen-treated Hif-2α+/−;CreER mice (Table 6B). This is consistent with the reported importance of PHD2/HIF-2α in erythropoietic control (Arsenault et al. 2013; Franke et al. 2013), but confirms that erythropoietic stimulation and changes in ventilatory control occur independently.

**Essential role of HIF-2α in hypoxia-induced cell proliferation within the CB**

Increased ventilatory sensitivity after hypoxic exposure has been associated with growth of the CB in a range of species (Kay & Laidler, 1977; McGregor et al. 1984). CBs from Phd2+/− mice show hyperplasia in the absence of hypoxic
exposure, which may contribute to the exaggerated HVR (Bishop et al. 2013). We therefore analysed the role of HIF-α isoforms in the cellular proliferative response to hypoxia in CBs by measuring BrdU incorporation during hypoxic exposure (10% oxygen for 7 days) (as described by Pardal et al. 2007; Bishop et al. 2013). Responses were compared in mice with either constitutive or conditional loss of HIF-1α or 2α.

In control mice (wild-type, or tamoxifen-treated Hif-1αf/f or 2αf/f mice without the Cre allele), striking

**Figure 3. Effect of conditional or constitutive inactivation of Hif-1α on the hypoxic ventilatory response in mice before and after ventilatory acclimatisation to chronic hypoxia**

Minute ventilation before, during and after an acute hypoxic exposure to 10% oxygen with 3% carbon dioxide (open bars). Comparisons were tamoxifen-treated Hif-1αf/f;CreER versus Hif-1αf/f littermates (n = 7 pairs)(A–D); Hif-1α+/- versus wild-type littermates (n = 6 pairs) (E–H). Animals were studied immediately before (0 h, A, E) or after (48 h, B, F; 7 days, C, G) exposure to chronic hypoxia (CH). D and H, hypoxic ventilatory responses (HVR) calculated from changes in minute ventilation before and after exposure to CH for the indicated times. For mice bearing the conditionally inactivated allele, HVR values are shown before and after tamoxifen (TMX). Values shown are mean ± SEM.

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incorporation of BrdU was observed after 7 days at 10% oxygen, consistent with previous descriptions (Pardal et al. 2007; Bishop et al. 2013). Acute conditional inactivation of HIF-2α resulted in near total loss of BrdU staining, which was reduced to the low background levels observed in normoxic animals (Fig. 5B). In contrast, conditional inactivation of HIF-1α had no discernible effect (Fig. 5B). Effects of heterozygous loss of HIF-α were less striking: with HIF-1α no discernible effect was apparent, whereas with HIF-2α, reduced BrdU staining was observed that

Figure 4. Effect of conditional or constitutive inactivation of Hif-2α on the hypoxic ventilatory response in mice before and after ventilatory acclimatisation to chronic hypoxia

Minute ventilation before, during and after an acute hypoxic exposure to 10% oxygen with 3% carbon dioxide (open bars). Comparisons were tamoxifen-treated Hif-2α<sup>fl/fl</sup>;CreER versus Hif-2α<sup>fl/fl</sup> littermates (n = 7 pairs) (A–D); Hif-2α<sup>+/−</sup> versus wild-type littermates (n = 6 pairs) (E–H). Animals were studied immediately before (0 h, A, E) or after (48 h, B, F; 7 days, C, G) exposure to chronic hypoxia (CH). D and H, hypoxic ventilatory responses (HVR) calculated from changes in minute ventilation before and after exposure to CH for the indicated times. For mice bearing the conditionally inactivated allele, HVR values are shown before and after tamoxifen (TMX). Mean ± SEM; **P < 0.01, ***P < 0.001.

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Table 4. Acute hypoxic ventilatory responses of mice after conditional inactivation of Hif-1α or Hif-2α followed by chronic hypoxia exposure

(A) Hypoxic ventilatory response (ml min$^{-1}$ g$^{-1}$)

| Acute hypoxic stimulus | Chronic hypoxia duration | Hif-1α$^{ff}$ | Hif-1α$^{ff};$CreER | t test P value | ANOVA P value |
|------------------------|--------------------------|--------------|---------------------|----------------|----------------|
| 10% O$_2$/3% CO$_2$    | 0 h                      | 2.82 ± 1.05  | 3.38 ± 0.26         | 0.500          | <0.0001        |
|                        | 48 h                     | 6.13 ± 0.39  | 6.03 ± 0.48         | 0.901          |                |
|                        | 7 days                   | 6.59 ± 0.64  | 6.93 ± 0.47         | 0.682          |                |
| ANOVA P value          |                          | 0.6305       |                     | 0.8227         |                |
| 10% O$_2$              | 0 h                      | 0.99 ± 0.51  | 0.39 ± 0.22         | 0.358          | 0.0015         |
|                        | 48 h                     | 2.58 ± 0.72  | 1.31 ± 0.38         | 0.053          |                |
|                        | 7 days                   | 2.27 ± 0.62  | 2.51 ± 0.36         | 0.290          |                |
| ANOVA P value          |                          | 0.0699       |                     | 0.6683         |                |

(B) Hypoxic ventilatory response (ml min$^{-1}$ g$^{-1}$)

| Acute hypoxic stimulus | Chronic hypoxia duration | Hif-2α$^{ff}$ | Hif-2α$^{ff};$CreER | t test P value | ANOVA P value |
|------------------------|--------------------------|--------------|---------------------|----------------|----------------|
| 10% O$_2$/3% CO$_2$    | 0 h                      | 4.68 ± 0.37  | 2.83 ± 0.36         | 0.0060         | <0.0001        |
|                        | 48 h                     | 7.41 ± 0.66  | 3.89 ± 0.55         | 0.0022         |                |
|                        | 7 days                   | 8.55 ± 0.48  | 4.14 ± 0.45         | 0.0004         |                |
| ANOVA P value          |                          | 0.0002       |                     | 0.0212         |                |
| 10% O$_2$              | 0 h                      | 0.73 ± 0.64  | -0.13 ± 0.51        | 0.24479        | 0.0013         |
|                        | 48 h                     | 2.10 ± 0.39  | 0.78 ± 0.76         | 0.08107        |                |
|                        | 7 days                   | 3.72 ± 0.27  | 1.16 ± 0.43         | 0.00144        |                |
| ANOVA P value          |                          | 0.0061       |                     | 0.2287         |                |

Hypoxic ventilatory responses of: A, Hif-1α$^{ff};$CreER versus Hif-1α$^{ff};$ and B, Hif-2α$^{ff};$CreER versus Hif-2α$^{ff}$ mice after tamoxifen-induced recombination (0 h), followed by 48 h and 7 days of exposure to 10% oxygen. Values shown are mean changes in minute ventilation (± SEM) in response to the indicated acute hypoxic stimulus; n = 7 littermate pairs for each genotype. Comparisons were made using two-way ANOVAs (right-hand column P value = chronic hypoxia factor; bottom row P value = genotype factor; right column, bottom row P value = chronic hypoxia/genotype interaction factor), followed by t tests (with Holm–Sidak correction) for analysis of individual time points; P < 0.05 highlighted in bold type.

did not reach statistical significance (Fig. 5A). The effect of conditional HIF-1α and HIF-2α inactivation on CB morphology was further tested in animals that were maintained under normoxia. HIF-1α or HIF-2α inactivation did not significantly alter CB volume, Type I cell number or Type I cell density, and tissues from all genotypes appeared to have qualitatively normal histological structure (Fig. 5C).

Discussion

Our findings indicate that, despite the long-term changes in CB morphology that are associated with constitutive heterozygous inactivation of PHD2, acute conditional inactivation of components of the PHD–HIF system in the adult have very major effects on ventilatory responses to hypoxia.

Specifically, inducible inactivation of PHD2 increased ventilatory sensitivity to hypoxia, whereas combined inducible inactivation of PHD2 and HIF-2α compensated for this effect, and inducible inactivation of HIF-2α largely ablated enhanced ventilatory sensitivity after chronic hypoxic exposure. These effects on ventilatory sensitivity were large and extended, in the case of inducible HIF-2α inactivation, to a significant reduction in basal HVR. By contrast, the effects of inducible inactivation of HIF-1α were modest.

Our work does not exclude developmental or longer term effects of the PHD–HIF system on ventilatory sensitivity to hypoxia, but the effects of acute conditional inactivation were as large as, or larger than, those of constitutive heterozygous inactivation of different components, possibly reflecting the potential for biallelic inactivation in the inducible models. As with the studies of combined inactivation of Phd2 and Hif-α genes in the acute model, heterozygous inactivation of HIF-2α had substantially greater effects on the enhanced HVR in Phd2$^{+/−}$ animals than heterozygous inactivation of HIF-1α. In each model effects of HIF-2α inactivation on the enhanced HVR observed after chronic hypoxia, and
Table 5. Acute hypoxic ventilatory responses of Hif-1α+/− or Hif-2α+/− mice before and after chronic hypoxia exposure

(A) Hypoxic ventilatory response (mL min⁻¹ g⁻¹)

| Acute hypoxic stimulus | Chronic hypoxia duration | WT | Hif-1α+/− | t test P value | ANOVA P value |
|------------------------|--------------------------|----|-----------|---------------|---------------|
| 10% O₂/3% CO₂          | 0 h                      | 3.89 ± 0.79 | 3.24 ± 0.53 | 0.562         | 0.0001        |
|                        | 48 h                     | 7.13 ± 0.62 | 5.74 ± 0.87 | 0.220         |               |
|                        | 7 days                   | 7.66 ± 1.02 | 8.46 ± 0.80 | 0.475         |               |
| ANOVA P value          |                          | 0.4470      |             |               | 0.4363        |
| 10% O₂                 | 0 h                      | 1.50 ± 0.31 | 1.20 ± 0.80 | 0.771         | 0.0021        |
|                        | 48 h                     | 3.88 ± 0.75 | 2.63 ± 0.76 | 0.250         |               |
|                        | 7 days                   | 4.24 ± 0.98 | 4.86 ± 0.75 | 0.563         |               |
| ANOVA P value          |                          | 0.5947      |             |               | 0.4986        |

(B) Hypoxic ventilatory response (mL min⁻¹ g⁻¹)

| Acute hypoxic stimulus | Chronic hypoxia duration | WT | Hif-2α+/− | t test P value | ANOVA P value |
|------------------------|--------------------------|----|-----------|---------------|---------------|
| 10% O₂/3% CO₂          | 0 h                      | 3.32 ± 0.70 | 2.41 ± 0.65 | 0.378         | <0.0001       |
|                        | 48 h                     | 5.99 ± 0.65 | 3.56 ± 0.40 | 0.020         |               |
|                        | 7 days                   | 6.85 ± 0.90 | 4.96 ± 0.82 | 0.067         |               |
| ANOVA P value          |                          | 0.0527      |             |               | 0.3433        |
| 10% O₂                 | 0 h                      | 2.18 ± 0.55 | 0.59 ± 0.88 | 0.129         | 0.0009        |
|                        | 48 h                     | 3.61 ± 0.50 | 1.51 ± 0.63 | 0.048         |               |
|                        | 7 days                   | 5.43 ± 1.09 | 4.19 ± 0.45 | 0.233         |               |
| ANOVA P value          |                          | 0.0068      |             |               | 0.8566        |

Hypoxic ventilatory responses of: A, Hif-1α+/− versus WT; and B, Hif-2α+/− versus WT mice after 0 h, 48 h and 7 days of exposure to 10% oxygen. Values shown are mean changes in minute ventilation (±SEM); n = 6 littermate pairs for each genotype. Comparisons were made using two-way ANOVAs (right-hand column P value = chronic hypoxia factor; bottom row P value = genotype factor; right column, bottom row P value = chronic hypoxia/genotype interaction factor), followed by t tests (with Holm–Sidak correction) for analysis of individual time points; P < 0.05 highlighted in bold type.

After PHD2 inactivation, were similar, strongly supporting the hypothesis that effects of hypoxia are mediated by PHD2 activity on HIF-2α.

The importance of HIF-2α in these responses is consistent with high expression of this isoform in sympato-adrenal tissues (Tian et al. 1998). It is also consistent with findings in human monogenic diseases and analyses of associated phenotypes in mouse models of the relevant human mutations. Thus, the hypomorphic Vhl allele associated with Chuvash polycythaemia, and with increased ventilatory sensitivity to hypoxia, manifests somewhat greater dysregulation of HIF-2α than HIF-1α (Ang et al. 2002; Smith et al. 2006; Hickey et al. 2010). Similarly, ‘knock-in’ mouse models of inherited human polycythaemia [‘loss of function’ Phd2P249R/− (Arsenault et al. 2013) and ‘gain of function’ Hif-2αG536W/G536W (Tan et al. 2013) mice] manifest altered (albeit more minor) effects on ventilatory sensitivity consistent with our findings.

Other work has emphasised the importance of HIF-1α, at least in the setting of constitutive heterozygous inactivation of Hif-α alleles. This work described reduced VAH in Hif-1α+/− animals following 72 h under hypobaric hypoxia (0.4 atmospheres) (Kline et al. 2002; Yuan et al. 2013). Although trends towards reduced VAH following inactivation of HIF-1α were observed in several of our datasets, these did not reach significance in any one comparison, and were generally much less than those following inactivation of HIF-2α. In contrast with our findings, Peng et al. (2011) describe increased sensory discharges in hypoxia from CBs isolated from Hif-2α+/− mice and ventilatory instability with sighing and apnoea in the intact animals. They also describe an interplay between HIF-1α heterozygosity and HIF-2α heterozygosity, such that double heterozygosity for HIF-1α and HIF-2α compensated for the effects of HIF-1α heterozygosity on ventilatory sensitivity (Yuan et al. 2013). We are not clear as to the reasons for these differences from our findings. Although we did not observe ventilatory instability in our Hif-2α+/− animals, it is possible that such a phenotype might be related to differences in background, and might obscure, or even reflect, the abnormalities of ventilatory control in response to hypoxia that we observed.

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One difference in our studies was the use of 10% oxygen with 3% carbon dioxide to compensate for hyperventilation-induced hypocapnia. This stimulus was used instead of the computer-regulated end-tidal clamping of carbon dioxide that has been used to clarify the measurement of ventilatory sensitivity to hypoxia in humans (Howard & Robbins, 1995), but is not feasible in mice. Our calculations indicated that, at the levels of hyperventilation that were induced, this manoeuvre provides approximate compensation for hypocapnia, and thus much more closely induces pure hypoxaemia than simple inhalation of 10% oxygen. Consistent with results in humans, HVRs were much better sustained when these attempts to compensate for hypocapnia were made than when they were not, and the effects of interventions on the size of the HVR were more clearly seen. Nevertheless, we cannot exclude that some animals have been subjected to an additional hypercapnic stimulus under these conditions, potentially acting outside the CB. We therefore analysed HVRs both in response to 10% oxygen with 3% carbon dioxide and in response to 10% oxygen alone, and have provided all datasets. In general, responses to both stimuli showed similar trends from interventions, although responses to 10% oxygen were smaller and more variable and the effects of interventions less frequently reached statistical significance.

Importantly, however, because ventilatory sensitivity in mice is reported to vary with genetic background (Han et al. 2002; Balbir et al. 2007), our observations on the dominant importance of PHD2 and HIF-2α were observed clearly using differently derived inactivating HIF-α isoforms and on the cellular proliferation that is observed in the CB in response to sustained hypoxia. Hypoxia-induced cellular proliferation as assessed by BrdU staining was specifically, and almost totally, ablated by acute inactivation of Hif-2α. In contrast, CBs from animals with either acute or constitutive heterozygous inactivation of Hif-1α were indistinguishable from controls.

Although changes in HVR and in these proliferative responses were altered in parallel, it is not yet precisely clear how they are connected. Nevertheless, quantitative differences in the effects of HIF-2α heterozygosity on HVR and on cellular proliferation, taken together with the rapid (within hours) effects of sustained hypoxia on HVR (reviewed by Robbins, 2007), suggest that enhanced HVR is not simply a reflection of increased CB mass. Further work will be required to understand how the role(s) of HIF-2α in each process are interlinked. Interestingly, cellular proliferation within the CB has been demonstrated to depend on an interplay between the Type I neurosecretory ‘glomerus’ cells and Type II ‘supporting’ cells (Pardal et al. 2007; Platero-Luengo et al. 2014). Proliferation originates in stem-like Type II cells that demonstrate to depend on an interplay between the Type I neurosecretory ‘glomerus’ cells and Type II ‘supporting’ cells (Pardal et al. 2007; Platero-Luengo et al. 2014). Proliferation originates in stem-like Type II cells that did then develop into Type I cells (Pardal et al. 2007), but is dependent on paracrine responses from hypoxia-activated Type I cells (Platero-Luengo et al. 2014). Although the rapid electrophysiological events that activate Type I cells in response to hypoxia are believed to be independent of HIF and HIF hydroxylase activity, our data clearly indicate that the integrity of HIF-2α is required at some stage in the events linking hypoxic exposure to proliferation and differentiation of cells within the CB.

It is also likely that other components of the HIF hydroxylase system are important in defining CB morphology through developmental or adaptive processes. For instance, inactivation of PHD3 leads to altered CB cell morphology, probably through defective developmental culling of sympathoadrenal neurones (Bishop et al. 2008), but Phd3−/− mice show normal HVR (Bishop et al. 2013). Furthermore, inactivation of VHL in Type I cells by TH-driven Cre recombinase results in atrophy of the CB (Macias et al. 2014), suggesting either that the very high levels of HIF-α associated with complete inactivation of VHL impair CB cell viability or that some other
Figure 5. Hypoxia-induced cellular proliferation and CB morphology in mice with conditional or constitutive inactivation of Hif-1α or Hif-2α.

Immunostaining for BrdU performed on CB sections from: A, Hif-1αα+/− or Hif-2αα+/− compared to wild-type (WT) littermates; and B, tamoxifen-treated Hif-1ααf/f;CreER or Hif-2ααf/f;CreER compared to Hif-1ααf/f or Hif-2ααf/f littermate controls. All mice were treated with BrdU and exposed to 10% oxygen for 7 days prior to being killed and preparation of tissues. Representative images from each genotype are shown in Aa and Ba where BrdU-positive cells are stained brown (scale bar = 0.04 mm). Ab–c and Bb–c, Quantification of BrdU-positive cells in CBs of Hif-1αα+/− (Ab) or Hif-2αα+/− (Ac) compared to WT littermates and tamoxifen-treated Hif-1ααf/f;CreER (Bb) or Hif-2ααf/f;CreER (Bc) compared to controls of similar genotype but lacking the CreER allele (n = 6 pairs per genotype).

C, immunostaining for tyrosine hydroxylase (TH) performed on CB sections from Hif-1ααf/f;CreER or Hif-2ααf/f;CreER compared to Hif-1ααf/f or Hif-2ααf/f littermate mice, 8–9 weeks after tamoxifen treatment. Morphometric analysis of CB volume, TH-positive cell number and TH-positive cell density in CBs of Hif-1ααf/f;CreER (Ca) or Hif-2ααf/f;CreER (Cb) compared to controls lacking the CreER allele (n = 3 pairs per genotype). Cc, representative images from each genotype with TH-positive cells stained brown (scale bar = 0.04 mm). Values shown are mean ± SEM; ****P < 0.0001.
consequence of defective VHL function has the same effect.

Nevertheless, despite these complexities, the current work reveals the importance of specific components of the HIF hydroxylase system (the PHD2 – HIF-2α enzyme–substrate couple) in mediating both anatomical and physiological responses that underlie ventilatory acclimatisation to hypoxia.

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Additional information

Competing interests

C.W.P. and P.J.R. are scientific co-founders and hold equity in ReOx Ltd, a university spin-out company that seeks to develop therapeutic inhibitors of the HIF hydroxylases.

Author contributions

E.J.H., P.J.T., P.A.R., C.W.P., K.J.B., P.J.R. and T.B. conceived and designed the experiments. E.J.H., L.G.N., P.J.T., R.L., J.W.F., G.D., I.R., P.A.R., C.W.P., K.J.B., P.J.R. and T.B. collected, analysed and interpreted the data. E.J.H., P.J.R. and T.B. drafted the article. E.J.H., L.G.N., P.J.T., R.L., J.W.F., G.D., I.R., P.A.R., C.W.P., K.J.B., P.J.R. and T.B. revised the manuscript critically for important intellectual content. All authors have read and approved the final copy. All experiments were carried out at the University of Oxford.

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