

The increase in pulmonary arterial pressure caused by hypoxia depends on iron status

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Hypoxia is a major cause of pulmonary hypertension. Gene expression activated by the transcription factor hypoxia-inducible factor (HIF) is central to this process. The oxygen-sensing iron-dependent dioxygenase enzymes that regulate HIF are highly sensitive to varying iron availability. It is unknown whether iron similarly influences the pulmonary vasculature. This human physiology study aimed to determine whether varying iron availability affects pulmonary arterial pressure and the pulmonary vascular response to hypoxia, as predicted biochemically by the role of HIF. In a controlled crossover study, 16 healthy iron-replete volunteers undertook two separate protocols. The 'Iron Protocol' studied the effects of an intravenous infusion of iron on the pulmonary vascular response to 8 h of sustained hypoxia. The 'Desferrioxamine Protocol' examined the effects of an 8 h intravenous infusion of the iron chelator desferrioxamine on the pulmonary circulation. Primary outcome measures were pulmonary artery systolic pressure (PASP) and the PASP response to acute hypoxia (Δ PASP), assessed by Doppler echocardiography. In the Iron Protocol, infusion of iron abolished or greatly reduced both the elevation in baseline PASP ($P < 0.001$) and the enhanced sensitivity of the pulmonary vasculature to acute hypoxia ($P = 0.002$) that are induced by exposure to sustained hypoxia. In the Desferrioxamine Protocol, desferrioxamine significantly elevated both PASP ($P < 0.001$) and Δ PASP ($P = 0.01$). We conclude that iron availability modifies pulmonary arterial pressure and pulmonary vascular responses to hypoxia. Further research should investigate the potential for therapeutic manipulation of iron status in the management of hypoxic pulmonary hypertensive disease.

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Pulmonary hypertensive disorders frequently complicate hypoxic lung disease and worsen patient survival (Barbera *et al.* 2003; Chaouat *et al.* 2005; McLaughlin & McGoon, 2006). For example, up to 90% of patients with advanced chronic obstructive pulmonary disease (COPD) have moderate to severe pulmonary hypertension that worsens survival despite maximal therapy (Oswald-Mammosser *et al.* 1995; Scharf *et al.* 2002; Mal, 2007). Hypoxia-induced pulmonary hypertension is also a major cause of morbidity among high altitude residents, while in non-residents it directly precipitates high altitude pulmonary oedema, the most common cause of death related to high altitude (Hackett & Roach, 2001; Penalzoza & Arias-Stella, 2007).

Hypoxia causes pulmonary hypertension through hypoxic pulmonary vasoconstriction and vascular remodelling (Barbera *et al.* 2003). It has recently become apparent that these processes are regulated at least in part by the hypoxia-inducible factor (HIF) family of transcription factors, which coordinate intracellular responses to hypoxia by directly or indirectly regulating the expression of several hundred genes (Semenza, 2004; Smith *et al.* 2008). In mice, heterozygous deficiency for functional HIF genes markedly inhibits the development of hypoxia-induced pulmonary hypertension and vascular remodelling (Yu *et al.* 1999; Brusselmans *et al.* 2003), and impairs hypoxic responses in the pulmonary arterial myocytes responsible for these processes (Shimoda *et al.* 2001). In patients diagnosed with the rare recessive disease Chuvash polycythaemia, HIF-mediated gene activation is

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pathologically increased and is associated with pulmonary hypertension (Bushuev *et al.* 2006; Smith *et al.* 2006) and a grossly exaggerated pulmonary vasoconstrictive response to hypoxia (Smith *et al.* 2006). Other rare HIF-activating mutations have also been linked to the development of pulmonary hypertension (Gale *et al.* 2008).

HIF is synthesized continuously and is primarily regulated through oxygen-dependent proteosomal degradation. HIF is targeted for proteolysis by hydroxylation of specific residues in the HIF- α subunit (Ivan *et al.* 2001; Jaakkola *et al.* 2001). This crucial oxygen-sensitive step is catalysed by HIF hydroxylase enzymes belonging to the iron- and 2-oxoglutarate-dependent dioxygenase superfamily, and requires iron as an obligate cofactor. In cultured cells HIF degradation is both inhibited by iron chelation with desferrioxamine (DFO) and potentiated by supra-physiological iron supplementation (Wang & Semenza, 1993; Knowles *et al.* 2003), raising the question of what effect variations in iron availability have on HIF-signalling at the systemic level. Previous studies in humans have demonstrated that DFO stimulates production of the HIF-regulated hormone erythropoietin (Ren *et al.* 2000)

and mildly elevates pulmonary arterial pressures (Balanos *et al.* 2002).

The known biochemistry of HIF regulation and its involvement in pulmonary physiology together suggest that clinical iron status may modify the effects of hypoxia on the pulmonary circulation. Such an interaction would have important implications for patients with hypoxia-induced pulmonary hypertension. This study of healthy iron-replete individuals aimed to determine the effects of increasing and decreasing iron availability on the pulmonary vasculature and its response to hypoxia. The primary outcome measures were pulmonary artery systolic pressure (PASP) and the change in PASP stimulated by exposure to acute hypoxia (Δ PASP), assessed by Doppler echocardiography.

Methods

There were two protocols to the study, which are illustrated schematically in Fig. 1. The *Iron Protocol* compared the effects of intravenous iron with those of a control infusion on the subsequent responses to 8 h of sustained hypoxia. This protocol tested the hypothesis that increasing iron availability would attenuate the increases

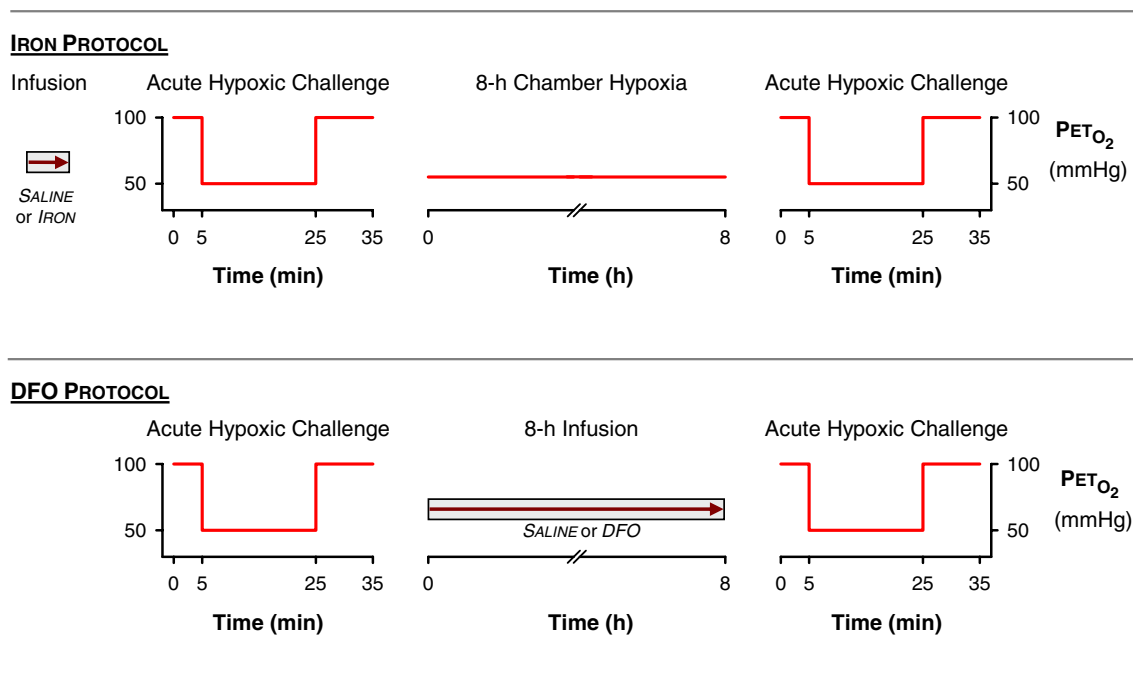


Figure 1. Schematic representation of experimental protocols

Each protocol was conducted over two separate days. For the Iron Protocol, a control infusion of normal saline was administered on the first experimental day and an infusion of iron sucrose was given on the second experimental day. For the DFO Protocol, a control infusion of normal saline was administered on one experimental day and an infusion of desferrioxamine (DFO) was given on the other experimental day. Pulmonary artery pressure was assessed echocardiographically during the acute hypoxic challenges. $P_{ET}O_2$ denotes end-tidal partial pressure of oxygen.

in PASP and Δ PASP that normally accompany sustained exposure to hypoxia. The *DFO Protocol* investigated the effect of reducing iron availability on PASP and Δ PASP, by comparing the effects of an 8 h intravenous infusion of DFO with those of a control infusion. Sixteen healthy volunteers with normal iron status participated in the study (age 25 ± 3 year, mean \pm S.D.; four men and four women in each protocol). Participants were taking no medications or supplements, and provided written informed consent. The Oxfordshire Clinical Research Ethics Committee approved the study, which was conducted in accordance with the *Declaration of Helsinki*.

Iron Protocol

Each participant was studied twice with the studies separated by at least 1 week. The first protocol began with a control infusion of normal saline. Following the infusion, PASP and Δ PASP were measured during an acute hypoxic challenge. Subjects were then exposed to 8 h of sustained isocapnic hypoxia (end-tidal partial pressure of oxygen, P_{ETO_2} , of 55 mmHg) in a purpose built hypoxia chamber (Howard *et al.* 1995). After the 8 h exposure, PASP and Δ PASP were again determined during an acute hypoxic challenge. The second protocol began with an infusion of iron(III)-hydroxide sucrose (200 mg over 105 min; Syner-Med (Pharmaceutical Products) Ltd, Purley, UK) and was otherwise identical to the first protocol.

The order of the saline and iron infusions was not randomised between protocols as the duration of the potential downstream effects of iron administration is unknown. The plasma half-life of iron sucrose is 5–6 h (Danielson *et al.* 1996) and the immediately bioavailable labile iron fraction is approximately 5% (Van Wyck *et al.* 2004).

DFO Protocol

Each participant was studied on two days separated by at least 1 week. Participants received an 8 h infusion of desferrioxamine mesylate (4 g per 70 kg body weight; Ciba-Geigy, Cheshire, UK) on one experimental day and a control infusion of normal saline on the other. The infusion order was randomised. PASP and Δ PASP were measured during an acute hypoxic challenge before and after the 8 h infusions.

Acute hypoxic challenges: measurement of PASP

Plasma erythropoietin concentration was measured immediately prior to each acute hypoxic challenge. The apparatus and techniques used in the acute hypoxic challenges have been extensively validated and described in detail previously (Balanos *et al.* 2005; Smith *et al.*

Table 1. Baseline venous blood analyses

Analysis (normal range)	DFO Protocol	Iron Protocol
Haemoglobin (12–17 g dl ⁻¹)	14.0 \pm 1.0	14.0 \pm 1.5
Haematocrit (0.36–0.50 l l ⁻¹)	0.42 \pm 0.03	0.42 \pm 0.04
Serum iron (11–31 μ mol l ⁻¹)	21.9 \pm 10.1	16.1 \pm 5.6
Serum ferritin (10–300 μ g l ⁻¹)	29.3 \pm 14.6	72.7 \pm 81.6

Mean \pm S.D. values are shown. Where normal ranges vary with sex, the widest range is given.

2006). Challenges were conducted with the subject reclining in the left lateral position and breathing through a mouthpiece. Gas control was achieved by means of dynamic end-tidal forcing (Robbins *et al.* 1982). For each challenge an initial 5 min of euoxia (P_{ETO_2} of 100 mmHg) preceded a period of 20 min of isocapnic hypoxia (P_{ETO_2} 50 mmHg). This was followed by a final 10 min of euoxia. End-tidal partial pressure of carbon dioxide was maintained close to each subject's baseline value. A Sonos 5500 echocardiography machine (2–4 MHz transducer; Hewlett-Packard, Boston, MA, USA) was used to monitor PASP continuously. Using a standard Doppler technique, the maximum systolic pressure gradient across the tricuspid valve was determined and PASP was calculated using the modified Bernoulli equation and an estimated right atrial pressure of 5 mmHg (Swenson *et al.* 2002; Smith *et al.* 2006). Heart rate and ventilation were recorded, and cardiac output was determined echocardiographically every 2 min (Howson *et al.* 1986; Balanos *et al.* 2005).

Statistical analyses

Differences between experiment days were assessed statistically using Student's paired *t* test (Microsoft Excel), and were considered significant at the $P < 0.05$ level. All values are expressed as means \pm S.D. unless otherwise stated.

Results

All subjects were initially iron-replete (see Table 1). All infusions were well tolerated. Neither iron nor DFO significantly affected ventilation, heart rate or cardiac output, either during euoxia or in response to hypoxia.

Iron Protocol

Figure 2 shows that, of itself, infusion with iron did not affect PASP or Δ PASP. However, pretreatment with iron prevented the rise in PASP normally induced by 8 h of sustained hypoxia (increase in PASP of

23% \pm 13% with saline control *versus* 2% \pm 3% with iron, $P < 0.001$). Iron also substantially attenuated the increase in Δ PASP normally induced by sustained hypoxia (increase of 124% \pm 77% with saline control *versus* increase of 59% \pm 67% with iron, $P = 0.002$). The initial plasma erythropoietin concentration measured prior to the infusions was not significantly different on the two experimental days (13.2 \pm 1.4 mIU ml⁻¹ prior to control infusion and 13.3 \pm 0.4 mIU ml⁻¹ prior to iron infusion; $P = 0.91$). The elevation in plasma erythropoietin concentration generated by hypoxia was not significantly affected by iron (increase of 67% \pm 35% with saline control *versus* increase of 61% \pm 40% with iron, $P = 0.79$).

DFO Protocol

Figure 3 shows that an 8 h infusion of DFO significantly increased basal PASP (increase of 0% \pm 3% with saline control *versus* increase of 10% \pm 4% with DFO, $P < 0.001$). DFO also increased pulmonary vascular reactivity to acute hypoxia (1% \pm 12% reduction in Δ PASP with saline control *versus* 36% \pm 26% increase with DFO, $P = 0.01$). The initial plasma erythropoietin concentration measured prior to the infusions was not significantly different on the two experimental days (10.4 \pm 5.8 mIU ml⁻¹ prior to control infusion and 8.3 \pm 4.2 mIU ml⁻¹ prior to DFO infusion; $P = 0.14$). DFO generated a significant rise in plasma erythropoietin

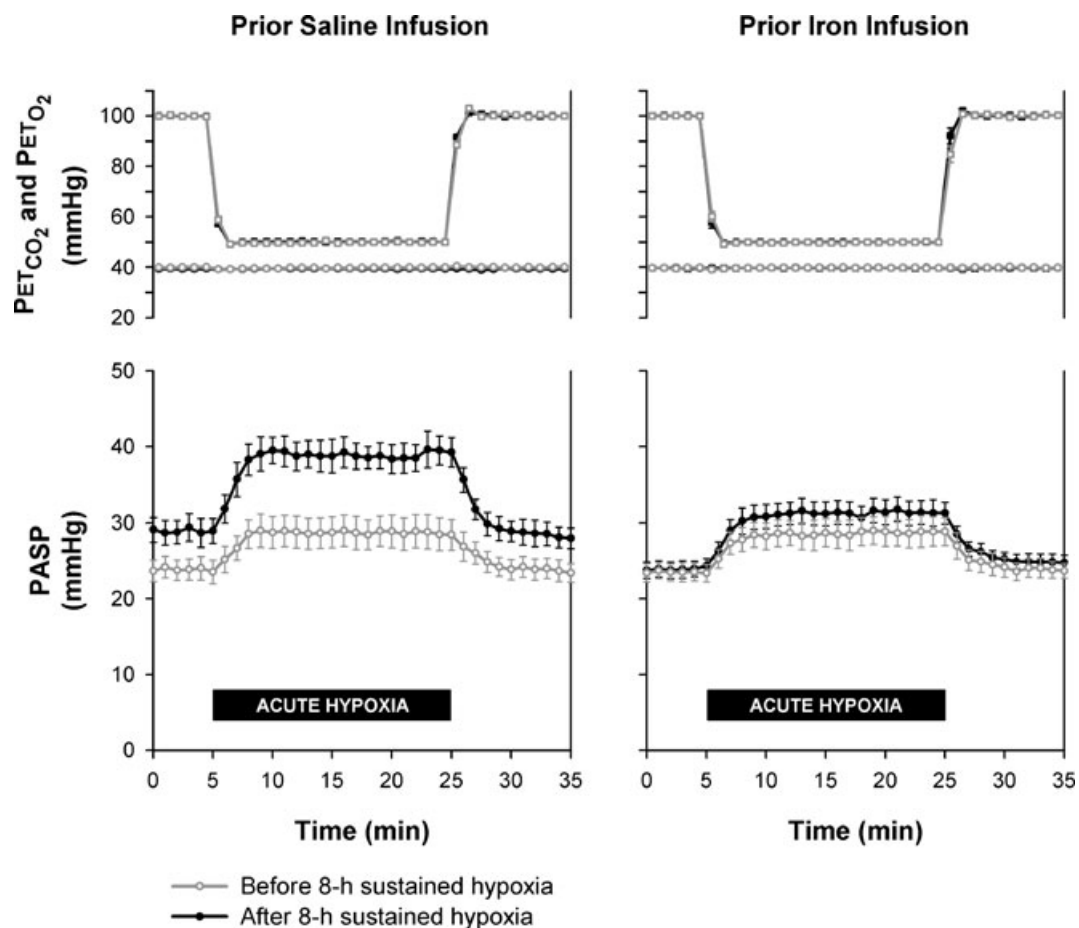


Figure 2. Effects of 8 h of sustained hypoxia on pulmonary artery systolic pressure with and without prior infusion of iron

Upper panels show end-tidal partial pressures of oxygen (P_{ETO_2}) and carbon dioxide (P_{ETCO_2}) during acute hypoxic challenges. Lower panels show corresponding measurements of pulmonary artery systolic pressure (PASP). Values are means and error bars indicate s.e.m. The control measurements demonstrate the normal pulmonary vascular response to sustained hypoxia, consisting of an increase in PASP and a sensitized PASP response to subsequent acute hypoxia (Δ PASP). Intravenous infusion of iron prior to the 8 h hypoxic exposure prevented the increase in baseline PASP ($P < 0.001$) and markedly attenuated the degree to which Δ PASP was sensitized ($P = 0.002$).

(control increase of $2\% \pm 65\%$ versus increase of $794\% \pm 566\%$ with DFO, $P = 0.03$).

Discussion

This study establishes the existence of a novel and substantial interaction between iron, hypoxia and the pulmonary circulation. We are not aware of any previous work describing such a link in animals or humans.

The results from the Iron Protocol demonstrate that, in iron-replete individuals, administration of iron abolishes the elevation in pulmonary arterial pressure that is normally induced following an 8 h exposure to hypoxia, and markedly inhibits the accompanying increase in acute hypoxic pulmonary vasoreactivity. This introduces the potential for benefit from iron therapy in disorders caused by pulmonary vasoconstrictive responses to hypoxia, such as COPD-related pulmonary hypertension and high altitude pulmonary oedema (HAPE).

The results from the DFO Protocol demonstrate that, for a given level of hypoxia, acute reduction of iron availability increases pulmonary arterial pressure. This raises the possibility that clinical iron deficiency might exacerbate hypoxically induced pulmonary hypertension, for example in cor pulmonale. Indeed, this might explain why the initial pulmonary antihypertensive benefits of venesection for secondary polycythaemia tend not to be sustained with continued venesection (Lewis *et al.* 1952; Hecht *et al.* 1955; Rakita *et al.* 1965; Segel & Bishop, 1966; Weisse *et al.* 1975).

Overall this study demonstrates the importance of iron in pulmonary vascular responses to hypoxia and suggests that even modest alterations in iron balance might be clinically significant in this respect. Although prolonged administration might eventually be limited by iron overload, it is nonetheless remarkable for a pharmacological agent as familiar, safe and inexpensive as iron to offer promise for an entirely new indication,

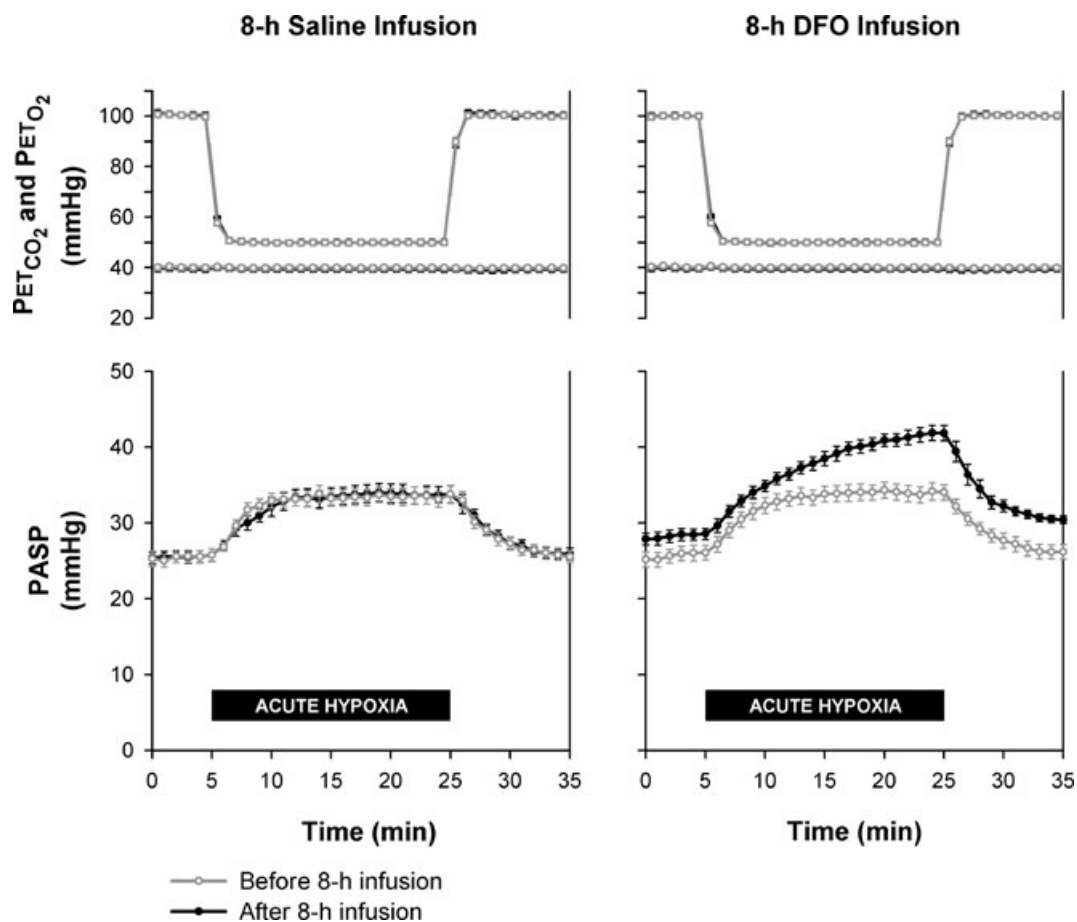


Figure 3. Effects of an 8 h infusion of desferrioxamine on pulmonary artery systolic pressure

Upper panels show end-tidal partial pressures of oxygen (P_{ETO_2}) and carbon dioxide (P_{ETCO_2}) during acute hypoxic challenges. Lower panels show corresponding measurements of pulmonary artery systolic pressure (PASP). DFO denotes desferrioxamine. Values are means and error bars indicate s.e.m. Infusion of DFO increased PASP ($P < 0.001$) and sensitized the pulmonary vascular response to acute hypoxia ($P = 0.01$).

and further studies are warranted to determine whether careful adjustment of iron balance has any place in the management of hypoxic pulmonary hypertensive disease. Perhaps most immediately, potential protective effects of iron loading should be investigated for HAPE, which afflicts non-acclimatized individuals ascending rapidly to high altitude. HAPE is associated with an excessive hypoxic pulmonary vasoconstrictive response that is characteristic of HAPE-susceptible individuals (Hackett & Roach, 2001) and that might be attenuated by parenteral iron, as was observed in this study.

Our hypothesis – that alterations in iron availability affect the pulmonary vascular response to hypoxia – was predicated on the known effects of iron manipulation on HIF hydroxylation, and on evidence implicating HIF in pulmonary vascular responses *in vivo*. The positive results we obtained would therefore imply that iron availability indeed modifies HIF hydroxylation in the pulmonary circulation, which appears to be limited by normal physiological levels of iron. This is difficult to prove formally as there is no means of directly quantifying HIF in the human lung, and peripheral concentrations of HIF-regulated gene products such as erythropoietin do not necessarily reflect HIF activity in the pulmonary vasculature. However, it is of interest that iron chelation and iron supplementation emulated the pulmonary vascular manifestations of up- and down-regulation of HIF that are seen in Chuvash polycythaemia (Smith *et al.* 2006) and HIF^{+/-} mice (Yu *et al.* 1999; Brusselmans *et al.* 2003), respectively.

An alternative explanation for our findings is that the responses were due to some novel iron-dependent oxygen sensor, possibly another member of the iron-dependent dioxygenase enzyme family. Up to 40 such enzymes may sense changes in iron and oxygen availability and might mediate HIF-independent responses to these stimuli (Elkins *et al.* 2003; Elvidge *et al.* 2006; Ratcliffe, 2006; Ozer & Bruick, 2007). A further theoretical explanation is that iron availability influenced pulmonary vascular tone through the formation of oxygen free radicals. However, the precise role of reactive oxygen species in hypoxic pulmonary vasoconstriction is controversial (Aaronson *et al.* 2006). Furthermore, it is likely that any such hypothetical effect would be rapid, yet PASP and Δ PASP were normal when measured shortly after the iron infusion. Nevertheless, involvement of reactive oxygen species cannot be excluded as the time course of the observed effects could reflect, for example, that of iron uptake into pulmonary arterial myocytes.

We conclude that iron availability modifies pulmonary arterial pressures and pulmonary vasoconstrictive responses to acute and sustained hypoxia, and suggest that this interaction may derive from respective inhibition and potentiation of HIF hydroxylation in the lung. Until further evidence is forthcoming it may be prudent to avoid iron deficiency in hypoxic patients with end-stage

pulmonary hypertension, and in HAPE-susceptible individuals returning to high altitude. Further research should investigate the link between iron status and pulmonary arterial pressure, and explore the therapeutic potential of manipulating iron and HIF in hypoxic pulmonary hypertensive disease.

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