The von Hippel-Lindau Chuvash mutation in mice causes carotid body hyperplasia and enhanced ventilatory sensitivity to hypoxia.

Authors. Mary E. Slingo¹, Philip J. Turner¹, Helen C. Christian¹, Keith J. Buckler¹, Peter A. Robbins¹.

Author contributions. MES, PJT, KJB and PAR designed the study. HCC contributed to study design. MES and PJT conducted the ventilatory experiments. MES, PJT and KJB conducted the immunohistochemistry. MES, PJT and HCC conducted the electron microscopy. MES, PJT, HCC and PAR analyzed the data and contributed to preparation of the paper. MES, KJB and PAR wrote the paper.

Affiliation. ¹. Department of Physiology, Anatomy and Genetics, Sherrington Building, Parks Road, University of Oxford, Oxford OX1 3PT, UK.

Running title. von Hippel-Lindau Chuvash mutation and ventilatory control.

Corresponding author. Peter A. Robbins. Department of Physiology, Anatomy and Genetics, Sherrington Building, Parks Road, University of Oxford, Oxford OX1 3PT, UK. Email: peter.robbins@dpag.ox.ac.uk. Tel: +44 1865 272471.
Abstract

The hypoxia-inducible factor (HIF) family of transcription factors coordinates diverse cellular and systemic responses to hypoxia. Chuvash polycythemia (CP) is an autosomal recessive disorder in humans in which there is impaired oxygen-dependent degradation of HIF, resulting in long-term systemic elevation of HIF levels at normal oxygen tensions. CP patients demonstrate the characteristic features of ventilatory acclimatization to hypoxia, namely an elevated baseline ventilation and enhanced acute hypoxic ventilatory response. We investigated the ventilatory and carotid body phenotype of a mouse model of CP, using whole-body plethysmography, immunohistochemistry, and electron microscopy. In keeping with studies in humans, CP mice had elevated ventilation in euoxia and a significantly exaggerated acute hypoxic ventilatory response when exposed to 10% oxygen, with or without the addition of 3% carbon dioxide. Carotid body immunohistochemistry demonstrated marked hyperplasia of the oxygen-sensing Type I cells, and the cells themselves appeared enlarged with more prominent nuclei. This hypertrophy was confirmed by electron microscopy, which also revealed that the Type I cells contained an increased number of mitochondria, enlarged dense-cored vesicles, and markedly expanded rough endoplasmic reticulum. The morphological and ultrastructural changes seen in the CP mouse carotid body are strikingly similar to those observed in animals exposed to chronic hypoxia. Our study demonstrates that the HIF pathway plays a major role, not only in regulating both euoxic ventilatory control and the sensitivity of the response to hypoxia, but also in determining the morphology of the carotid body.

Keywords

High altitude, carotid body, HIF, ventilation, VHL
Introduction

The acute hypoxic ventilatory response (AHVR) describes the rapid increase in ventilation that occurs upon exposure to hypoxia. However, if the hypoxic stimulus continues for more than several minutes, then ventilation decreases towards, but does not reach, pre-hypoxic levels. This hypoxic ventilatory decline (HVD) cannot be explained simply by the respiratory alkalosis resulting from the initial rapid increase in ventilation, since it has also been demonstrated under conditions of isocapnia (21). If hypoxia continues, the process of ventilatory acclimatization to hypoxia follows, which comprises both a progressive increase in ventilation together with increased sensitivity of AHVR to further hypoxic stimuli (6, 16, 17, 36).

A substantial body of evidence suggests that the carotid bodies, containing both the oxygen-sensing Type I cells and afferent nerve fibres to the brainstem, are critical for evoking AHVR and ventilatory acclimatization to hypoxia (8, 11, 15, 23, 26, 38). Exposure to chronic hypoxia results in an increase in the size and number of Type I cells, the diameter of their cytoplasmic dense-cored vesicles (DCV) and the quantity of their mitochondria and rough endoplasmic reticulum (ER) (20, 24, 25, 30, 31, 34, 42, 43). What is less well known is what the underlying mechanism(s) driving these changes may be.

It is now recognized that many diverse cellular and systemic responses to hypoxia are coordinated by the hypoxia-inducible factor (HIF) family of transcription factors. The stability of HIF-α subunits is regulated by oxygen-dependent prolyl hydroxylation, which enables recognition by the von Hippel-Lindau (VHL) ubiquitin E3 ligase and subsequent degradation via the ubiquitin-proteasome pathway (19, 35). Chuvash polycythemia (CP) is a rare autosomal recessive disorder in which a single point mutation in VHL causes diminished binding affinity for both hydroxylated HIF-1α and HIF-2α, resulting in increased expression
of HIF target genes under euoxic conditions (1, 10). Patients with CP have elevated baseline
ventilation (with consequent respiratory alkalosis) and an abnormally high AHVR (40). In
other words, they demonstrate the characteristic features of individuals acclimatized to
hypoxia.

In keeping with the human disease, a recently-engineered mouse model of CP demonstrated
increased euoxic expression of both HIF-1-specific and HIF-2-preferential target genes
within diverse tissues (13, 14, 28). The CP mice also exhibited elevated ventilation in euoxia
but the increase in AHVR appeared to be absent, leading the authors to conclude that the
model did not recapitulate this aspect of the human disorder (14). However, a concern with
this study is that the authors measured averaged ventilation over 10 minutes of 12% oxygen,
and thus the associated hypocapnia and HVD may well have masked the true phenotype. The
purpose of the present study was to use a form of hypoxia and data analysis in which the
confounding effects of hypocapnia and HVD have been minimized. Using this approach, we
were able to show that AHVR is markedly increased in the CP mouse. Furthermore, we
demonstrate striking morphological and ultrastructural changes within the carotid body that
are similar to those observed after exposure to chronic hypoxia. These findings together
indicate the major role that HIF has to play in both the calibration and homeostasis of the
respiratory system and in determining the morphology of the carotid body.
Methods

Animals. Male CP and wild type (WT) mice (from the same breeding colony of mice heterozygous for the VHL Chuvash mutation), aged between 4 and 6 months, were used for all comparisons. Original CP breeding pairs were donated, and the original mutation was generated as described previously (13). All animal procedures were compliant with both the UK Home Office Animals (Scientific Procedures) Act 1986 and the Local Ethical Review Procedures (University of Oxford Medical Sciences Division Ethical Review Committee). Mice were housed within individually ventilated cages, in room air, with free access to food and water.

Ventilatory measurements. Respiratory rate and tidal volume (corrected to body weight) were measured in awake animals using individual whole-body plethysmographs (600 ml volume; Model PLY4211, Buxco, UK). Minute ventilation was calculated as the product of respiratory rate and tidal volume. Before commencement of ventilatory measurements, mice were allowed to breathe room air within the plethysmography chamber for at least 30 min. Baseline ventilation measurements during euoxia (room air) were then made over a 5-min period, after which mice were exposed to a 5-min hypoxic stimulus before returning to room air for a further 5 min. The hypoxic stimulus (utilizing pre-mixed gas delivered to each chamber at 2 L/min) comprised either 10% oxygen with balance nitrogen, or 10% oxygen with 3% carbon dioxide and balance nitrogen. Two technical replicates, on separate days, were performed and subsequently averaged. For quantitation, baseline euoxic respiratory rate, tidal volume and minute ventilation were determined during the 90 s prior to the onset of hypoxia. Corresponding values for the hypoxic stimuli were determined during the first 60 s of stable hypoxia (i.e. excluding the first 30 s of hypoxia). The AHVR was defined as the difference between hypoxic and euoxic minute ventilation.
Carotid body immunohistochemistry. Carotid bifurcations were harvested from mice that had been killed by overdose of inhaled anesthetic (halothane); they were then fixed in 4% paraformaldehyde overnight then transferred to 70% ethanol. Bifurcations were subsequently processed, wax-embedded and sectioned to 4 μm thickness. Following de-waxing and antigen retrieval, sections were blocked with goat serum for one hour at room temperature, then incubated overnight at 4 °C with a rabbit polyclonal antibody to tyrosine hydroxylase (TH, to identify Type I cells; Abcam Biochemicals, UK). Sections were washed in PBS then incubated with goat anti-rabbit secondary antibody (Abcam Biochemicals, UK). Stereological estimation of TH-positive tissue volume and cell number was performed on all sections throughout the carotid body using ImageJ software (NIH, USA).

Carotid body electron microscopy. Carotid bifurcations were removed as before and immersion-fixed in 3% paraformaldehyde/0.05% glutaraldehyde in phosphate buffer (pH 7.2) for 2 hours at room temperature and stored overnight at 4 °C in a ten-fold dilution of the fixative. The next day bifurcations were contrasted with uranyl acetate (2% (w/v) in distilled water), dehydrated in ethanol and embedded in LR Gold resin (Agar Scientific, UK). For the purpose of creating high quality images, further bifurcations were embedded in Spurr’s resin; bifurcations were post-fixed in osmium tetroxide (1% w/v in 0.1 M phosphate buffer) stained with uranyl acetate (2% w/v in distilled water), dehydrated through increasing concentrations of ethanol (70-100%) and embedded in Spurr's resin (Agar Scientific, UK).

Semi-thin sections were initially prepared to identify the carotid body and subsequently ultrathin sections (50-80 nm) were cut using a Reichart-Jung ultracut microtome and mounted on nickel grids (Agar Scientific, UK). For identification of Type I cells, sections of LR Gold-embedded tissue were immunogold-labelled for TH by incubation for 2 hours at room temperature with TH antibody (Abcam Biochemicals, UK) followed by a 1 hour incubation with a 15 nm protein A immunogold-conjugate (1:60; British Biocell, UK). All antibodies
were diluted in 0.1 M PBS (containing 0.1% egg albumin). Specificity of antibody labelling was confirmed in negative control sections in which the primary antibody was replaced with non-immune serum. Sections were counterstained with lead citrate and uranyl acetate and examined on a JOEL 1010 transmission electron microscope (JOEL Inc., USA).

For analysis of cell morphology six micrographs of TH-positive cells per animal were taken at a magnification of x4000 and scanned into Adobe Photoshop (Adobe, USA, Version 5.5) and analysed using Axiovision (Zeiss, UK, Version 4.5) image analysis software. The analyst was blind to the sample code. The following parameters were calculated: nuclear and total cell areas; mitochondria number and DCV granule diameter. Expansion of the rough ER was assessed visually and graded on a scale of 0-4 (0, no expansion; 4 the most expansion). The ER estimates do not provide absolute measurements but do provide a basis for comparison.

**Hematological measurements.** Prior to harvesting of carotid bodies, the heart was removed (for an independent study) and blood collected immediately from the chest cavity into heparinized capillary tubes. Hematocrits were measured using a hematocrit centrifuge (Hettich, Germany); hemoglobin was measured using a HemoCue Hb 201+ (HemoCue, UK).

**Statistical analysis.** Data are presented as mean ± SEM. Differences between groups were evaluated using un-paired Student’s t-tests with $p$ values < 0.05 considered as statistically significant.
Results

The purpose of this study was to determine the ventilatory and carotid body phenotype of the CP mouse, in comparison to the WT.

General characteristics of the CP mouse model. The general characteristics of the mice studied are summarized in Table 1. To confirm the presence of polycythemia in the CP mice we measured hemoglobin and hematocrit and demonstrated a modest increase in both parameters, consistent with previous reports (13, 14). The CP mice also had a markedly lower body weight compared to WT controls, a feature that had not been commented upon previously (13, 14, 28). However, large-scale studies of CP patients have demonstrated reduced body weight and/or body mass index (12, 28, 45).

CP mice have increased ventilation and hypoxic sensitivity. During euoxia, CP mice had an elevated baseline ventilation (Figures 1 and 2). This was similar in character to that observed in previous studies in both humans and mice (14, 40), and involved mainly an increase in tidal volume (Figures 1 and 2, middle). Acute exposure to 10% oxygen elicited an immediate increase in ventilation (the AHVR, Figure 1) which was 2.2-fold greater in the CP mice compared to WT (3.0 ± 0.7 ml/min/g versus 1.4 ± 0.3, p < 0.05; Figure 1B, right). However, this increase in ventilation was poorly sustained (Figure 1A).

The hypocapnic attenuation of AHVR in humans can partly be prevented by clamping of the end-tidal carbon dioxide level (16, 17, 40). Since this procedure is not possible in mice, we sought to offset the hypocapnia of increased ventilation by the addition of a low concentration of carbon dioxide to the hypoxic stimulus. The choice of 3% carbon dioxide was based upon, first, published data demonstrating that its addition prevents a major rise in arterial pH or fall in PCO₂ when mice are exposed to 7% oxygen (18) and, second, that it has
been shown previously that CP patients do not have an increased hypercapnic ventilatory sensitivity (39).

Exposure of mice to 10% oxygen with 3% carbon dioxide evoked a larger and better-sustained increase in ventilation (Figures 2 and 3) similar to that seen in humans in response to isocapnic hypoxia (40). CP mice demonstrated a striking increase in ventilation with an AHVR that was 1.6-fold greater than that seen in the WT (7.9 ± 0.3 ml/min/g versus 4.8 ± 0.5, *p* < 0.01; Figure 2B, right). This difference remains statistically significant even if normalization to body weight is not performed. These results confirm that the CP mice manifest an elevated baseline ventilation and increased ventilatory sensitivity to hypoxia.

**CP mice exhibit carotid body Type I cell hypertrophy and hyperplasia.** Since ventilatory acclimatization and the AHVR are dependent upon the carotid body, and chronic hypoxia evokes marked changes in the morphology and ultrastructure of the Type I cells, we performed immunohistochemistry and electron microscopy on carotid bodies from CP mice. Immunostaining for TH revealed that the CP carotid bodies were strikingly different to those from WT mice (Figure 4A and 4B). There was a 2.7-fold increase in the number of Type I cells and a 4.4-fold increase in the total volume of TH-positive tissue per carotid body (Figure 4C). The cells also appeared enlarged with more prominent nuclei. The latter finding was confirmed by electron microscopy studies which demonstrated that both the cell and nuclear areas were increased, indicating hypertrophy (Figures 5 and 6). Furthermore, Type I cells contained an increased number of mitochondria, enlarged dense-cored vesicles, and markedly expanded rough endoplasmic reticulum (Figures 5 and 6). Thus, CP mice show striking Type I cell hyperplasia and hypertrophy, with increased mitochondrial number and evidence of improved capacity for protein synthesis (rough ER expansion) and neurotransmitter storage (DCV).
Discussion

This study demonstrates that the Chuvash mutation in mice results in features that are characteristic of ventilatory acclimatization to hypoxia, namely an elevated baseline ventilation and enhanced AHVR. These findings are consistent with those seen in patients with CP (40). The clear increase in AHVR demonstrated in our study appears to contradict previous reports that the CP mouse model fails to recapitulate this aspect of the human disorder (14). However, our use of a shorter hypoxic stimulus together with the addition of 3% carbon dioxide readily explains this discrepancy. Both hypocapnia and HVD during the averaged 10-minute exposure used previously (14) would have masked the true ventilatory response to hypoxia.

The enhanced hypoxic ventilatory sensitivity in the CP mouse was accompanied by marked changes in carotid body morphology. Immunohistochemistry revealed striking increases in Type I cell number and total TH-positive tissue volume, changes which have also been reported in animals exposed to chronic hypoxia (24, 31). We sought to investigate further these changes within the carotid body, using transmission electron microscopy to examine the ultrastructure of the Type I cell. We demonstrated Type I cell hypertrophy with increased cell and nuclear area, findings that have been repeatedly shown in the carotid bodies from animals exposed to chronic hypoxia (20, 24, 25, 30, 31, 34, 42, 43). In addition, we have shown increased number of mitochondria and evidence of improved capacity for protein synthesis (expansion of the rough ER) and neurotransmitter storage (increased DCV diameter).

We are aware of several studies documenting the carotid body and/or ventilatory phenotypes of genetic disorders of the HIF pathway (4, 9, 22, 32, 33, 41, 46). Mice with heterozygous deletion of HIF-1α (with associated increased HIF-2α levels in the carotid body) have a normal AHVR and carotid body morphology, but manifest markedly impaired ventilatory
responses to either sustained or intermittent chronic hypoxia (22, 33, 46). In contrast, heterozygous null HIF-2α mice also have normal carotid body structure (yet with increased HIF-1α levels), but exhibit irregular breathing patterns and increased ventilatory sensitivity to hypoxia (32, 46). It has been proposed that it is the relative balance, rather than absolute levels, of the HIF-α isoforms within the carotid body that establishes the set point for hypoxic sensing and that HIF-2α may serve an inhibitory role (46). In keeping with this, both patients and mice with HIF-2α gain-of-function mutations do not exhibit increased ventilatory sensitivity to hypoxia (9, 41). However, the VHL Chuvash mutation in mice and humans results in increased expression of both HIF-1-specific and HIF-2-preferential target genes (1, 10, 13, 14, 28); thus the mechanism underlying the ventilatory and carotid body phenotypes is likely to be complex. This point is emphasized by the finding that mice with heterozygous deficiency of PHD2, with consequent increased euoxic levels of HIF-1α and HIF-2α (27), also exhibit carotid body hyperplasia and augmented hypoxic ventilatory sensitivity (4).

It is tempting to speculate that the changes within the carotid body of CP mice underlie the elevated ventilation and hypoxic sensitivity. That these changes mimic those seen in response to both chronic hypoxia and heterozygous deficiency of PHD2 adds weight to this argument. However, further work is needed to understand fully the response both of the carotid body and of individual Type I cells to acute hypoxia. The CP mice were clearly very different to WT in terms of their ventilatory and carotid body phenotypes, despite demonstrating only a moderate increase in hemoglobin and hematocrit. The latter is unlikely to have been a confounding influence, since other animal studies have demonstrated that the hypoxic ventilatory response is either inhibited (3) or unchanged (7) acutely by polycythemia.

Humans exposed to very long-term hypoxia (e.g. through a life-time’s residency at high altitude) ventilate less than individuals acclimatized to high altitude and have a blunted, rather than enhanced, AHVR (5, 29, 37, 44) despite having enlarged carotid bodies (2). As
such, it is somewhat surprising that the CP patients and mice, in whom there is life-long up-regulation of HIF, more closely resemble individuals recently acclimatized to high altitude, rather than those exposed to life-long hypoxia. This suggests that the changes in ventilatory control that occur in response to very long-term hypoxia are not solely dependent upon HIF or the carotid body.

In conclusion, mice with the Chuvash mutation have physiology that is similar to CP patients, including an increased AHVR. In addition, they also possess markedly altered carotid body morphology.
Acknowledgements

We thank Celeste Simon for the gift of the mice, and Lynne Scott, Patrick Pollard and Tammie Bishop for their help in various forms.

Grants

This work was supported by the Wellcome Trust (Grant Number WT090123MA) and Medical Research Council (Grant Number G101134).

Disclosures

The authors have no conflicts of interest to disclose.


47
Figure captions

**Figure 1. Ventilatory responses to 10% oxygen.** (A) Respiratory rate (left), tidal volume (middle) and minute ventilation (right) in response to a five minute exposure to 10% oxygen (black bar). (B) Corresponding mean values for euoxia, hypoxia and the difference between them. Results are mean ± SEM; * (p < 0.05); ** (p < 0.02); *** (p < 0.01); **** (p < 0.001).

**Figure 2. Ventilatory responses to 10% oxygen with 3% carbon dioxide.** (A) Respiratory rate (left), tidal volume (middle) and minute ventilation (right) in response to a five minute exposure to 10% oxygen with 3% carbon dioxide (open bar). (B) Corresponding mean values for euoxia, hypoxia and the difference between them. Results are mean ± SEM; * (p < 0.05); *** (p < 0.01); **** (p < 0.001).

**Figure 3. Plethysmography traces in euoxia and hypoxia.** Representative plethysmography traces of WT and CP mice in both euoxia and hypoxia (10% oxygen with 3% carbon dioxide). Three-second euoxia traces were taken from the final 30 s prior to the hypoxic stimulus; three-second hypoxia traces were taken after 30 s of the hypoxic stimulus had elapsed. Tracings represent the measured ‘box flow’ of the plethysmograph, which is used to calculate physiological flow. a.u. (arbitrary units). Note the increased ventilation of the CP mouse in both euoxia and hypoxia, despite a lower body mass.

**Figure 4. Immunohistochemical analysis of carotid bodies.** Representative low- and high-power sections of wild type (A) and Chuvash (B) carotid bodies (CB) immunostained for tyrosine hydroxylase (TH) (green). Note the larger-sized and more densely-stained CBs from the CP mouse compared with WT (C) Morphometric analysis of TH-positive cell number (per CB) and total volume of TH-positive tissue within the CB. Results are mean ± SEM (n = 3 for each group); *** (p < 0.01); **** (p < 0.001); SCG (superior cervical ganglion).

**Figure 5. Electron micrographs of Type I cells.** Representative electron micrographs of wild-type (A) and Chuvash (B) Type I cells. Note the expanded rough ER, enlarged dense-cored vesicles (DCV)
and increased number of mitochondria in the CP cells. Nucleus (1); peri-vascular space (2); dilated, abundant rough ER (3); DCV (4); mitochondrion (5). Scale bar = 500 nm.

**Figure 6.** Electron microscopy analysis of Type I cells. Morphometric analysis of the ultrastructure of Type I cell area, nuclear area, mitochondrial number, rough ER expansion, and DCV diameter. Results are mean ± SEM (n = 3 for each group); *** (p < 0.01).
Table 1. Body weight and hematological parameters. Table shows the body weights, hemoglobin and hematocrit values for male wild type and Chuvash mice (aged between 4 and 6 months, n = 6 for each group). Results are mean ± SEM.