Iron homeostasis and pulmonary hypertension: iron deficiency leads to pulmonary vascular remodelling in the rat

Cotroneo et al Iron deficiency and pulmonary hypertension
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Word count: 6926


Key words: pulmonary vascular remodelling, iron deficiency, glycolysis, HIF, STAT3
Abstract

**Rational:** Iron deficiency without anaemia is prevalent in patients with idiopathic pulmonary arterial hypertension and associated with reduced exercise capacity and survival.

**Objectives:** We hypothesised that iron deficiency is involved in the pathogenesis of pulmonary hypertension and iron replacement is a possible therapeutic strategy.

**Methods:** Rats were fed an iron-deficient diet (IDD, 7mg/kg) and investigated over 4 weeks.

**Measurements and main results:** Iron deficiency was evident from depleted iron stores (decreased liver, serum iron and ferritin), reduced erythropoiesis and significantly decreased transferrin saturation and lung iron stores after 2 weeks IDD. IDD rats exhibited profound pulmonary vascular remodelling with prominent muscularization, medial hypertrophy and perivascular inflammatory cell infiltration, associated with raised pulmonary artery pressure and right ventricular hypertrophy. IDD rat lungs demonstrated increased expression of HIF1α and HIF2α, NFAT and survivin, and STAT3 activation, which promote vascular cell proliferation and resistance to apoptosis. Biochemical examination showed reduced mitochondrial complex I activity and mitochondrial membrane hyperpolarisation in mitochondria from IDD rat pulmonary arteries. Along with upregulation of the glucose transporter, GLUT1, and glycolytic genes, hkl and pdk1, lung \(^{18}\)FDG uptake was significantly increased in IDD rats. The hemodynamic and pulmonary vascular remodelling were reversed by iron replacement (ferric carboxymaltose, 75mg/Kg) and attenuated in the presence of iron deficiency by dichloroacetate and imatinib, two putative treatments explored for PAH that target aerobic glycolysis and proliferation, respectively.

**Conclusions:** These data suggest a major role for iron in pulmonary vascular homeostasis and support the clinical evaluation of iron replacement in patients with pulmonary hypertension.
**Introduction**

There is considerable interest in the role of iron homeostasis in pulmonary hypertension. Iron supplementation has been reported to ameliorate, and iron chelation to augment, the rise in pulmonary artery pressure seen in healthy volunteers on exposure to altitude\(^1\), \(^2\). Several independent clinical centres have observed that iron deficiency without anaemia is common in patients with idiopathic pulmonary arterial hypertension (IPAH), and associated with reduced exercise capacity and survival\(^3\), \(^4\), \(^5\), \(^6\). These data suggest that iron supplementation may be beneficial in patients with IPAH, and clinical studies are in progress to evaluate this hypothesis\(^7\).

Counter to this argument, studies in rats suggest that iron deficiency may protect against the development of pulmonary hypertension\(^8\). Both contemporaneous treatment with deferoxamine and dietary iron restriction attenuate the development of pulmonary hypertension in rats exposed to chronic hypoxia or monocrotaline, respectively\(^9\), \(^10\).

We set out to understand the effect of iron deficiency in the absence of the confounding effects of another pulmonary vascular insult. Specifically, we examined the impact of iron deficiency on pulmonary haemodynamics, pulmonary vascular structure and cardiac hypertrophy over a 4-week time course. After observing striking remodelling of the pulmonary vasculature, we explored whether this may be driven by perturbation of mitochondrial biochemistry and a shift in metabolic phenotype. We then investigated reversibility of the pulmonary vascular pathology by iron replacement and probed the potential mechanisms using targeted therapeutic interventions, the tyrosine kinase inhibitor, imatinib\(^11\) and the metabolic modulator dichloroacetate (DCA)\(^12\).

**Methods**

**Animals and experimental design**

Adult male Sprague-Dawley rats (body weight 200-250g; Charles River, UK) were used. All experiments were conducted in accordance with the UK Home Office regulations. Experiments were designed as follows:
a) Iron deficient rat model - time course study: rats were divided into 4 groups (n≥6) and fed with either a standard diet (CTR) or an iron-deficient diet (IDD, 7mg iron/kg; TD.80396, Harlan, UK) for 2 (IDD 2WK), 3 (IDD 3WK) and 4 weeks (IDD 4WK).

b) Iron supplement treatment study: rats were divided into 3 groups (n=6) and fed with (i) a standard diet (CTR), (ii) IDD for 4 weeks (IDD 4WK), or (iii) IDD for 4 weeks plus ferric carboxymaltose treatment (IDD 4WK+Fe) (Ferinject®, iv. 75mg/Kg; twice a week for two weeks, Vifor Pharma) started after two weeks of IDD.

c) DCA and imatinib treatment study: rats were divided into 3 groups (n=6) and fed with (i) IDD for 4 weeks (IDD 4WK), (ii) IDD for 4 weeks with DCA treatment (drinking water, 70mg/kg/day, sigma) (IDD 4WK+ DCA) and (iii) IDD with imatinib treatment (oral gavage, 100mg/kg/day, LC laboratory) (IDD 4WK+Imatinib). Treatments were initiated after two weeks of IDD and maintained for the remaining two weeks while the rats remained on the IDD.

d) 18FDG PET imaging study: Positron emission tomography with fluorine-18–labeled 2-fluoro-2-deoxyglucose ligand (18FDG-PET) was performed in rats fed with either a standard diet (n=5) or an IDD (n=4) for 4 weeks.

**Haemodynamic measurements and tissue collection**

Rats were anaesthetised (Hypnorn 1ml/Kg i.m., Midazolam 0.8 ml/Kg i.p). Right atrial pressure (RAP), right ventricle systolic pressure (RVSP) and pulmonary arterial pressure (PAP) were measured with a pre-curved catheter inserted through the right jugular vein. Systemic blood pressure (SBP), left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVDP) were assessed via carotid artery cannulation. Cardiac output (CO) was measured by thermodilution13 (Supplementary data). Pulmonary vascular resistance (PVR) was calculated using the standard equation: (mean PAP – LVDP) / CO. Similarly, systemic vascular resistance (SVR) was calculated as (mean SBP – RAP)/ CO. All haemodynamic data were recorded and analysed with a PowerLab Data Acquisition system (AD Instruments).
At the end of procedure blood was collected to obtain serum. Tissues (lung, heart, liver, skeleton muscle) were flushed with saline to clear blood and snap-frozen in liquid nitrogen, and stored at -80 °C. Hearts were dissected and weighted; the ratio of right ventricle (RV) over left ventricle (LV) plus the septum was used as an index of RV hypertrophy (RVH). The left lung was fixed in 10% formalin and paraffin-embedded for histological examination.

**In vivo** $^{18}$FDG-PET

In vivo $^{18}$FDG-PET was performed using a Siemens Inveon small animal multimodality PET/CT system (Siemens Healthcare Molecular Imaging). A protocol of 60mins dynamic scanning with kinetic analysis established in our lab was employed\(^\text{14}\). The rate of glycolysis was calculated as follows: Rate of glycolysis = [Glucose]*$\dot{K}_i$ ( [Glucose], plasma glucose concentration; $K_i$, FDG influx rate). We assumed the relative glycolytic flux of FDG and glucose (lumped constant) remain unchanged between groups\(^\text{14, 15}\).

**Iron markers**

Hemoglobin levels (Hb) were measured by HEMOCUE reader (Sweden) and haematocrit (Hct) using a microhaemotocrite device after centrifugation and blood glucose level using a Aviva Accu-Check monitor. Circulating iron and unsaturated iron binding capacity (UIBC) were measured in plasma (Abbott Diagnostics, Ireland). Transferrin saturation (Tsat) was calculated from the iron and total iron binding capacity (TIBC). Ferritin was measured using ELISA (Alpco Diagnostics, USA). For tissue iron measurements, 0.1g of tissue was digested in 70% nitric acid, with total iron assessed by means of Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS).

**Histology, immunohistochemistry and immunofluorescence**

Lung histology was examined by elastic Van Gieson (EVG) and haematoxylin and eosin (H&E) staining. Peripheral vessels $<$50 µm diameter were counted at x40 magnification and pulmonary vascular remodelling was expressed as the proportion of vessels with double elastic lamina ($>$75% of the circumference) to total vessels counted.
For immunohistochemistry examination, lung sections were stained for smooth muscle actin (SMA, 1/200; Sigma), or Von Willebrand factor (VWF, 1/100; Dako), or CD68 (1/200, Serotec), or β-catenin (1/100, Abcam), or nuclear factor of activated T-cells 1 (NFAT-1, 1/50, Novus Biological), with appropriate horseradish peroxidase conjugated secondary antibodies (1/200). We counted macrophages (CD 68+) as previously described.  

For double immunofluorescence, sections were incubated with GLUT1 (1/50, Abcam), carbonic anhydrase (CAIX, 1/100, Novus Biological) and SMA (1/200, Sigma), detected with secondary antibodies, Alexa 488 anti-mouse and Alexa 568 anti-rabbit (1/1000; Invitrogen), with mounting solution containing DAPI. Images were obtained with a Leica laser confocal microscope (TCS SP2 AOBs).

**Western blotting**

Protein samples (25 µg), extracted from lung homogenates, were run on a precast gel (Invitrogen) and transferred to a nitrocellulose membrane. Western blotting was performed as per manufacture’s suggestions (rabbit anti-survivin antibody 1/1000, ProteinTech; rabbit anti HIF2α 1/1000, mouse anti HIF1α 1/1000, mouse anti NFAT-1, 1/1000, Novus Biological; mouse anti Bcl-2 1/1000, BD Bioscience; anti STAT3 1/2000, anti phospho-STAT3 1/2000, Abcam). Proteins were detected by Novex enhanced chemiluminescent (ECL) kit (Invitrogen, Paisley, UK). Optical densities of individual bands were measured and protein expression were standardised with β actin.

**Real Time-PCR analysis**

Total RNA was extracted by RNeasy Mini Kit (Quiagen) and reversed transcribed with M-MLV Reverse Transcriptase (Promega). Real Time PCR for GLUT1, pyruvate dehydrogenase kinase 1 (pdk1) and hexokinase 1 (hk1) was performed using pre-designed, gene-specific TaqMan primer and probe sets (Applied Biosystems). Gene expression data were normalized with the housekeeping ubc1 expression and analysis was carried out using the relative expression software tool (REST).
Mitochondrial isolation and measurement of complex I activity

Mitochondria were isolated from control or IDD rat pulmonary arteries (PA, 2nd to 6th division). Briefly, freshly dissected tissues were homogenized in ice-cold isolation buffer (250 mM sucrose, 20 mM HEPES, pH 7.2, 1 mM EDTA, and 0.5% BSA) and centrifuged at 2500x g to pellet nuclear material. The supernatant was centrifuged at high-speed (9600 x g for 15 min at 4°C); the pellet was washed twice in isolation buffer by centrifugation at 9600x g. Mitochondria were frozen and stored at -80°C until use. Mitochondrial complex I activity was measured using an immunocapture enzyme activity assay according to manufacturer’s instructions (Abcam).

Mitochondrial inner membranes were also isolated from mitochondria from control and IDD rat pulmonary arteries (n=3) and purified by centrifugation. Patch clamp recordings were performed to measure the mitochondrial inner membrane peak conductance levels (Supplemental material).

Cell culture

Human pulmonary artery smooth muscle cells (PASMCs) were serum-deprived and then stimulated with 50ng/ml platelet-derived growth factor (PDGF) with or without iron chelator deferoxamine (DFO, 200µM, Sigma) for 48 hours. Final cell count or WST-1 based colorimetric assay was performed (Roche).

A separate set of PASMCs were cultured with or without 200µM DFO for 24 hours and then incubated with fluorescence probes for 30 minutes at 37°C in the dark. Mitochondrial membrane potential was determined using 20nM tetramethylrhodamine (TMRE; Life technologies). Reactive oxygen species (ROS) production was detected using 5µM CellROX Green (Life technologies). Hoechst (Life technologies) was used for nucleus staining. Images were obtained with a Zeiss Axio microscope.

Statistics
Data are presented as mean ± SEM. Normal distribution was verified employing the Kolmogorov-Smirnov test and variance of homogeneity was tested by the Levene test. Differences between groups were assessed by either Student t-test or appropriate ANOVA followed by Bonferroni post hoc test for multiple group comparisons. A value of p<0.05 was considered statistically significant. All statistical analyses were performed with Prism 5 (GraphPad Software, Inc.).

Results

Establishment of iron deficient rat model

Rats were maintained on an IDD for up to 4 weeks and iron status evaluated (Table 1; Online Figure Ia). Both stored and circulating iron levels were reduced following 2 weeks IDD, as reflected in the significantly decreased liver (<30% of control) and serum (<20% of control) iron levels, blood ferritin and Tsat, while UIBC increased (p<0.05). This was accompanied by reduced Hb (109g/L versus 147 g/L) and Hct (38% versus 48%) in IDD rats, maintained through to 4-weeks. Lung and skeletal muscle tissue iron levels were significantly reduced over the 4 weeks (69% and 63% of control, respectively), while RV and LV tissue iron levels were reduced to 78% and 90% of control level.

Iron deficient rat develops pulmonary hypertension

IDD rats developed progressive pulmonary hypertension over the 4-week time course. Mean PAP and RVSP were elevated significantly by 3 weeks in IDD rats compared with controls, but SBP was not affected ((Figure 1a & b; Online Figure Ib). RVH was evident at 4 weeks in IDD rats (Figure 1c). LV and total heart weight (corrected for body weight) were also increased in the 2-week iron deficient rat (Online Figure 1c & d), as previously reported17. There was a trend of increasing in LV systolic pressure (Online Figure 1e). Cardiac output was significantly increased at 4 weeks compared to the control group (135%, P<0.05) (Figure 1d, Online Figure If). PVR was significantly increased in 4-week IDD rats, but not SVR (Figure 1e & f).

Histological examination of lung sections revealed profound pulmonary vascular remodelling in iron deficient rats (Figure 2, Online Figure IIA). SMA staining demonstrated tunica media hypertrophy in
vessels with diameter <50 µm or 50 to 100µm (Figure 2a & b, Online Figure IIa), with an increased proportion of peripheral vessels (<50µm) demonstrating muscularization (Figure 2c). These structural changes were accompanied by a reduction in lumen area, protruding endothelial cells and concertinered appearance to the inner elastic lamina (Figure 2a & b). Prominent infiltration of macrophages (CD68+) with a preferential accumulation surrounding the peripheral remodelled vessels was also evident in iron deficient rats (Figure 2d & e, Online Figure IIb).

**Increased HIF1α and HIF2α expressions, and STAT3 activation in the iron deficient rat lung**

To better understand the molecular drivers of pulmonary vascular remodelling, we first investigated HIF1α and HIF2α levels. Iron is required for the optimal activity of prolyl hydroxylases (PHDs), which act to degrade hypoxia inducible factors. Western blots show an increase in both HIF1α and HIF2α levels in the iron deficient rat lung (Figure 3a). Consistent with HIF1α stabilisation under iron deficient conditions, expression of CAIX, a downstream response of increased HIF1α, was increased in the remodelled vasculature (Figure 3b, Online Figure IIIa).

Following up on the finding of inflammatory cells in IDD lungs, and reports that iron deficiency can cause a proinflammatory state, we also measured levels of signal transducers and activators of transcription-3 (STAT3). This cytoplasmic latent transcription factor is activated by phosphorylation in response to cytokines and recently proposed to act a signalling hub in PAH. Levels of phosphorylated STAT3 were increased in IDD rat lungs (Figure 3 c-e), indicating STAT3 activation. The nuclear factor of activated T cells, NFAT, increases the transcription of multiple inflammatory mediators such as interleukins and tumor necrosis factor, and contributes directly to recruitment of inflammatory cells in remodeled vessels. Increased NFAT expression was evident in the IDD rat lung with increased expression in remodelled vessels (Figure 3 f, g).

Both STAT3 and NFAT activation have been associated with altered/suppressed mitochondrial function, potentiating a hyperproliferative and antiapoptotic cell phenotype, and in keeping with
this, we observed a 3-fold increase in the antiapoptotic regulator Bcl2 in the IDD rat lung (Online Figure IIIb).

**Iron deficiency induces alteration of mitochondrial function in vitro and in vivo**

To investigate further mitochondrial activity in the presence of reduced iron, we first measured the direct effects of iron deficiency on mitochondrial function in human PASMCs in culture. A global index of mitochondrial function is the mitochondrial membrane potential. We observed hyperpolarization of the mitochondrial membrane potential ($\Delta \Psi_m$) as measured by the positively charged dye TMRE (Figure 4a) and inhibition of reactive oxygen species (mROS) generation (Figure 4b). Accompanying this, iron chelation increased metabolic activity and cell proliferation (Figure 4c, d).

Iron is a critical substrate for mitochondria and iron-sulfur clusters (Fe-S) exclusively formed in mitochondria are essential components of electron transport chain complexes and enzymes like succinate dehydrogenase and aconitase$^{24}$, impacting on mitochondrial function directly. We measured mitochondrial complex I activity, the first enzyme in the mitochondrial respiratory chain, by immunocapture of NAD+ in mitochondria extracted from pulmonary arteries. In IDD rats ($n=4$), complex I activity was reduced compared to control animals (Figure 4 e & f). Patch clamp measurements on mitochondrial inner membrane vesicles using a recently modified technique$^{25, 26}$ showed a drop in ion channel peak conductance levels in IDD rats ($51.2 \pm 10.5$, $n=5$) compared to controls ($109.2 \pm 10.5$, $n=5$), consistent with altered mitochondrial function (Online Figure IVa, b).

**Increased glucose uptake in the iron deficient rat lung**

We reasoned that perturbation of mitochondrial function would result in a glycolytic phenotype, detectable using $^{18}$FDG PET. Dynamic PET acquisition with kinetic analysis$^{14}$ demonstrated a significant increase in lung $^{18}$FDG uptake in 4 week IDD animals compared to controls (Figure 5a, b & c). The calculated glycolysis rate ($[\text{glucose}] \times K_i$) was also significantly elevated in IDD rat lungs (Figure 5d). Blood glucose levels in non-fasting 4 week IDD rats were significantly increased
compared to controls (IDD 4WK 10.5 ± 2.1; CTR 8.3 ± 0.11; mmol/L, p<0.05) (Figure 5e), as previously observed\textsuperscript{20, 21}.

Consistent with increased \textsuperscript{18}FDG uptake, the remodelled pulmonary vessels demonstrated prominent GLUT1 expression by immunofluorescence (Figure 5f). Real time PCR analysis of IDD rat lungs showed increased expression of glut1 (>2 fold) along with hkl and pdk1 (3 and 2 fold respectively) (Figure 5g).

**Iron supplementation treatment attenuates pulmonary hypertension in iron deficient rat**

To demonstrate reversibility of the pulmonary vascular phenotype, we administered intravenous iron (Ferinject) to 2 week IDD rats\textsuperscript{7}. Iron supplementation for 2 weeks elevated circulating iron indices and tissue iron levels and restored erythropoiesis in IDD rats to control levels (Table 1).

Iron treatment reduced PAP (22 ± 1.6 vs 27 ± 0.6, mmHg, p<0.05), normalised RV mass (Figure 6a, b), and reduced pulmonary vascular muscularization (Figure 6c, Figure 2a & b) and perivascular macrophage accumulation (Figure 6e, f & g; Online Figure IIa) compared to 4 week IDD rats. Blood glucose concentration was also reduced to control levels (7.8 mmol/L ± 0.7) (Figure 6f).

**DCA and imatinib attenuate pulmonary hypertension in iron deficient rat**

To examine the role of metabolic dysfunction in the development of pulmonary hypertension in IDD rats, rats with established iron deficiency were treated with DCA, an inhibitor of pyruvate dehydrogenase kinase (PDK)\textsuperscript{11, 12} for 2 weeks while remaining on the IDD. Inhibition of PDK facilitates mitochondrial oxidative phosphorylation and has been reported to attenuate pulmonary hypertension in a number of experimental models. DCA attenuated the rise in PAP (Figure 7a) and the associated RV hypertrophy (Figure 7b) and pulmonary vascular remodelling (Figure 7c; Online Figure IIa, b) seen in iron deficiency. Cardiac output was also decreased to control levels (Figure 7d).
An alternative strategy for probing the metabolic phenotype in our model is to treat with imatinib, a tyrosine kinase inhibitor that downregulates GLUT1. Two weeks treatment with imatinib also reduced PAP and remodelling in the presence of persistent iron deficiency (Figure 7a-d).

**Increased oncogenic protein survivin expression in iron deficient rat lung**

Survivin, an inhibitor of apoptosis, is expressed in established human and experimental PAH and has been etiologically associated with the development of PAH27. Survivin expression was increased in the IDD rat lung and tracked pulmonary remodelling severity. Both DCA and imatinib reduced survivin expression in the lung, consistent in other animal models28, 29 (Figure 7e & f). Survivin has been identified as a target gene for Wnt/β-catenin signalling30. β-catenin was observed to be expressed mainly in the vascular cell membrane in control rat lungs, but translocated to the nucleus of vascular cells (e.g. endothelial cell and SMC), indicating Wnt pathway activation in IDD rat lungs (Figure 7g).

**Discussion**

We report for the first time profound vascular remodelling of pulmonary arterioles as a consequence of chronic iron deficiency in rats. The pulmonary vascular changes were associated with raised PAP, PVR and RV hypertrophy and accompanied by activation of HIF, STAT3, and mitochondrial dysfunction associated with a glycolytic phenotype. Both the hemodynamic and pulmonary vascular changes were reversed by iron replacement. Significantly, pulmonary remodelling was attenuated in the presence of persistent iron deficiency by DCA and imatinib11, 12, two putative investigational treatments for PAH that target aerobic glycolysis and cell proliferation, respectively.

Iron deficiency was achieved by restricting dietary iron to 7 mg/kg per day, a regimen employed by other investigators31-33. By two weeks, liver iron stores and serum iron and ferritin were substantially reduced (<30% of control level) and remained low. Hb and Hct were also reduced but to a lesser degree (25%). Hb remained >100g/L for the duration of the experiment, above the 70-90g/L range regarded as moderate anaemia in the rat2. Lung tissue iron levels were significantly reduced in IDD rats (69% of control), while RV iron levels were reduced to 78% of control level.
The pulmonary vascular remodelling observed in the IDD rats is striking and appears more severe than described in most reports of monocrotaline and hypoxia-induced pulmonary hypertension. There are notable similarities with human PAH\textsuperscript{34-38}, specifically medial hypertrophy coupled with an abnormal intimal endothelium and perivascular inflammatory cell infiltration. In contrast to the pulmonary hypertension phenotype, systemic pressure and systemic vascular resistance were not affected in IDD rats. That said, cardiac output was elevated in 2 week and 4 week IDD rats (112\% and 135\% of control respectively), most likely in response to the reduced Hct and Hb from iron deficiency, and increased shear stress from increased pulmonary blood flow may contribute to the pulmonary vascular changes observed.

Support for a direct effect of iron deficiency on pulmonary vascular remodelling comes from the effect of iron chelation on PASMC proliferation in culture. In vivo, vascular remodelling was accompanied by increased expression of HIF1\(\alpha\) and HIF2\(\alpha\) protein, and downstream targets, CAIX and GLUT1, biochemical signatures of iron deficiency-induced “tissue hypoxia”\textsuperscript{39} and integral to the pulmonary vascular remodelling observed in human IPAH and several experimental models of pulmonary hypertension\textsuperscript{40-42}.

Inflammation is also recognised as a mediator of pulmonary vascular remodelling in many presentations of pulmonary hypertension and may participate in that associated with iron deficiency. The relationship between inflammation and iron deficiency is complex but there is evidence that the low hepcidin levels associated with iron deficiency can cause a proinflammatory state\textsuperscript{43}. The presence of macrophages in the IDD rat lung is relevant here, as is the activation of STAT3 and NFAT\textsuperscript{22, 23}; both integrate signalling from cytokines but also growth and angiogenic factors and effect actions through a number of downstream targets, including Bcl2 and survivin.

Collectively, the activated HIF, STAT3 and NFAT signalling would be expected to support a proproliferative and anti-apoptotic state. An important manifestation of this cellular phenotype is
mitochondrial dysfunction, associated with a metabolic shift to glycolysis, referred to as the “Warburg effect” in oncology and postulated to play a central role in the pathogenesis of PAH\textsuperscript{12, 14, 24}. We found that iron chelation induces hyperpolarization of the mitochondrial membrane potential and the inhibition of ROS generation in PASMCs in culture. There is also clear evidence for mitochondrial dysfunction \textit{in vivo}. Mitochondria from IDD rat PAs demonstrated decreased mitochondrial complex I activity along with reduced baseline inner membrane conductance levels in comparison to control specimens. Consistent with increased expression of \textit{glut1} and the glycolysis enzymes, \textit{hk1} and \textit{pdk1}, in IDD rat lungs, parenchymal \textsuperscript{18}FDG uptake was increased, along with glycolysis as measured by dynamic PET scanning.

The pulmonary hypertension phenotype induced by iron deficiency is reversible with iron supplementation. We chose intravenous ferric carboxymaltose as it has previously been used in heart failure\textsuperscript{44, 45} and is currently under study in a clinical trial in PAH\textsuperscript{7}. Of greater interest, the elevated PAP and vascular remodelling were attenuated by treatment with the metabolic modulator, DCA\textsuperscript{12}, and the anti-proliferative tyrosine kinase inhibitor, imatinib\textsuperscript{11}, in the presence of persistent iron deficiency. DCA acts by inhibition of PDK to restore oxidative phosphorylation\textsuperscript{46} and so reverses the metabolic shift induced by iron deficiency. Imatinib, through inhibition of PDGF, influences glucose metabolism by translocalization of GLUT1 transporters from the cell membrane into the cytosol and by inhibiting glycolysis and promoting mitochondrial oxidative glucose utilization\textsuperscript{47}.

Our observations that iron deficiency leads to pulmonary hypertension is congruent with studies in humans that suggest iron deficiency augments the pressure response to hypoxia at altitude\textsuperscript{3, 4} and is associated with a poor prognosis in PAH\textsuperscript{3-6}. It is at odds with two studies in rats, both of which suggest iron deficiency is protective against hypoxia-induced and monocrotaline-induced pulmonary hypertension\textsuperscript{9, 10}. However, both these studies have confounding factors. Polycythemia is an important contributor to hypoxia-induced pulmonary hypertension; the lower haemoglobin associated with iron deficiency in that model would be expected to be beneficial, as indeed it is in Tibetans acclimatised to high altitude\textsuperscript{48, 49}. In the case of monocrotaline, inflammation plays a major role in the genesis of the
vascular lesion and iron deficiency can alter the inflammatory response. Examination of the effects of iron deficiency in the absence of hypoxia or monocrotaline provide a more potent approach to unmask the effects of tissue iron depletion alone.

An important observation is that the lung, like the liver, is susceptible to iron depletion during dietary iron deficiency, more so than the myocardium. Iron deficiency is important in heart failure and has received considerable attention in recent years\textsuperscript{50}. Iron supplementation has been shown to improve well-being and exercise capacity in patients with left heart failure\textsuperscript{44, 45}. It remains to be seen whether it improves survival. In this study we did not study cardiac function in detail. Both the LV and RV showed evidence of hypertrophy in the iron deficient rat. But in keeping with pulmonary hypertension, the RVH index (RV/LV+sep) was increased and responded to iron replacement, as well as treatment with DCA and imatinib.

In summary, we have shown that chronic iron deficiency in the absence of other disease activates HIF, STAT3, glycolysis, NFAT and survivin and leads to a shift in pulmonary vascular cellular bioenergetics towards aerobic glycolysis. The accompanying mitochondrial dysfunction, apoptosis resistance and inflammatory cell infiltration contribute to the striking pulmonary vascular remodelling in the iron deficient rat (Figure 8). As iron deficiency can exert pathological effects on the pulmonary vasculature, our observations lend support to further clinical evaluation of iron supplementation for iron deficient patients with PAH.
Acknowledgement

We would like to thank Maria Weinert from Division of Brain Sciences, Imperial College London for her kind support on mitochondria isolation.

Sources of funding

This research was supported by a grant from British Heart Foundation RG/10/16/28575

List of contributions

Conception and design: LZ, MRW, JW; Data acquisition and analysis: EC, AA, LW, KNA, OD, SB, MB, LZ; Interpretation of data: LZ, MW, EC, KNA; Data Drafting the manuscript for important intellectual content: LZ, EC, MRW; Final approval and revision of the version to be published: LZ, MRW.

Disclosures

None
Reference List


Legends

Figure 1. Time course of development of pulmonary hypertension phenotype. a) Mean pulmonary arterial pressure (PAP); b) Systemic pressure; c) Right ventricular (RV) hypertrophy (RV/ LV+sep; d) Cardiac output; e) Pulmonary vascular resistance (PVR); f) Systemic vascular resistance (SVR). Rats were fed with an iron deficient diet for 2 weeks (IDD 2WK), 3 weeks (IDD 3WK) and 4 weeks (IDD 4WK) (n≥6). Group comparisons were made with one-way ANOVA and Bonferroni post hoc test. ***P<0.001, **P<0.01 and *P<0.05 vs control (CTR); #p<0.05 between other groups.

Figure 2. Histological examination of lung peripheral vasculature showing vascular remodeling in iron deficient rats at 2 weeks (IDD 2WK), 3 weeks (IDD 3WK), and 4 weeks (IDD 4WK), and IDD rats treated with ferric carboxymaltose (IDD 4WK+Fe) compared with controls (CTR). Smooth muscle actin (SMA), Von Willebrand factor (VWF), elastic Van Gieson (EVG), haematoxylin and eosin (HE) of a) vessels < 50µm and b) vessels 50 to 100µm. Bar=50µm. c) Percentage of muscularized vessels (n≥4); d) Total macrophages (CD68+) per mm² in lungs of IDD 4WK rats compared to CTR (n=6); e) Number of macrophages (CD68+) per mm² in close proximity to vessels <50µm in lungs of controls and IDD rats (n=6). Section were counterstained with Gill’s haematoxylin; (400x, Bar=50µm). Groups’ comparison was made with one-way ANOVA and Bonferroni post hoc test. *p<0.05 vs control (CTR); #p<0.05 between other groups.

Figure 3. a) Representative western blot bands and summary data of lung HIF1α and HIF2α protein expression (normalized to β-actin) in control, 2 weeks iron deficient (IDD 2WK) and 4 weeks iron deficient (IDD 4WK) rats (n=5 each group). b) Representative pictures of carbonic anhydrase IX (CA IX, green) and smooth muscle actin (SMA, red) double immunofluorescence of lung sections of IDD 4WK and CTR animals showing CA IX expression in the remodelled vessels. Bar=50µm. c-f) Representative western blot bands and summary data of lung STAT3 and phospho-STAT3 protein expression (normalized to β-actin) in control and IDD 4WK rats (n=5 each group). g) Lung nuclear factor of activated T-cell (NFAT) protein expression in CTR (n=5), IDD 2WK and IDD 4WK rats (n=5). h) NFAT localization in the remodeled vessels of IDD 4WK compared to CTR, Bar=50µm. All
western quantification were normalised to β-actin and presented as mean±SEM of fold change compared to control. *p<0.05 vs control group by one-way ANOVA and Bonferroni post hoc test on selected columns.

Figure 4. Effects of iron chelator deferoxamine and ferric carboxymaltose on human pulmonary smooth muscles cells (hPSMCs) in culture. a-d) Representative confocal images and mean data of a) mitochondrial membrane potential measured by the positively charged dye TMRE and b) ROS production (nuclei are stained blue by Hoechst) in live hPSMCs; c) cell count and d) metabolic activity in hPSMCs following deferoxamine (DFO, 200µm) and iron treatment (Fe, 200µm) compared with untreated controls (CTR). e) Representative mitochondrial complex I activity, from immunocaptured of NAD+ and recorded over 30mins. f) Summary complex I activity results expressed as mean OD/min and presented as fold change compared to control (n=4 each group, ***p<0.0001 vs control).

Figure 5. a) Lung 18FDG influx rate (Kᵢ) from Patlak analysis in control (CTR) and 4 week iron deficient rats (IDD 4WK) (n=6). b) Representative static images from dynamically acquired PET images 60 minutes after ¹⁸FDG injection. c) Measurement of lung ¹⁸FDG uptake by gamma counter in CTR and IDD 4WK; %ID/gm, percentage of injected dose per gram of tissue. d) Rate of glycolysis calculated from influx rate. e) Blood glucose concentration (non-fasted) in CTR and IDD 4WK (n≥3). f) Representative lung sections with double immunofluorescence of glucose transporter 1 (GLUT-1) in the remodelled vessels of IDD 4WK rat; smooth muscle actin (SMA, green), GLUT-1, red; Bar=100µm. g) Levels of expression of glucose transporter 1 (glut1), pyruvate dehydrogenase kinase 1 (pdk1) and hexokinase 1 (hk1) in IDD 4WK rat lungs; data are presented as fold changed compared to controls (baseline) (n=3); fold changes were calculated using the REST software. Each group were compared to control using Student’s t-test, *p<0.05.

Figure 6. Ferric carboxymaltose ameliorates PAH phenotype. a) Mean PAP; b) RV Hypertrophy; c) Percentage of muscularized vessels (n≥5); d) Total macrophages (CD68⁺) per mm² in lung (n=6); e) Number of macrophages (CD68⁺) per mm² in close proximity to vessels in lung (<50µm) (n=6); f) blood glucose levels. Data from 4 weeks iron deficient rat (IDD 4WK) and IDD rat treated with ferric
carboxymaltose (IDD 4WK+Fe) are compared with controls (CTR). Group comparisons were made with one-way ANOVA and Bonferroni post hoc test. *p<0.05 vs control (CTR); #p<0.05 vs IDD 4WK+Fe.

Figure 7. Effects of DCA and imatinib treatments on hemodynamics, pulmonary vasculature and survivin expression. a) Mean PAP; (b) RV hypertrophy; c) Percentage of muscularized vessels (n≥4); d) Cardiac output (n≥6); (e) Representative pictures of EVG and SMA staining on lung sections; and f) Representative bands and summary data of lung survivin protein expression levels in control (n=5), 2 weeks iron deficient (IDD 2WK), 4 weeks iron deficient (IDD 4WK), 4 weeks iron deficient + dichloroacetate (DCA), and 4 weeks iron deficient + imatinib (n=4). Western blot data are normalized to β-actin. and presented as mean±SEM of fold change compared to control. Groups were compared by one-way ANOVA and Bonferroni post hoc test on selected columns, *p<0.05 vs control, #p<0.05 between other groups. g) Representative pictures show β-catenin localization in the nuclei of smooth muscle cells (arrow) and endothelial cells (open arrow) in iron deficient lung, Bar=50μm.

Figure 8. Cellular changes in the iron deficient state. Chronic iron deficiency activates hypoxia induced factor (HIF1α), signal transducers and activators of transcription-3(STAT3), lung nuclear factor of activated T-cell (NFAT) and survivin; and leads to a shift of pulmonary vascular cellular bioenergetics towards aerobic glycolysis, a proinflammatory state and mitochondrial dysfunction. The consequent pulmonary vascular cell apoptosis resistance, hyper-proliferation and inflammatory cell infiltration contribute to the striking pulmonary vascular remodelling in the iron deficient rat. Treatments targeted at restoring normal mitochondrial activity with an inhibitor of pyruvate dehydrogenase kinase (PDK) known to promote mitochondrial oxidative phosphorylation, dichloroacetate (DCA), and an antiproliferative tyrosine kinase inhibitor which acts to downregulate glucose transporter (GLUT1), imatinib, attenuate pulmonary hypertension and vascular remodeling in the presence of persistent chronic iron deficiency.
Table 1. Iron markers and tissue iron levels. Iron deficient rats were fed with an iron restricted diet (7mg iron/kg) for 2 weeks (IDD 2WK), 3 weeks (IDD 3WK) and 4 weeks (IDD 4WK) (n≥3). Iron replete rats were fed with an iron deficient diet for 4 weeks, with ferric carboxymaltose treatment introduced for the last 2 weeks (75 mg/Kg, twice a week) (IDD 4WK+Fe) (n≥3). Group comparisons were made with one-way ANOVA and Bonferroni post hoc test. *p<0.05 vs control (CTR); # p<0.05 vs 4 weeks iron deficient (IDD 4WK).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IDD 2WK</th>
<th>IDD 3WK</th>
<th>IDD 4WK</th>
<th>IDD 4WK+Fe</th>
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<tbody>
<tr>
<td><strong>Serum Iron (μmol/L)</strong></td>
<td>37±1.9</td>
<td>6±0.7*</td>
<td>4.7±0.4*</td>
<td>5.2±0.1*</td>
<td>78.95±5.4**</td>
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<td><strong>Tf Sat (%)</strong></td>
<td>39±3.1</td>
<td>6±0.7*</td>
<td>5±0.4*</td>
<td>5±0.1*</td>
<td>NA</td>
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<td><strong>Blood Ferritin (ng/ml)</strong></td>
<td>284±23</td>
<td>104±21*</td>
<td>12±0.5*</td>
<td>115±57*</td>
<td>6398±279**</td>
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<tr>
<td><strong>Hb (g/L)</strong></td>
<td>147±5.1</td>
<td>109.3±6.5*</td>
<td>106.3±2*</td>
<td>103.2±9*</td>
<td>172.7±3.6**</td>
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<td><strong>HTC (%)</strong></td>
<td>48.6±03</td>
<td>38.3±2*</td>
<td>31.6±06*</td>
<td>35.1±1.4*</td>
<td>49±1.3#</td>
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<tr>
<td><strong>Fe Lung (μg/g of tissue)</strong></td>
<td>44.6±2</td>
<td>34.4±1.6</td>
<td>28.9±5.4*</td>
<td>30.7±2.4*</td>
<td>205.3±18.8**</td>
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<tr>
<td><strong>Fe Liver (μg/g of tissue)</strong></td>
<td>71.6±7</td>
<td>18.9±1*</td>
<td>20.1±1.2*</td>
<td>22.4±0.6*</td>
<td>3182±122.4**</td>
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<tr>
<td><strong>Fe Right Ventricle (μg/g of tissue)</strong></td>
<td>59±7.4</td>
<td>46.6±0.9</td>
<td>46.6±3.2</td>
<td>46.6±0.7</td>
<td>134.3±4.4**</td>
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<tr>
<td><strong>Fe Left Ventricle (μg/g of tissue)</strong></td>
<td>62±10</td>
<td>57.7±4.7</td>
<td>57.7±3.1</td>
<td>55.7±9.6</td>
<td>132.2±11.3**</td>
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<tr>
<td><strong>Fe Skeletal Muscle (μg/g of tissue)</strong></td>
<td>21.1±2.5</td>
<td>14.2±2.2</td>
<td>13±1*</td>
<td>13.3±1*</td>
<td>29.3±2.9#</td>
</tr>
</tbody>
</table>

* p<0.05 compared to control  
# p<0.05 compared to IDD 4WK
Figure 1.
Figure 2.

Muscularised vessels (<50µm)

Total Lung Macrophages

Macrophages (vessels <50µm)
Figure 3.
Figure 4.
Figure 5.

(a) Lung $^{18}$FDG influx rate ($K_I$)

(b) CTR vs. IDD 4WK

(c) Lung $^{18}$FDG uptake (Gamma count)

(d) Rate of Glycolysis (Lung)

(e) Blood Glucose

(f) SMA, GLUT-1, MERGE

(g) Fold change compared to control
Figure 6

(a) mean PAP

(b) RV/LV+Septum

(c) Muscularized vessels (<50μm)

(d) Total Lung Macrophages

(e) Macrophages (Vessels <50μm)

(f) Blood Glucose
Figure 7.
Figure 8.

Iron deficiency state

- GLUT1
- HIF1α
- NFAT
- Survivin
- STAT3
- PDK
- PDH
- DCA
- Krebs Cycle
- Fe-S
- mROS
- ETC
- NADH
- NAD+

Metabolic shifts:
- A. Metabolic shift to glycolysis
- B. Proinflammatory state
- C. Mitochondrial dysfunction

Processes:
- Apoptosis resistance
- Proliferation
- Inflammation

Pathways:
- Glucose → Glycolysis → Lactate
- Glucose → PDK → PDH → Krebs Cycle
- HIF1α → NFAT → Survivin

Drugs:
- Imatinib

Pulmonary vascular remodelling
Novelty and Significance

What Is Known?

- Pulmonary arterial hypertension is commonly associated with iron deficiency.
- Iron deficiency, even in the absence of anaemia, is associated with a poor clinical outcome in pulmonary arterial hypertension.

What New Information Does This Article Contribute?

- Chronic dietary iron deficiency induces structural changes in the pulmonary vasculature of the rat, associated with raised pulmonary arterial pressure and right heart hypertrophy.
- The vascular changes are associated with altered mitochondrial function and bioenergetics.
- Iron deficiency may have a direct effect on pulmonary vascular homeostasis and contribute adversely to the vascular pathology of pulmonary arterial hypertension.

To address whether iron deficiency is simply a biomarker of morbidity or actively contributes to the vascular pathology of pulmonary arterial hypertension, we studied rats on a chronic iron deficient diet. Pulmonary hypertension was evident after 2 weeks and histological examination of the lungs showed striking remodelling of pulmonary arterioles, with medial hypertrophy, abnormal intimal endothelium and perivascular inflammatory cell infiltration. The pulmonary vasculature exhibited the biochemical signature of mitochondrial dysfunction, with a shift in cellular metabolism towards glycolysis. The pulmonary hypertension and vascular remodeling were attenuated in the presence of persistent chronic iron deficiency by treatments targeted at restoring normal mitochondrial activity (dichloroacetate) and inhibiting cellular proliferation (imatinib). Our observations suggest that iron availability has a direct impact on pulmonary vascular homeostasis and has implications for correcting iron deficiency in patients with pulmonary hypertension.
Supplemental Material

Electrophysiology

Mitochondrial inner membranes were isolated from control or IDD rat pulmonary arteries (n=3) and purified by centrifugation (Sacchetti et al., 2013). Patch clamp recordings were performed by forming a giga-ohm seal in intracellular solution (in mM: 120 KCl, 8 NaCl, 0.5 EGTA, 10 HEPES, pH 7.3) using an Axopatch 200B amplifier (Axon Instruments) at room temperature (22–25°C), Vm held at voltages between -100 mV to +100 mV. Recording electrodes were pulled from borosilicate glass capillaries (WPI) with a final resistance in the range of 50-100MΩ. Signals were filtered at 5kHz using the amplifier circuitry. Data were analyzed using pClamp 10.4 software (Axon Instruments). Membrane currents under different experimental conditions were assessed by measuring peak membrane current minus the baseline current. In this experiment, the baseline membrane conductance was measured as the peak amount of current (pA) from the closed state. All current measurements were adjusted for the holding voltage, assuming a linear current-voltage relationship. The resulting conductances are expressed in pS according to the equation G = V/DI where G is conductance (pS), V is membrane holding voltage (mV) and DI is peak membrane current in pA - baseline current. Group data were quantified in terms of conductance. Data were expressed as mean ± SEM.

Online Figure I. Effect of iron deficient diet (7mg iron/kg; TD.80396, Harlan, UK) on (a) unsaturated iron binding capacity (UIBC); (b) right ventricle (RV) systolic pressure; (c) right ventricle (RV) over body weight (bw) ratio; (d) left ventricle (LV+septum mass) over body weight (bw) ratio; (e) LV systolic pressure. Animals were fed for 2 (IDD 2WK), 3 (IDD 3WK) and 4 weeks (IDD 4WK) and compared to controls (CTR). (f) Effect of ferric carboxymaltose (75mg/Kg) on cardiac output; rats were fed with an iron deficient diet for 2 weeks, than ferric carboxymaltose was introduced along with the iron deficient diet for 2 additional weeks (IDD 4WK+Fe). Data are compared to 4 weeks iron deficient diet only (IDD 4WK) and controls (CTR). Group comparisons were made with one-way ANOVA and Bonferroni post hoc test. **P<0.01 and *p<0.05 vs control (CTR).
Online Figure II. a) Representative (200x) lung sections (EVG and SMA staining) demonstrating pulmonary vascular remodelling (red arrow) in 2, 3 and 4-week IDD rats (IDD 2WK, IDD 3WK, IDD 4WK). b) Representative (200x) lung sections indicate macrophage (CD68+) infiltration in control (CTR), 4 week IDD rats (IDD 4WK) and 4 week IDD rats with ferric carboxymaltose treatment (IDD 4WK+Fe). Sections were counterstained with Gill’s haematoxylin.
**Online Figure III.** a) Representative pictures indicate increased carbonic anhydrase (CAIX) expression in remodelled pulmonary vessels in IDD 4WK rat compared to control. Bar=50μm. b) Representative western blot bands and summary data of lung Bcl2 protein expression (normalized to β-actin) in control and 4 weeks iron deficient (IDD 4WK) rats (n=5 each group). **p<0.01 vs control.

**Online Figure IV.** Effect of iron deficiency on mitochondrial inner membrane peak conductance levels. a) Representative patch clamp recording of control and iron deficient (IDD) rat pulmonary artery mitochondrial inner membrane vesicle at the indicated holding voltages (100 mV). Closed state of channel activity is indicated by the dotted red lines. b) Group data from patch clamp recordings of the peak conductance of control (n=5) and IDD (n=5), *p=0.0366.