Citation for published version (APA):
Formenti, F., Beer, P. A., Croft, Q. P. P., Dorrington, K. L., Gale, D. P., Lappin, T. R. J., ... Robbins, P. A. (2011). Cardiopulmonary function in two human disorders of the hypoxia-inducible factor (HIF) pathway: von Hippel-Lindau disease and HIF-2alpha gain-of-function mutation. *Faseb Journal, 25*(6), 2001-2011. https://doi.org/10.1096/fj.10-177378
Cardiopulmonary function in two human disorders of the hypoxia-inducible factor (HIF) pathway: von Hippel-Lindau disease and HIF-2α gain-of-function mutation

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ABSTRACT The hypoxia-inducible factors (HIFs; isoforms HIF-1α, HIF-2α, HIF-3α) mediate many responses to hypoxia. Their regulation is principally by oxygen-dependent degradation, which is initiated by hydroxylation of specific proline residues followed by binding of von Hippel-Lindau (VHL) protein. Chuvash polycythemia is a disorder with elevated HIF. It arises through germline homozygosity for hypomorphic VHL alleles and has a phenotype of hematological, cardiopulmonary, and metabolic abnormalities. This study explores the phenotype of two other HIF pathway diseases: classic VHL disease and HIF-2α gain-of-function mutation. No cardiopulmonary abnormalities were detected in classic VHL disease. HIF-2α gain-of-function mutations were associated with pulmonary hypertension, increased cardiac output, increased heart rate, and increased pulmonary ventilation relative to metabolism. Comparison of the HIF-2α gain-of-function responses with data from studies of Chuvash polycythemia suggested that other aspects of the Chuvash phenotype were diminished or absent. In classic VHL disease, patients are germline heterozygous for mutations in VHL, and the present results suggest that a single wild-type allele for VHL is sufficient to maintain normal cardiopulmonary function. The HIF-2α gain-of-function phenotype may be more limited than the Chuvash phenotype either because HIF-1α is not elevated in the former condition, or because other HIF-independent functions of VHL are perturbed in Chuvash polycythemia.—Formenti, F., Beer, P. A., Croft, Q. P. P., Dorrington, K. L., Gale, D. P., Lappin, T. R. J., Lucas, G. S., Maher, E. R., Maxwell, P. H., McMullin, M. F., O’Connor, D. F., Percy, M. J., Pugh, C. W., Ratcliffe, P. J., Smith, T. G., Talbot, N. P., Robbins, P. A. Cardiopulmonary function in two human disorders of the hypoxia-inducible factor (HIF) pathway: von Hippel-Lindau disease and HIF-2α gain-of-function mutation. FASEB J. 25, 2001–2011 (2011). www.fasebj.org

Key Words: pulmonary hypertension • ventilation • metabolism • polycythemia

The hypoxia-inducible factor (HIF) family of transcription factors orchestrates a coordinated response to variations in oxygen availability by directly or indirectly altering the expression of hundreds of genes (1–4). These factors are composed of two subunits: the oxygen-regulated HIFα (isoforms are HIF-1α, HIF-2α, or HIF-3α) and the constitutively expressed HIFβ (5). In the presence of oxygen at physiological levels, HIFα
MATERIALS AND METHODS

Participants

VHL study

Six patients with classic VHL disease (VHL patients) and 6 healthy control participants took part in the study. The patients were free from any associated neoplasia at the time of the study and had no other medical disorders. VHL patients were recruited through their respective physicians. Control participants were recruited through advertisement and were chosen to match VHL patients for sex, age, height, weight, and body mass index.

HIF2 study

Three patients with gain-of-function mutations in HIF-2α (HIF2 patients) and 6 healthy control participants took part in the study. The patients had not been venesected within several weeks of the experiment and had no other medical disorders. They were identified from previous studies (25, 28) and recruited through their respective physicians. The number of patients recruited was limited by the rarity of the condition. Control participants were recruited through advertisement and were chosen to match HIF2 patients for sex, age, height, weight, and body mass index.

In both studies, all participants were informed about the aims, procedure, and details of the study and signed a consent form before taking part in the experiments. The studies conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee.

Experimental protocol

Experiments were performed during the course of a single day. Participants were asked to avoid vigorous exercise and alcohol and caffeine consumption for 48 h prior to the experiments; they were also asked to eat a light breakfast on the morning of the experimental day. Room temperature was kept at 21°C throughout. Air-breathing end-tidal partial pressures of oxygen (P\textsubscript{ET}O\textsubscript{2}) and carbon dioxide (P\textsubscript{ET}CO\textsubscript{2}) were measured before venous and arterial blood samples were taken. Participants were then asked to undertake four 20-min hypoxia response protocols, separated by three 20-min intervals. Each protocol started with 5 min of isocapnic euoxia (P\textsubscript{ET}CO\textsubscript{2} 100 mmHg), followed by 10 min of isocapnic hypoxia and ended with a final 5 min of isocapnic euoxia. The first two protocols examined the responses to mild hypoxia (P\textsubscript{ET}O\textsubscript{2} 70 mmHg), similar to those experienced on a commercial flight. The last two protocols examined the responses to moderate hypoxia (P\textsubscript{ET}O\textsubscript{2} 50 mmHg), similar to those associated with acute exposure to an altitude of ~3500 m. P\textsubscript{ET}CO\textsubscript{2} was maintained close to each participant’s baseline level throughout each protocol. For the HIF2 study only, participants undertook an incremental exercise test to exhaustion after the hypoxia response protocols had been completed. For one HIF2 patient, the experiments were repeated on a second occasion after the patient had undergone a program of venesection to reduce his hematocrit to within the normal range.

Experimental technique

Pulmonary arterial systolic pressure and cardiac output were estimated through a continuous recording of Doppler echocardiography (Vivid-4; GE Healthcare, Little Chalfont, UK) with a 2-dimensional S3 probe (1.5–3.6 MHz). In the first and third protocols, pulmonary arterial systolic pressure was estimated by measurement of the maximum gradient of blood pressure across the tricuspid valve. This technique has been
RESULTS

VHL study

Details of the specific mutation for each VHL patient, together with the physical characteristics of patients and control participants, are given in Supplemental Table S1. The physical characteristics of patients and control participants were well matched. VHL patients did not differ significantly from control participants in arterial Paco₂, arterial pH, hemoglobin, hematocrit, mean corpuscular volume, serum iron, ferritin, and transferrin (Supplemental Table S2). In 3 VHL patients and in 1 control participant, the arterial Po₂ was lower than is predicted for their age, but average arterial Po₂ did not differ significantly between VHL patients and control participants.

Pulmonary measurements

VHL patients did not differ significantly from control participants in either baseline ventilation or in the increments in ventilation with mild or moderate hypoxia. The responses are illustrated in Supplemental Fig. S1.

An element of tricuspid regurgitation was detectable echocardiographically in 5 of the 6 VHL patients, but in only 2 of the control participants. VHL patients did not differ significantly from control participants in either baseline pulmonary arterial systolic pressure or in the increments in that pressure with mild or moderate hypoxia. The responses are illustrated in Supplemental Fig. S1. These responses were also similar to those of the control participants for the HIF2 study.

Systemic vascular measurements

The systemic vascular responses to hypoxia are illustrated in Supplemental Fig. S2. VHL patients did not differ significantly from control participants in either baseline values for heart rate, blood pressure, and cardiac output or in the increments in these variables with mild or moderate hypoxia.

Summary of VHL study

Figure 1 illustrates the deviation of each participant's baseline value (or incremental response) from the mean value for the control group, expressed in units of sd of the control group. There was considerable overlap between VHL patients and control participants for all variables.

HIF2 study

Details of the specific mutations for each HIF2 patient, together with the physical characteristics of patients and control participants, are given in Supplemental Table S3. The physical characteristics of the patients and control participants were well matched. The results obtained from the analysis of the venous and arterial blood samples are given in Table 1. Compared with the control participants, HIF2 patients had lower arterial Paco₂ (−10.6±5.3 mmHg, \( P<0.01 \)), higher hemoglobin concentration (+2.4±0.9 g/dl, \( P<0.03 \)), higher hematocrit (+0.08±0.03 L/L, \( P<0.03 \)), and higher value for transferrin (+0.8±0.3 g/L, \( P<0.003 \)). HIF2 patients did not differ significantly from control participants in arterial pH, mean corpuscular volume, serum iron, and ferritin.

Pulmonary measurements

HIF2 patients did not differ significantly from control participants in either baseline ventilation or increment in ventilation with mild or moderate hypoxia. The responses are illustrated in Fig. 2.

Echocardiographic measurements of pulmonary arterial systolic pressure were possible in 2 HIF2 patients and 4 control participants. Compared with the control participants, the HIF2 patients had significantly higher pulmonary arterial systolic pressures at baseline (35±2 vs. 24±1 mmHg, HIF2 patients vs. control participants, \( P<0.003 \)), and a significantly greater increase in pulmonary arterial systolic pressure in response to moderate hypoxia (11±1 vs. 5±1 mmHg, \( P<0.004 \)). HIF2 patients did not differ significantly from control participants in the increase in pulmonary arterial systolic pressure with mild hypoxia. The responses are illustrated in Fig. 2.
Systemic vascular measurements

Figure 3 illustrates the systemic vascular responses to hypoxia. At baseline, compared with the control participants, the HIF2 patients had significantly higher heart rate (71 ± 2 vs. 56 ± 8 beats/min, \( P < 0.02 \)) and significantly greater cardiac output (5.5 ± 1.0 vs. 4.1 ± 0.5 L/min, \( P < 0.02 \)), while they did not differ significantly in blood pressure. HIF2 patients did not differ significantly from control participants in the increments in
Summary of cardiopulmonary responses for HIF2 study

Figure 1 presents a summary of the variations in both baseline values and in the incremental responses to hypoxia for the various cardiopulmonary measurements. For baseline pulmonary arterial systolic pressures and for the increments in pulmonary arterial systolic pressure with moderate hypoxia, no overlap was observed in responses between the control participants and the HIF2 patients. For other variables, there was some overlap between the two groups, although the cardiac output of 2 of the 3 HIF2 patients was >3 SD from the mean of the control participants.

Incremental exercise test to exhaustion

Figure 4 illustrates the responses obtained during the incremental exercise test to exhaustion. The rise in venous lactate with work rate appeared to be similar between HIF2 patients and control participants. Two of the 3 HIF2 patients appeared somewhat limited in their maximum exercise capacity, but the third patient exercised very comparably with the control participants. Overall, there were no significant differences between HIF2 patients and control participants for the maximum work rate achieved and the associated lactate level attained (2.0±0.9 vs. 2.9±0.9 W/kg, NS; and 4.3±2.2 vs. 5.4±2.7 mM, NS, respectively). The hyperventilation and associated hypocapnia at rest persisted throughout the period of incremental exercise to exhaustion.

Effects of venesection on one of the HIF2 patients

One of the HIF2 patients (patient 2) was studied both before and after venesection, which reduced the hemoglobin and hematocrit to values within the normally accepted range, serum iron to 7.8 μM, ferritin to 8.9 μg/L, and increased transferrin concentration to 4.24 g/L. (Supplemental Table S4).

Venesection in this patient had no clear effect on ventilation and pulmonary arterial systolic pressure (Supplemental Fig. S3); ventilation appeared normal, while pulmonary arterial systolic pressure remained above normal. Venesection had no clear effect on heart rate, blood pressure, and cardiac output (Supplemental Fig. S4) at baseline and with hypoxia. Also, venesection had no clear effect on the accumulation of lactate in venous blood, ventilation, and end-tidal pressure of carbon dioxide (Supplemental Fig. S5), and on the maximum work rate and maximum venous blood lactate concentration achieved during the incremental exercise test to exhaustion; all these parameters remained within the normally accepted range (Supplemental Table S4).

DISCUSSION

In patients with classic VHL disease, the tumors that occur always have a somatic mutation of the wild-type allele for VHL, and, for this reason, it has generally been assumed that a single normal allele for VHL is sufficient to maintain normal cellular function. Recently, however, it has been shown that freshly isolated neutrophils from VHL patients displayed delayed apoptosis and enhanced bacterial phagocytosis, consistent with a pseudohypoxic phenotype (24). Haploinsufficiency resulting in HIF activation was further supported by the presence of elevated levels of PHD3 transcript in these cells, which is a known HIF target gene (41, 42). From these observations, the researchers concluded that, in humans, “heterozygous VHL defects are sufficient to perturb normal responses” (24), at least in some cells.

Despite this conclusion, erythrocytosis is not a feature of VHL disease, except in the context of cerebellar or renal tumors. In these cases, erythrocytosis is due to unregulated erythropoietin production from the tumor tissue, where both copies of VHL are mutated. Removal of the tumor tissue corrects the erythrocytosis, confirming that the underlying regulation is normal in the context of a single wild-type allele for VHL (43). In a relatively small number of VHL patients, the present study did not detect any of the major cardiopulmonary abnormalities present in the CP phenotype. Thus, a single functional allele for VHL appears to be sufficient.
Figure 2. End-tidal gas control, ventilation, and pulmonary arterial systolic pressure during mild and moderate hypoxia. A, B) $P_{ET}O_2$ and $P_{ET}CO_2$ in mild hypoxia (A) and moderate hypoxia (B). $P_{ET}O_2$ was well matched between groups. $P_{ET}CO_2$ was lower for the HIF2 patients, reflecting their lower baseline air-breathing $P_{ET}CO_2$. C, D) Ventilation, given at body temperature and pressure, saturated with water vapor, in mild (C) and moderate hypoxia (D). Baseline ventilation and the increase in ventilation provoked by hypoxia were not significantly different between the two groups. E, F) Pulmonary arterial systolic pressure. Compared with control participants, HIF2 patients had elevated pulmonary arterial systolic pressures at baseline (21±2 vs. 35±1 mmHg, *P*<0.003), and increased sensitivity to moderate (F) but not mild (E) hypoxia (5.5±1.3 vs. 11.5±0.7 mmHg, *P*<0.004). Values are expressed as means ± sd. Horizontal black bars indicate 10-min periods of mild (A, C, E) and moderate (B, D, F) exposures to hypoxia.
to ensure either normality, or at least near normality, for these components of the CP phenotype.

In common with CP, gain-of-function mutations in HIF-2α/H9251 were first identified through excessive erythrocytosis (14, 28). This similarity between the two conditions suggests that, in CP, elevation of HIF-2α alone may be sufficient to generate the erythrocytosis. Consistent with this, erythrocytosis in a mouse model of CP can be reversed by loss of the HIF-2α allele in VHL\(^{R/R}\)HIF2-α\(^{+/−}\) mice (where R indicates...
the Chuvash allele), but not by loss of the HIF-1α allele in VHL$^{R/R}$HIF1-α$^{-/-}$ mice (46).

Components of the CP phenotype (18) identified in the HIF2 patients in this study include the pulmonary hypertension present under air breathing conditions and the elevation of pulmonary ventilation in relation to metabolism, as indicated by arterial and end-tidal hypocapnia. Indeed, clinically severe pulmonary hypertension has already been noted in two HIF2 patients from one family (25). Again, these findings suggest that elevation of HIF-2α alone in CP may be sufficient to generate these components of the phenotype. Consistent with this notion, pulmonary hypertension, which is also present in the mouse model of CP, was reduced in severity in VHL$^{R/R}$HIF2-α$^{-/-}$ mice, but not in VHL$^{R/R}$HIF1-α$^{-/-}$ mice (46). Overall, these findings suggest that HIF-1α may have little physiological role in the regulation of basal ventilation, pulmonary blood pressure, and red blood cell production.

In the present study, two novel phenotypic components were identified in the HIF2 patients, namely elevations in basal heart rate and cardiac output, which...
have not been demonstrated in CP. This raises the possibility that the HIF2 phenotype is not simply a subset of the CP phenotype. However, a previous study of the CP phenotype (18) noted trends toward higher cardiac outputs and heart rates in CP that did not quite reach statistical significance. As such, it would seem unlikely that these features are indeed unique to the HIF2 phenotype.

Other aspects of the CP phenotype appeared to be either reduced in magnitude or absent in the HIF2 patients. However, a serious limitation of the present study is the very small number of HIF2 patients that was available for investigation. Thus, negative findings may simply arise as a type II statistical error through lack of power, and, therefore, cannot be used to differentiate HIF2 patients from CP patients. However, as previous studies of CP patients from our laboratory have used very similar protocols, and as some aspects of the CP phenotype are quite extreme, it is possible that significant differences exist between the CP and HIF2 patients, despite the very small numbers in both groups. These comparisons are made below.

For the ventilatory responses to hypoxia, CP patients (18) could not be distinguished from HIF2 patients (CP vs. HIF2: mild hypoxia at 70 Torr, 4.5 ± 2.3 vs. −0.1 ± 1.7 L/min, P < 0.054; moderate hypoxia at 50 Torr, 21 ± 11 vs. 10 ± 8 L/min, P < 0.29). For the pulmonary vascular responses to hypoxia, CP patients could be distinguished from HIF2 patients (CP vs. HIF2: mild hypoxia at 70 Torr, 12 ± 2 vs. 4 ± 4 mmHg, P < 0.053; moderate hypoxia at 50 Torr, 33 ± 1 vs. 11 ± 0 mmHg, P < 0.001). Apart from the cardiopulmonary phenotype, CP patients exhibit an early and above-normal accumulation of lactate in the venous blood during incremental exercise, and a reduced maximum work rate capacity (19). In that study of CP patients, 3 of 5 patients and 3 of 5 control participants exercised hard enough to generate a significant metabolic stress, as evidenced by a venous lactate concentration >5 mM (47), and this occurred at 157 ± 12 W for CP patients and 257 ± 12 W for controls (P < 0.001). Only one HIF2 patient exercised sufficiently hard to generate a venous lactate concentration >5 mM, but this occurred at 270 W—a value that differs significantly (P < 0.02) from the distribution of values from the CP patients. The absence of effect of the HIF-2α mutation on metabolic function is consistent with studies undertaken in cell culture, which suggest that HIF-2α plays no role in regulating the expression of genes that affect glycolytic function (48). While metabolic abnormalities have been observed in HIF-2α-null mice, these changes are only part of a very severe and extensive overall phenotype associated with complete HIF-2α inactivation (49).

In summary, the HIF2 phenotype appeared to recapitulate those aspects of the CP phenotype that are most closely related to the regulation of absolute (or basal) values for variables within the hematological, cardiovascular, and respiratory systems. These include the hematocrit, the (euoxic) pulmonary vascular resistance (as reflected by the pulmonary hypertension), the ratio of ventilation to metabolic rate (as reflected by arterial/end-tidal PCO2), the cardiac output, and possibly cardiac sympathetic tone (as reflected by an increased heart rate). Aspects of the CP phenotype that are not so well replicated by the HIF2 phenotype are the increased sensitivity of the pulmonary vasculature to acute hypoxia and the excessive production of lactic acid during exercise. The limited number of available patients has made it impossible to draw definitive conclusions with respect to the ventilatory sensitivity to hypoxia. While it may be tempting to ascribe those aspects of the CP phenotype that were not observed in the HIF2 patients to an elevation of HIF-1α, it has been reported that VHL possesses a number of HIF-independent effects (44, 45) and so this is not necessarily correct.

Homoglobin concentration, hematocrit, serum iron, and ferritin were all significantly reduced in one HIF2 patient after venesection. Despite the decrease in blood viscosity that would have occurred, no associated reduction in pulmonary arterial systolic pressure was detected. It is possible that the elevated HIF-2α levels in this patient contributed to maintaining a high pulmonary blood pressure, despite a marked decrease in hematocrit. However, in a study of venesection in patients with excessive erythrocytosis from chronic mountain sickness, pulmonary arterial blood pressure increased rather than decreased, suggesting that iron depletion can exacerbate hypoxic pulmonary hypertension (50). This underlines that the effects of venesection are not yet clear (51), and an important question is the extent to which patients with CP or HIF-2α gain of function erythrocytosis should be venesected.

The authors thank the patients and volunteers who took part in the studies. F.F. was funded by a University College Oxford postdoctoral research scholarship and by the Fell Fund from the Department of Physiology, Anatomy, and Genetics, University of Oxford, Oxford, UK. This work was supported by the Wellcome Trust (grant 075876 to P.A.R.), by the Dunhill Medical Trust (to K.L.D.), by the Royal Society (grant TG092416 to F.F.), and by the NIHR Cambridge Biomedical Research Centre, Cambridge, UK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. F.F., P.A.B., Q.P.P.C., K.L.D., D.P.G., T.R.J.L., G.S.L., E.R.M., P.H.M., M.F.M., D.F.O., M.J.P., C.W.P., P.J.R., T.G.S., N.P.T., and P.A.R. agree with the manuscript’s results and conclusions; F.F., C.W.P., and P.A.R. designed the experiments and the study; F.F. analyzed the data; F.F., Q.P.P.C., K.L.D., D.F.O., T.G.S., and N.P.T. collected data and conducted experiments for the study; F.F., P.A.B., D.P.G., T.R.J.L., G.S.L., E.R.M., P.H.M., M.F.M., M.J.P., T.G.S., and P.A.R. enrolled patients; F.F., P.J.R., and P.A.R. wrote the manuscript; K.L.D. and P.A.R. supervised and obtained funding; P.J.R., P.H.M., and C.W.P. are scientific cofounders of, and hold equity in, ReOx Ltd, a university spinoff company that is seeking to make HIF hydroxylase inhibitors. The other authors declare no competing financial interests.
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Received for publication December 1, 2010. Accepted for publication February 24, 2011.