Experimental **Physiology**

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This information is current as of April 17, 2008

This is the final published version of this article; it is available at:
http://ep.physoc.org/cgi/content/full/93/1/104

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Effects of hydralazine on the pulmonary vasculature and respiratory control in humans

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This study sought: (1) to clarify the effects of hydralazine on both the pulmonary vasculature and respiratory control in euoxia and hypoxia in healthy humans; and (2) to determine whether hydralazine alters the expression of genes regulated by hypoxia-inducible factor 1 (HIF-1). Ten volunteers participated in two 2 day protocols. Hydralazine (25 mg) or placebo was administered at 1 pm and 11 pm on the first day, and at 1 pm on the second day. In the mornings and afternoons of both days, we measured plasma vascular endothelial growth factor (VEGF) and erythropoietin (EPO) concentrations (both HIF-1-regulated gene products), systemic arterial blood pressure, and changes in heart rate, cardiac output, maximal systolic pressure difference across the tricuspid valve (ΔP_{max}) and ventilation in response to 20 min of isocapnic hypoxia. Recent hydralazine: (1) decreased diastolic blood pressure; (2) increased heart rate and cardiac output in euoxia and hypoxia whilst having no effect on ΔP_{max}; and (3) increased the ventilatory sensitivity to hypoxia. Hydralazine had no effect on plasma EPO or VEGF concentration. We conclude that hydralazine increases the sensitivity of the ventilatory response to hypoxia, but lacks any effect on the pulmonary vasculature at the dose studied. It did not affect the expression of HIF-1-regulated genes.

Hydralazine is a vasodilator (Stunkard *et al.* 1954) whose mode of action remains uncertain. It has been employed in the treatment of pulmonary hypertension, where some authors have found that it reduces pulmonary vascular resistance (Packer *et al.* 1982; Keller *et al.* 1984; Groves *et al.* 1985), but others have found that it has no such effect (Lupi-Herrera *et al.* 1984). One possible explanation for these differences is that they simply relate to heterogeneity within the disease states studied, for example underlying differences in ventilation–perfusion matching. Despite the uncertainty surrounding the effects of hydralazine on the pulmonary circulation, there is nevertheless agreement that hydralazine improves oxygenation through its effect as a respiratory stimulant (Rubin *et al.* 1982; Miller *et al.* 1983; Keller *et al.* 1984; Corriveau *et al.* 1987; Corriveau *et al.* 1988). However, this effect of hydralazine complicates the interpretation of its effects on the pulmonary circulation still further, because, in those studies where a reduction in pulmonary vascular resistance was detected, it cannot be determined whether it arose from a direct effect of

hydralazine on the vasculature or from an indirect effect through the increase in oxygenation.

The first purpose of this study was to clarify the effects of hydralazine on both the pulmonary vasculature and respiratory control in humans. The study sought to do this in normal, healthy volunteers so that the complicating effects of heterogeneous disease states would be removed. Furthermore, it sought to do this under tightly regulated conditions for partial pressures of O_2 (P_{O_2}) and CO_2 (P_{CO_2}) so that any secondary effects of hydralazine on the pulmonary vasculature arising through differences in oxygenation would be removed. Finally, the study sought to examine the effects of hydralazine under conditions of both euoxia and acute hypoxia, allowing any effects to be separated into those that were hypoxia independent and those that were hypoxia dependent.

A second purpose of this study was to investigate, in humans, the suggestion of Knowles *et al.* (2004) that hydralazine may act by stabilizing the transcription factor, hypoxia-inducible factor 1 (HIF-1). Hypoxia-inducible

factor 1 is a transcription factor which induces the expression of a wide variety of genes (Hu *et al.* 1998; Palmer *et al.* 1998; Ang *et al.* 2002). Under conditions of hypoxia, the availability of molecular oxygen is reduced and HIF-1α is stabilized. This increases the expression of HIF-1 target genes and physiological responses to the reduced oxygen concentration are achieved. At the integrative level, HIF-1 partially influences many processes. In particular, mice heterozygously deficient for HIF-1 show reduced pulmonary vasoconstriction (Shimoda *et al.* 2001) in response to hypoxia and reduced ventilatory acclimatization to hypoxia (Kline *et al.* 2002). Humans with Chuvash polycythaemia, a genetic disorder that gives rise to elevated levels of HIF-1, have a reduced systemic arterial blood pressure (Gordeuk *et al.* 2004) and exhibit exaggerated pulmonary vascular and respiratory responses to acute hypoxia (Smith *et al.* 2006). Thus, if Knowles *et al.* (2004) are correct in their suggestion that hydralazine stabilizes HIF-1, then this could explain both any differential effects of hydralazine on the systemic and pulmonary circulations and its effects as a respiratory stimulant.

The evidence provided by Knowles *et al.* (2004) in support of their hypothesis was obtained not only from cell culture, but also from experiments involving the administration of hydralazine to mice, which then developed an elevation in plasma vascular endothelial growth factor (VEGF), the product of an increase in expression of a HIF-1-regulated gene. The present study sought to determine whether such increases in HIF-1-regulated gene products could also be detected in humans following administration of hydralazine. Furthermore, the actions of drugs which induce alterations in gene expression tend to be somewhat slower in onset and offset than drugs which have more direct modes of action. Accordingly, we also sought to determine whether the physiological effects of hydralazine were slow in onset following the first dose of the drug and whether or not they persisted after most of the hydralazine had been eliminated from the body.

Methods

Participants

Ten healthy volunteers (6 men, 4 women), with an average age of 24 ± 4 years (mean $\pm$ s.d.), took part in the study. Each gave written informed consent before participating. The study was approved by the Oxfordshire Clinical Research Ethics Committee. Each volunteer visited the laboratory twice before undertaking any of the main experimental protocols to ensure that they were familiar with the laboratory and its procedures. On these visits, the volunteers' normal end-tidal P_{CO_2} (P_{ET,CO_2}) values were measured and it was confirmed that they were suitable for echocardiographic assessment of tricuspid regurgitation.

Protocols

Each volunteer attended the laboratory for two main protocols (Fig. 1), one in which hydralazine (Sovereign Medical, Basildon, UK) was administered orally (hydralazine protocol, HP), and one in which placebo was administered orally (control protocol, CP). The order of the two protocols was randomized. Each protocol lasted two successive days and the protocols were separated from one another by at least 1 week. Either 25 mg hydralazine (hydralazine protocol) or placebo (control protocol) was administered at 1 pm and 11 pm on the first day, and at 1 pm on the second day of the respective protocols. This gave effectively four measurement periods for each protocol. Measurements made in the morning (AM) on the first day (day 1) provided control measurements without hydralazine (AM1 measurements). Measurements made in the afternoon (PM) of the first day were made ∼1 h after the first dose of hydralazine had been administrated (PM1 measurements). Thus the PM1 measurements were made before any effects of the drug on gene expression would have had time to develop. Measurements made in the morning of the second day (day 2) were made against a background of hydralazine administration on the previous day, but without a recent dose so that circulating levels would be low (AM2 measurements). This reasoning is based on the known half-life in humans of hydralazine following a single dose, which rarely exceeds 70 min (Shepherd *et al.* 1980). Measurements made in the afternoon of the second day were made against a background of recent hydralazine administration about 1 h previously, so that circulating levels would not be low (PM2 measurements).

In each of the four periods, initial measurements were made of the volunteer's blood pressure and P_{ET,CO_2} to gauge the effect of hydralazine as a vasodilator and respiratory stimulant. Blood was drawn to determine the effects of hydralazine on plasma EPO and VEGF, two substances whose expression is regulated by HIF-1. In each of the four measurement periods, the respiratory and cardiovascular responses to an acute hypoxic challenge were also determined. During this procedure, end-tidal P_{O_2} (P_{ET,O_2}) was held at 100 Torr for the first 10 min, then at 50 Torr for 25 min, and finally at 100 Torr again for 5 min. The P_{ET,CO_2} was held constant at 3 Torr above the volunteer's normal value throughout the exposure to hypoxia.
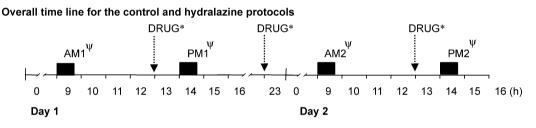
Control of end-tidal gases during the hypoxic exposure and respiratory measurements

During the exposures to hypoxia, volunteers lay horizontal on a couch and breathed through a mouthpiece with the nose occluded. Ventilatory volumes were measured

by a turbine volume-measuring device (Cardiokinetics Ltd, Salford, UK), and flows by a pneumotachograph (Fleisch, Lausanne, Switzerland), both in series with the mouthpiece. Respired gases were sampled via a fine catheter close to the mouth and analysed continuously for P_{CO_2} and P_{O_2} by mass spectrometry (Airspec, Bognor Regis, UK). A pulse oximeter (Datex-Ohmeda, Helsinki, Finland) was used to monitor arterial O_2 saturation. Inspired and expired volumes, end-inspiratory and end-expiratory P_{CO_2} and P_{O_2}, and saturation were detected in real time by a computer and logged breath by breath. A dynamic end-tidal forcing system (Robbins *et al.* 1982) was used to control the end-tidal gases in the manner required for the determination of the acute hypoxic ventilatory response (AHVR). Before the start of each experiment, a cardiorespiratory model was used to construct a forcing function that contained the breath-by-breath values for inspiratory P_{CO_2} and P_{O_2} predicted to produce the desired end-tidal sequences. During the experiment, a computer-controlled gas-mixing system was used to generate this sequence in a modified manner. The

modifications resulted from feedback control based on the deviations of the measured values for P_{ET,CO_2} and P_{ET,O_2} from their desired values.

Measurements of arterial blood pressure, pulmonary blood pressure and cardiac output

Arterial blood pressure was measured using automated sphygmomanometry (Omron, Kyoto, Japan), with volunteers seated. An average of three measurements was taken for each measurement period. Maximal systolic pressure difference across the tricuspid valve was used to estimate pulmonary artery pressure. The majority of people have detectable regurgitation through their tricuspid valves during systole. Doppler echocardiography is able to detect the presence of this jet of blood and measure the velocity with which it travels back into the right atrium. On the assumption that the flow within the jet may be regarded as steady, Bernoulli's equation may be used to calculate the maximal pressure difference between the right ventricle and right atrium (ΔP_{max})

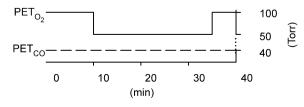**Figure 1. Diagrammatic representation of the two protocols**
In the hydralazine protocol, 25 mg hydralazine was administrated at the points marked 'drug'. In the control protocol, placebo was administrated at the points marked 'drug'. Terms: P_{ET,CO_2}, end-tidal P_{CO_2}; P_{ET,O_2}, end-tidal P_{O_2}; AM1, first day morning; PM1, first day afternoon; AM2, second day morning; PM2, second day afternoon.

from the density of blood (ρ) and the peak velocity of the jet (v). This gives the relationship $\Delta P_{\max} = \rho v^2/2$. Assuming right atrial pressure remains constant, changes in $\Delta P_{\max}$ will be equal to changes in the peak systolic pulmonary arterial pressure. These echocardiographic measurements were performed by using a Hewlett-Packard Sonos 5500 ultrasound machine with an S4 two-dimensional transducer (2–4 MHz) as previously described (Balanos *et al.* 2003). Heart rate and respiratory waveform were both recorded simultaneously.

Cardiac output was determined using Doppler echocardiography. An apical five-chamber view of the heart was used with Doppler mode to identify flow through the aortic valve during systole. We used pulsed-wave spectral mode, at a screen sweep speed of $100 \, \text{mm s}^{-1}$, to obtain the velocity profile of the aortic flow. Doppler sampling of the flow was taken immediately below the orifice of the aortic valve. The flow was quantified automatically using the velocity–time integral (VTI); this is the mean distance through which blood travels in the outflow tract during ventricular contraction. Each measurement of VTI was made from at least three velocity profiles taken towards the end of expiration. The diameter of the aortic valve was measured from a parasternal long axis view and the aortic valve area (A) was calculated. Stroke volume (SV) was calculated from VTI $\times A$; cardiac output was calculated from SV multiplied by heart rate (HR).

Measurement of plasma VEGF and EPO concentrations

For each of the four measurement periods, 10 ml of venous blood was collected in a tube coated with ethylenediamine tetra-acetic acid. The sample was immediately centrifuged at $1000g$ for 15 min. Plasma was removed and stored at $-80°$C until analysis. The concentrations of VEGF and EPO were measured using enzyme-linked immunosorbent assays (ELISA) for human specimens (Quantikine DVE00, R&D Systems Inc. for VEGF and Quantikine IVD DEP00, R&D Systems Inc. for EPO, Abingdon, UK), according to the manufacturer's instructions. The coefficient of variation for VEGF and EPO is stated by the manufacturer to be approximately 5% for measurements made on human plasma.

Modelling of hypoxic ventilatory responses

To obtain numerical values for the sensitivity of ventilation to the hypoxic exposures, a respiratory model was fitted to the data. The particular respiratory model employed was Model I of those described by Liang *et al.* (1997). This model separates the total ventilation, $\dot{V}_E$, into a central, hypoxia-independent component, $\dot{V}_C$, and a peripheral,

hypoxia-dependant component, $\dot{V}_P$, and may be written:

$$\dot{V}_E = \dot{V}_P + \dot{V}_C$$

$$T_P \left(\frac{d\dot{V}_P}{dt} \right) + \dot{V}_P = G_P \left[100 - S(t - D_P) + K_P \right]$$

$$T_h \left(\frac{dG_P}{dt} \right) + G_P = G_{100} - G_h \left[100 - S(t - D_P) \right]$$

where T_P is the time constant for development of the peripheral response, K_P is the peripheral drive in the absence of hypoxia, G_P is the peripheral chemoreflex sensitivity, and S is the saturation function calculated at time (t) delayed by the peripheral time delay, D_P. The parameter T_h is the time constant for the development of hypoxic ventilatory decline (HVD), G_{100} is the steady-state chemoreflex sensitivity in the absence of HVD (i.e. following conditioning at 100% arterial oxygen saturation), and G_h defines the magnitude of HVD as the ratio of the decrease in peripheral chemoreflex sensitivity to the decrease in S. Under conditions of steady air breathing, G_P is equal to $G_{100} - 2.3G_h$, since the algorithm of Severinghaus (Severinghaus, 1979) yields a value for arterial desaturation of 2.3% when P_{O_2} is equal to 100 Torr.

In order to allow for the autocorrelation that exists between measurements of ventilation on successive breaths, a model of the noise processes was fitted to the data in parallel with the fitting of the model of the ventilatory response to hypoxia (Liang *et al.* 1996). The model parameters were estimated by fitting the models to the data using a standard subroutine to minimize the sum of squares of the residuals (subroutine E04FDF, Numerical Algorithms Group, Oxford, UK).

Statistical analysis

Data are presented as means $\pm$ s.e.m. unless stated otherwise. Differences (in the effects of hypoxia) between the control protocol and the hydralazine protocol were analysed using ANOVA. The starting analysis had fixed factors of protocol (CP *versus* HP), and time (AM1, PM1, AM2 and PM2), and a random factor of volunteer. Whether hydralazine had a significantly different effect from placebo over time was assessed by the significance or otherwise of the interactive term between protocol and time.

Further analysis was conducted to determine whether the presence or absence of hydralazine had an effect by comparing AM1 with the pooled effect of PM1, AM2 and PM2 as a single factor. Further analysis was also conducted to determine whether a recent hydralazine administration had an effect by comparing AM1 with the pooled effect of PM1 and PM2 as a single factor. Statistical significance was assumed at $P < 0.05$.

Table 1. Average values for baseline cardiorespiratory variables and plasma concentrations of VEGF and EPO during the hydralazine and control protocols ($n = 10$)

	Control protocol				Hydralazine protocol			
	AM1	PM1	AM2	PM2	AM1	PM1	AM2	PM2
SBP	111 ± 3	110 ± 3	109 ± 3	111 ± 3	108 ± 3	110 ± 3	108 ± 2	111 ± 3
DBP	70 ± 2	68 ± 2	68 ± 2	66 ± 2	69 ± 2	$56 \pm 3^*$	63 ± 3	$57 \pm 3^*$
P_{ET,CO_2}	36.8 ± 0.6	36.9 ± 0.7	36.2 ± 0.9	37.3 ± 0.6	35.7 ± 0.9	36.5 ± 0.8	36.2 ± 1.1	37.7 ± 0.8
$\dot{V}_E$	10.4 ± 0.9	10.3 ± 1.1	9.3 ± 0.7	10.4 ± 1.4	9.5 ± 1.1	11.2 ± 1.2	9.3 ± 0.9	11.5 ± 1.5
VEGF	42.4 ± 7.0	49.1 ± 10.9	40.2 ± 7.3	53.5 ± 10.3	37.9 ± 6.6	59.1 ± 10.7	42.2 ± 8.4	53.4 ± 11.7
EPO	6.2 ± 0.6	7.5 ± 0.7	7.1 ± 1.1	8.4 ± 1.2	6.0 ± 0.7	7.5 ± 1.1	7.6 ± 1.1	7.4 ± 0.8

Values are means $\pm$ s.e.m. Variables and units: SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); P_{ET,CO_2}, end-tidal carbon dioxide partial pressure (Torr); $\dot{V}_E$, ventilation (l min^{-1}); VEGF, vascular endothelial growth factor (pg ml^{-1}); and EPO, erythropoietin (mI U ml^{-1}). Measurement periods: AM1, first day morning; PM1, first day afternoon; AM2, second day morning; and PM2, second day afternoon. $^*P < 0.05$, hydralazine protocol compared with control protocol.

Results

Baseline data

Systolic blood pressure was relatively constant during all measurement periods in both the control and hydralazine protocols, with no significant differences between the two protocols. However, diastolic blood pressure was significantly lower ($P < 0.05$, ANOVA) in the periods after recent hydralazine administration (Table 1).

The volunteers' natural air-breathing P_{ET,CO_2} was relatively constant throughout both protocols, and there were no significant differences between the control protocol and the hydralazine protocol for any of the measurement periods (Table 1). For the control protocol, the variation in the ventilation under air-breathing conditions was very small. For the hydralazine protocol, there was a tendency towards an increase in ventilation in the periods following recent hydralazine administration (PM1 and PM2), but this change did not reach a statistically significant level (Table 1).

Ventilatory responses to hypoxia

Figure 2 illustrates the average time course for P_{ET,O_2} and P_{ET,CO_2} across all volunteers for each measurement period for both protocols. The hypoxic stimulus was generated accurately in each measurement period. The background level of P_{ET,CO_2} was held very constant throughout. Overall, no discernable differences in gas control between the two protocols could be detected.

Figure 2 also illustrates the average time course for ventilation for each measurement period for each protocol. Recent hydralazine administration (PM1 and PM2) appears to have increased both the ventilation in euoxia and the sensitivity of the ventilatory response to hypoxia. For the measurement period 12 h after the second dose of hydralazine (AM2), ventilation appeared only slightly higher than in the control protocol.

The parameters obtained from fitting the respiratory model to the data, together with the results from the statistical comparisons, are shown in Table 2. These results confirm a significant effect of hydralazine on the ventilatory sensitivity to hypoxia (parameter G_{100}, also G_{EU}), but any effect on ventilation in the absence of hypoxia was more marginal. In particular, the effect of hydralazine on the calculated ventilation in the absence of hypoxia (parameter $\dot{V}_C$ of model) failed to reach significance, but there was a significant effect of hydralazine on ventilation calculated at a standard air-breathing P_{ET,O_2} of 100 Torr ($\dot{V}_{EU}$).

Cardiovascular responses

Figure 2 illustrates the average time course for the cardiovascular responses to the hypoxic stimuli for both protocols. Table 3 lists the mean values for the cardiovascular variables, together with the mean differences observed between the two protocols. Heart rate increased significantly with hypoxia in each measurement period in both protocols ($P < 0.05$, all comparisons). Hydralazine administration significantly increased HR in both euoxia and in hypoxia ($P < 0.05$, both comparisons), but it had no significant effect on the increase in HR from euoxia to hypoxia in any measurement period.

Cardiac output increased significantly in hypoxia for each measurement period in both protocols ($P < 0.01$, all comparisons). The presence of hydralazine significantly increased cardiac output in both euoxia and hypoxia ($P < 0.05$, both comparisons), and an overall effect of the presence of hydralazine was to increase the sensitivity of cardiac output to hypoxia ($P < 0.05$ for overall effect; individual comparisons did not reach statistical significance).

The ΔP_{max} increased significantly in hypoxia for each measurement period in both protocols ($P < 0.01$, all comparisons). The presence of hydralazine had no significant effects on the values for ΔP_{max} under conditions

of either euoxia or hypoxia, or indeed, the magnitude of the responses to hypoxia. The size of the standard errors suggests that an effect of hydralazine of ∼25% or more on the magnitude of the response to hypoxia would have been detected with our protocols.

Plasma EPO and VEGF concentrations

Plasma EPO concentrations were relatively stable for all measurement periods in both the control and hydralazine protocols (Table 1). The presence of hydralazine had no significant effect on plasma EPO concentration in any measurement period.

In both protocols on both days, there was a trend towards an increase in plasma VEGF concentration in the afternoon compared with the morning, although this did not reach a statistically significant level. Compared with the control protocol, hydralazine had no significant effects on plasma VEGF concentration in any measurement period.

Discussion

The main findings of this study are that in healthy humans: (1) hydralazine increases the ventilatory sensitivity to hypoxia; (2) hydralazine does not affect indices of pulmonary artery pressure in euoxia or hypoxia; and (3) hydralazine has no effect on the expression of VEGF and EPO.

The effect of hydralazine on ventilation

It has been demonstrated that hydralazine improves arterial oxygenation when it is used to treat pulmonary hypertension (Rubin *et al.* 1982; Miller *et al.* 1983; Keller *et al.* 1984; Corriveau *et al.* 1987, 1988). The improvement in arterial oxygenation following hydralazine may have resulted from an increase in minute ventilation in patients with cor pulmonale (Miller *et al.* 1983) and patients with chronic obstructive pulmonary disease (COPD; Corriveau *et al.* 1988). It also may have resulted from
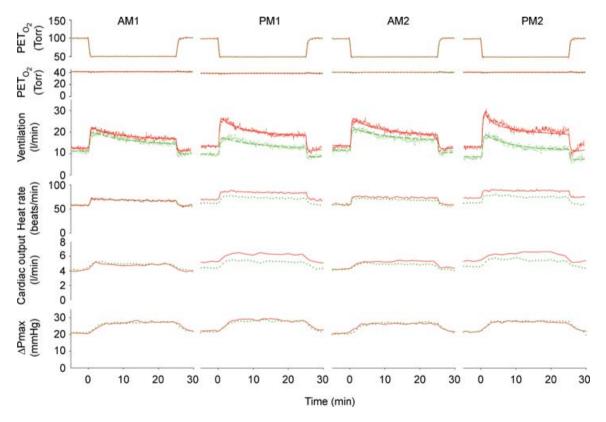

Figure 2. Mean values for end-tidal P_{O_2} (P_{ET,O_2}), end-tidal P_{CO_2} (P_{ET,CO_2}), ventilation, heart rate, cardiac output and maximal systolic pressure difference across the tricuspid valve ($\triangle P_{max}$) during hypoxic exposures (0–25 min) for the control protocol (dotted and dashed green lines) and the hydralazine protocol (continuous red lines)
Lines through data for ventilation indicate fit of respiratory model to data. Measurement periods as in legend to Fig. 1.

Table 2. Ventilatory responses to hypoxia: average parameter values for respiratory model for the hydralazine and control protocols ($n = 10$)

Protocol/time	G_{100} (l min^{-1} %$^{-1}$)	$\dot{V}_C$ (l min^{-1})	T_P (s)	D_P (s)	G_h (l min^{-1} %$^{-2}$)	T_h (s)	K_P (%)	$\dot{V}_{EU}$ (l min^{-1})	G_{EU} (l min^{-1} %$^{-1}$)
Control protocol (CP)									
AM1	0.88 ± 0.40	8.5 ± 3.4	15.0 ± 10.4	4.45 ± 1.61	0.07 ± 0.14	626 ± 352	2.0 ± 2.1	11.2 ± 3.0	0.71 ± 0.28
PM1	0.92 ± 0.63	7.6 ± 3.1	11.1 ± 10.3	6.43 ± 4.87	0.07 ± 0.14	765 ± 298	3.2 ± 3.9	11.3 ± 3.9	0.75 ± 0.43
AM2	1.14 ± 0.95	8.4 ± 3.4	5.2 ± 2.9	5.54 ± 1.95	0.11 ± 0.27	694 ± 343	2.2 ± 2.5	11.9 ± 3.5	0.89 ± 0.39
PM2	0.92 ± 0.48	8.4 ± 3.4	11.9 ± 9.5	5.12 ± 2.19	0.04 ± 0.02	567 ± 197	1.8 ± 2.6	11.4 ± 3.5	0.84 ± 0.43
Hydralazine protocol (HP)									
AM1	0.85 ± 0.5	8.5 ± 2.6	14.1 ± 9.6	4.60 ± 1.96	0.03 ± 0.02	744 ± 336	3.0 ± 2.8	12.6 ± 4.6	0.78 ± 0.46
PM1	1.32 ± 0.95	9.0 ± 5.8	11.1 ± 9.5	3.93 ± 1.80	0.05 ± 0.04	588 ± 303	3.9 ± 6.6	15.7 ± 7.4	1.22 ± 0.87
AM2	1.43 ± 1.24	10.3 ± 4.0	10.8 ± 7.1	5.53 ± 2.82	0.12 ± 0.26	726 ± 328	1.9 ± 3.8	13.5 ± 4.2	1.15 ± 0.78
PM2	1.48 ± 1.09	11.3 ± 5.3	14.3 ± 8.8	3.50 ± 1.40	0.06 ± 0.05	647 ± 386	1.5 ± 1.9	14.4 ± 6.1	1.35 ± 0.98
Difference between CP and HP									
AM1	−0.03 ± 0.43	0.0 ± 3.2	−0.9 ± 15.5	0.14 ± 2.35	−0.05 ± 0.13	118 ± 316	1.0 ± 3.7	1.4 ± 3.3	0.07 ± 0.28
PM1	0.41 ± 0.84	1.4 ± 4.4	−0.1 ± 9.9	−2.50 ± 5.25	−0.03 ± 0.15	−177 ± 343	0.8 ± 6.1	4.4 ± 4.1	0.47 ± 0.61
AM2	0.29 ± 0.60	1.9 ± 3.9	5.6 ± 7.8	−0.01 ± 3.39	0.01 ± 0.04	33 ± 158	−0.3 ± 3.3	1.6 ± 1.7	0.26 ± 0.53
PM2	0.56 ± 0.78	2.9 ± 2.6	2.3 ± 14.3	−1.62 ± 2.70	0.02 ± 0.04	80 ± 327	−0.3 ± 2.6	3.0 ± 3.7	0.51 ± 0.71
ANOVA on differences									
AM1 ^ 0	−	−	−	−	−	−	−	−	−
AM1 ^ PM1 ^ AM2 ^ PM2	−	−	−	−	−	−	−	−	−
AM1 ^ (PM1, AM2, PM2)	+	−	−	−	−	−	−	−	+ +
AM1 ^ (PM1, PM2)	+ +	−	−	−	+	−	−	+	+

Values are means ± s.e.m. Definitions: G_{100}, steady-state chemoreflex sensitivity to hypoxia in absence of any hypoxic ventilatory depression (arterial oxygen saturation is 100%); $\dot{V}_C$, hypoxia-independent (central chemoreflex) contribution to $\dot{V}_{EU}$; T_P, time constant for the peripheral chemoreflex responses to hypoxia; D_P, time delay for the peripheral chemoreflex; G_P, sensitivity to hypoxic ventilatory decline, expressed as the ratio of the decrease in the sensitivity of the peripheral chemoreflex to the decrease in conditioning arterial oxygen saturation; T_h, time constant associated with the development of hypoxic ventilatory decline; K_P, the peripheral drive in the absence of hypoxia; $\dot{V}_{EU}$, ventilation calculated at a standard air-breathing P_{ET,O_2} of 100 Torr; and G_{EU}, steady-state chemoreflex sensitivity during air breathing. The values of $\dot{V}_{EU}$ and G_{EU} are derived from parameters of the primary model. Measurement periods are as in Table 1. Analysis of variance was conducted on the differences between the control and hydralazine protocols, with the comparisons drawn indicated in the table; $+P < 0.05$ and $+ +P < 0.01$.

Table 3. Cardiovascular responses to hypoxia: average values for heart rate, cardiac output and maximal systolic pressure difference across the tricuspid valve (ΔP_{max}; $n = 10$)

Protocol/time	Heart rate (beats min^{-1})			Cardiac output (l min^{-1}).			ΔP_{max} (mmHg)		
	Euoxia	Hypoxia	Increase	Euoxia	Hypoxia	Increase	Euoxia	Hypoxia	Increase
Control protocol (CP)									
AM1	60 ± 9	69 ± 9	9 ± 5*	5.0 ± 0.6	6.0 ± 0.8	1.0 ± 0.6**	21.1 ± 4.5	27.1 ± 5.5	6.0 ± 1.9**
PM1	62 ± 8	75 ± 11	13 ± 8*	5.4 ± 0.9	6.4 ± 1.0	1.0 ± 0.7**	21.5 ± 4.2	28.2 ± 5.1	6.7 ± 1.5**
AM2	59 ± 8	70 ± 9	12 ± 7*	5.1 ± 0.8	6.1 ± 0.8	1.0 ± 0.6**	20.4 ± 4.2	26.9 ± 5.2	6.5 ± 1.9**
PM2	64 ± 7	77 ± 10	14 ± 7*	5.6 ± 0.9	6.5 ± 0.9	0.9 ± 0.4**	21.6 ± 4.4	28 ± 5.6	6.4 ± 1.9**
Hydralazine protocol (HP)									
AM1	59 ± 8	69 ± 9	10 ± 6*	5.0 ± 0.6	6.0 ± 0.9	1.0 ± 0.5**	20.7 ± 4.6	27.6 ± 5.9	6.9 ± 2.2**
PM1	70 ± 9	85 ± 13	15 ± 7*	6.5 ± 1.0	7.8 ± 1.1	1.3 ± 0.5**	22.2 ± 4.1	28.9 ± 5.3	6.7 ± 2.9**
AM2	60 ± 7	77 ± 10	17 ± 8*	5.4 ± 0.6	6.8 ± 0.8	1.4 ± 0.6**	20.8 ± 3.9	26.5 ± 4.3	5.7 ± 2.7**
PM2	73 ± 7	88 ± 10	15 ± 7*	6.7 ± 0.9	9.3 ± 1.1	1.6 ± 0.5**	21.8 ± 4.1	27.6 ± 5.6	5.9 ± 2.0**
Difference between CP and HP									
AM1	−1 ± 2	0 ± 3	1 ± 2	−0.0 ± 0.2	−0.1 ± 0.2	−0.1 ± 0.2	0.4 ± 0.6	0.5 ± 0.7	1.0 ± 0.7
PM1	8 ± 2	10 ± 4	2 ± 3	1.1 ± 0.2	1.4 ± 0.3	0.4 ± 0.2	0.8 ± 0.5	0.7 ± 0.6	0.1 ± 0.6
AM2	1 ± 2	7 ± 3	5 ± 2	0.3 ± 0.3	0.7 ± 0.3	0.4 ± 0.3	0.4 ± 0.5	0.4 ± 0.9	0.8 ± 0.8
PM2	9 ± 3	11 ± 4	2 ± 2	1.1 ± 0.2	1.8 ± 0.3	0.7 ± 0.2	0.2 ± 0.3	0.4 ± 0.8	0.5 ± 0.7
ANOVA on differences									
AM1 ^ 0	−	−	−	−	−	−	−	−	−
AM1 ^ PM1 ^ AM2 ^ PM2	+	−	−	+	+	−	−	−	−
AM1 ^ (PM1, AM2, PM2)	+	+	−	+	+	+	−	−	−
AM1 ^ (PM1, PM2)	+ +	+	−	+	+	−	−	−	−

Values are means ± s.e.m. Measurement periods are as in Table 1. Significant increases with hypoxia; *$P < 0.05$ and **$P < 0.01$. ANOVA, analysis of variance conducted on the differences between the control and hydralazine protocols, with the comparisons drawn indicated in the table; $+P < 0.05$ and $++P < 0.01$.

an improvement of gas exchange in the lung in patients with COPD and pulmonary hypertension (Keller *et al.* 1984). However, none of these studies has unravelled the mechanisms underlying the improvements in ventilation and gas exchange following administration of hydralazine. Furthermore, in these studies, the patients had a mixture of pulmonary disorders associated with pulmonary hypertension and varying degrees of severity of diseases. Indeed, the patients tended to have suffered from long-term hypoxia and to already have a lowered arterial oxygen partial pressure before administration of hydralazine. Therefore, the effects of hydralazine observed in these patients do not necessarily imply that hydralazine will have any effects when administered to healthy individuals who have not been chronically hypoxic.

The present study provides evidence that the effect of hydralazine on ventilation occurs independently of any disease state of the respiratory system. Furthermore, the results demonstrate that, while there may be a modest effect of hydralazine under euoxic conditions, its most marked effect is to increase the sensitivity of the peripheral chemoreflex to hypoxia. This finding suggests a physiological mechanism by which hydralazine may improve oxygenation in hypoxaemic patients that is independent of any effect hydralazine may or may not have on the pulmonary vasculature to improve gas exchange. One such possible mechanism is that the fall in systemic (diastolic) blood pressure and increase in cardiac output in some way generated the hyperventilation and the increase in ventilatory sensitivity to hypoxia. If this were the case, then we might predict that similar observations might have been made in relation to other vasodilators. A recent study from our laboratory made use of trimetaphan, which acts as a vasodilator through blockade of autonomic ganglia (Liu *et al.* 2007). In that study, a fall in mean systemic arterial pressure of ∼10–20 mmHg was observed, but it was not associated with any significant change in ventilatory sensitivity to hypoxia. That experiment was conducted using the same techniques, and analysed using the same modelling approaches, as those of the present investigation, and therefore can be regarded as a 'hypotension control' for the hydralazine protocol used here. A similar observation was made using trimetaphan as a vasodilator in humans under halothane anaesthesia (Hannhart *et al.* 1983).

The lack of effect of hydralazine on the pulmonary circulation

The present study found no effect of hydralazine on our index of pulmonary artery pressure, but there are a number of potential criticisms that need to be considered before this finding can be translated into the conclusion that hydralazine lacks an effect on the pulmonary vasculature in humans.

The first potential criticism is that our measurement of pulmonary artery pressure was indirect, using Doppler ultrasound, and consequently the absence of an effect might relate to a problem with the technique as opposed to anything else. However, there is now a lot of experimental evidence: (1) to support the accuracy of this technique as an index of peak pulmonary systolic pressure in both euoxia (Yock *et al.* 1986) and hypoxia (Allemann *et al.* 2000); and (2) to show that peak systolic pressure is tightly related to the mean pulmonary arterial pressure (Yock *et al.* 1986; Chan *et al.* 1987; Ishii *et al.* 1996).

A second potential criticism is that it is possible that the dose of hydralazine administered was insufficient to have an effect on the pulmonary circulation. However, as a counter-argument to this, the dose clearly reduced systemic vascular tone, increased cardiac output and reduced diastolic blood pressure.

A third potential criticism is that our index of pulmonary artery pressure is not a direct measure of the effect of hydralazine on the pulmonary vasculature. The difficulty here is that ΔP_{max} is a measure of inflow pressure in the pulmonary circulation that is expected to be a function of both flow through the circulation and the degree of smooth muscle tone, or constriction, within the circulation. Thus, changes in cardiac output with hypoxia might confound our measurement of pulmonary vascular tone. In order to address this issue, Balanos and co-workers sought to determine the effect of variations in cardiac output occurring spontaneously in the absence of any hypoxia (Balanos *et al.* 2005). In 33 healthy volunteers breathing air at rest, they found that spontaneous changes in cardiac output generated a change in ΔP_{max} of $0.6 \text{ mmHg l}^{-1} \text{ min}^{-1}$. From this, they estimated that the change in cardiac output with hypoxia contributed ∼5% of the total change in ΔP_{max}, with ∼95% of the change in ΔP_{max} arising from hypoxic vasoconstriction alone. Thus, in healthy volunteers, ΔP_{max} appears to be a sensitive index of pulmonary smooth muscle activity, confounded only to a limited extent by concurrent changes in flow.

The effect of hydralazine on expression of HIF target genes

Ideally, this study would have determined the direct effect of hydralazine on HIF in a number of different tissues. However, while HIF is present in many tissues under both euoxic and hypoxic conditions (Stroka *et al.* 2001), ethical considerations prevent us from obtaining such tissue samples in most cases. Furthermore, HIF is a low-abundance protein that is highly labile, and therefore our ability to quantify HIF levels accurately is limited. As a result of these factors, the approach adopted in the present study was to assess plasma levels of circulating proteins whose expression is known to be regulated by HIF.

Two proteins were selected for study. The first of these was EPO. This was chosen on the grounds that significant increases in EPO have been shown to occur in humans in response both to hypoxia (Knaupp *et al.* 1992) and to iron chelation (Ren *et al.* 2000), which is also known to stabilize HIF in cell culture. The second protein, VEGF, was chosen on the grounds that the circulating levels of VEGF increased in mice in response to hydralazine administration (Knowles *et al.* 2004). In neither case did we observe an increase in plasma levels with hydralazine.

There are a number of possible explanations as to why hydralazine did not affect plasma levels of EPO and VEGF, apart from the notion that it does not act on the HIF pathway. First, the dose of hydralazine may have been inadequate. Although comparisons between species are very difficult, the dose employed by Knowles *et al.* (2004) in mice of $5 \, \mathrm{mg \, kg^{-1}}$ would appear substantially greater than our dose of 25 mg twice daily. Nevertheless, the dose we employed is fairly standard as a clinical dose, and it is one that had clear cardiovascular effects in our volunteers.

A second possibility is that the timing of our samples in relation to the hydralazine dose (PM1 and PM2 blood samples drawn 3 h after hydralazine doses) was not correct. However, in human volunteers exposed acutely to 5.5 h of hypoxia, EPO levels had increased by 80 min and continued to rise throughout the exposure (Eckardt *et al.* 1989). For the mice, VEGF induction in plasma was significant by 2 h after intravenous administration of hydralazine (Knowles *et al.* 2004).

A third possibility is that, whilst there may have been an increase in HIF in response to hydralazine, this increase did not result in an increase in circulating levels of EPO and VEGF. In general terms, there must always be a degree of uncertainty when inferring changes in gene expression from the measurement of circulatory levels of protein. However, in the case of EPO, it is well recognized that cellular hypoxia is the major regulating signal, that the hypoxia signal acts through the HIF pathway and that, in intact humans, both hypoxia and iron chelation produce a substantial increase in circulating EPO. However, for VEGF this is not necessarily the case. While there is clear evidence in cell culture to show that hypoxia induces an increase in the expression of VEGF (Ferrara, 2004), nevertheless Oltmanns *et al.* (2006) found that acute systemic hypoxia may decrease circulating VEGF levels. Furthermore, a recent study has demonstrated that substantial temporal dissociation between mRNA and protein levels may occur for VEGF in the skeletal muscle of rats (Milkiewicz *et al.* 2006).

In summary, there are a number of possible explanations for the negative findings of this study. However, we feel that the most likely is that hydralazine did not affect cellular levels of HIF with the dose employed in this study in humans.

The possible mechanisms underlying cardiorespiratory responses to hydralazine

Knowles *et al.* (2004) suggested that the effects of hydralazine on HIF may explain some of its vascular effects. However, in the present study, vascular and ventilatory effects of hydralazine were both observed, yet there was no change in proteins regulated by the HIF system. Therefore, in healthy humans, it would seem unlikely that the effect of hydralazine on cardiovascular and respiratory responses arises via an effect on the HIF system. This conclusion is further strengthened by the observation of the rapidity with which the cardiorespiratory effects of hydralazine appeared. It is hard to conceive that the substantial effects of hydralazine could have appeared by the PM1 measurement period simply through alterations of gene expression induced through the HIF system.

Alternative explanations for the effects of hydralazine include: (1) that it can directly inhibit the IP_3-induced release of Ca^{2+} from the sarcoplasmic reticulum in vascular smooth muscle cells (Gurney & Allam, 1995; Ellershaw & Gurney, 2001); (2) that it opens high-conductance Ca^{2+}-activated K^+ channels (Bang *et al.* 1998); and (3) that it interferes with the entry of Ca^{2+} into the cell via effects at the cell surface (McLean *et al.* 1978; Nasu & Yanagimoto, 1996). These potential mechanisms have a common feature in that they result in a lowering of intracellular Ca^{2+} within the smooth muscle cell. However, if hydralazine had a similar effect on the type 1 cells of the carotid body, it would be expected to lower the ventilatory sensitivity to hypoxia rather than to augment the sensitivity as was observed in the present experiments.

Conclusion

We conclude that hydralazine increases the sensitivity of ventilation to hypoxia, between 1 and 12 h after a single oral dose, whilst having minimal effect on pulmonary vascular tone. This effect occurs without obvious alterations in the expression of HIF-related gene products.

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Acknowledgements

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This information is current as of April 17, 2008

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