Iron Homeostasis and Pulmonary Hypertension

Iron Deficiency Leads to Pulmonary Vascular Remodeling in the Rat

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

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

**Methods and Results:** Rats were fed an iron-deficient diet (IDD, 7 mg/kg) and investigated for 4 weeks. 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 remodeling 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 hypoxia-induced factor-1α and hypoxia-induced factor-2α, nuclear factor of activated T cells and survivin, and signal transducers and activators of transcription-3 activation, which promote vascular cell proliferation and resistance to apoptosis. Biochemical examination showed reduced mitochondrial complex I activity and mitochondrial membrane hyperpolarization in mitochondria from IDD rat pulmonary arteries. Along with upregulation of the glucose transporter, glucose transporter 1, and glycolytic genes, hkl and pdkl, lung fluorine-18–labeled 2-fluoro-2-deoxyglucose ligand uptake was significantly increased in IDD rats. The hemodynamic and pulmonary vascular remodeling were reversed by iron replacement (ferric carboxymaltose, 75 mg/kg) and attenuated in the presence of iron deficiency by dichloroacetate and imatinib, 2 putative treatments explored for pulmonary arterial hypertension 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. (Circ Res. 2015;116:1680-1690. DOI: 10.1161/CIRCRESAHA.116.305265.)

**Key Words:** glycolysis ■ hypertonies, pulmonary ■ iron ■ STAT3 ■ vascular remodeling
vascular insult. Specifically, we examined the impact of iron deficiency on pulmonary hemodynamics, pulmonary vascular structure, and cardiac hypertrophy during a 4-week time course. After observing striking remodeling 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, and the metabolic modulator, dichloroacetate.

**Methods**

**Animals and Experimental Design**

Adult male Sprague–Dawley rats (body weight, 200–250 g; Charles River, United Kingdom) were used. All experiments were conducted in accordance with the UK Home Office regulations. Experiments were designed as follows:

1. Iron-deficient rat model—time course study: rats were divided into 4 groups (n=6) and fed with either a standard diet (control) or an iron-deficient diet (IDD, 7 mg iron/kg; TD.80396, Harlan, United Kingdom) for 2, 3, and 4 weeks.

2. Iron supplement treatment study: rats were divided into 3 groups (n=6) and fed with (1) a standard diet (control), (2) IDD for 4 weeks, or (3) IDD for 4 weeks plus ferric carboxymaltose (n=6) and fed with (1) a standard diet (control), (2) IDD for 4 weeks, or (3) IDD for 4 weeks plus ferric carboxymaltose

3. Dichloroacetate and imatinib treatment study: rats were divided into 3 groups (n=6) and fed with (1) IDD for 4 weeks, (2) IDD for 4 weeks with dichloroacetate treatment (drinking water, 70 mg/kg per day, Sigma), and (3) IDD with imatinib treatment (oral gavage, 100 mg/kg per day, LC laboratory). Treatments were initiated after 2 weeks of IDD and maintained for the remaining 2 weeks while the rats remained on the IDD.

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

**Hemodynamic Measurements and Tissue Collection**

Rats were anaesthetized (Hypnorm 1 mL/kg IM, midazolam 0.8 mL/kg IP). Right atrial pressure, right ventricular (RV) systolic pressure, and pulmonary arterial pressure (PAP) were measured with a precurved catheter inserted through the right jugular vein. Systemic blood pressure, left ventricular (LV) systolic pressure, and LV end-diastolic pressure were assessed via carotid artery cannulation. Cardiac output (CO) was measured by thermodilution (Online Data Supplement). Pulmonary vascular resistance was calculated using the standard equation: (mean PAP-LV end-diastolic pressure)/CO. Similarly, systemic vascular resistance was calculated as (mean systemic blood pressure–right atrial pressure)/CO. All hemodynamic data were recorded and analyzed with a PowerLab Data Acquisition system (AD Instruments).

At the end of procedure, blood was collected to obtain serum. Tissues (lung, heart, liver, and 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 over left ventricle plus the septum was used as an index of RV hypertrophy. The left lung was fixed in 10% formalin and paraffin-embedded for histological examination.

**In Vivo 18FDG-PET**

In vivo 18FDG-PET was performed using a Siemens Inveon small animal multimodality PET/computed tomographic system (Siemens Healthcare Molecular Imaging). A protocol of 60-minute dynamic scanning with kinetic analysis established in our laboratory was used. The rate of glycolysis was calculated as follows: rate of glycolysis=[glucose]/K_s, where [glucose] is plasma glucose concentration and K_s is FDG influx rate. We assumed that the relative glycolytic flux of FDG and glucose (lumped constant) remains unchanged between groups.

**Iron Markers**

Hemoglobin levels were measured by HEMOCUE reader (Sweden) and hematocrit using a microhematocrit device after centrifugation and blood glucose level using an Aviva Accu-Check monitor. Circulating iron and unsaturated iron binding capacity were measured in serum (Abbott Diagnostics, Ireland). Transferrin saturation was calculated from the iron and total iron binding capacity. Ferritin was measured using ELISA (Alpco Diagnostics). For tissue iron measurements, 0.1 g of tissue was digested in 70% nitric acid, with total iron assessed by means of inductively coupled plasma-mass spectroscopy.

**Histology, Immunohistochemistry, and Immunofluorescence**

Lung histology was examined by elastic Van Gieson, and hematoxylin and eosin staining. Peripher al vessels <50 μm diameter were counted at x40 magnification and pulmonary vascular remodeling 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 (1/200; Sigma), Von Willebrand factor (1/100; Dako), CD68 (1/200; Serotec), B-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 (CD68+) as previously described.

For double immunofluorescence, sections were incubated with e glucose transporter 1 (GLUT1; 1/50, Abcam), carbonic anhydrase (1/100; Novus Biological), and smooth muscle actin (1/200; Sigma), detected with secondary antibodies, Alexa 488 antimouse, and Alexa 568 antirabbit (1/1000; Invitrogen), with mounting solution containing 4',6-diamidino-2-phenylindole. Images were obtained with a Leica laser confocal microscope (TCS SP2 AOB5).

**Western Blotting**

Protein samples (25 μg), extracted from lung homogenates, were run on a 4% precast gel (Invitrogen) and transferred to a nitrocellulose membrane. Western blotting was performed as per manufacturer’s suggestions (rabbit anti-survivin antibody 1/1000, ProteinTech; rabbit anti–hypoxia-induced factor [HIF]–2α 1/1000, mouse anti–HIF1-α 1/1000, mouse anti–NFAT-1 1/1000, Novus Biological; mouse anti–Bcl-2 1/1000, BD Bioscience; anti–signal transducers and activators of transcription-3 [STAT3] 1/2000, anti–phospho-STAT3 1/2000, Abcam). Proteins were
detected by Novex enhanced chemiluminescent kit (Invitrogen, Paisley, United Kingdom). Optical densities of individual bands were measured and protein expression was standardized with β-actin.

**Real-Time Polymerase Chain Reaction Analysis**

Total RNA was extracted by RNeasy Mini Kit (Qiagen) and reverse-transcribed with M-MLV Reverse Transcriptase (Promega). Real-time polymerase chain reaction for glut1, pyruvate dehydrogenase kinase 1 (pdk1), and hexokinase 1 (hk1) was performed using predesigned, gene-specific TaqMan primer and probe sets (Applied Biosystems). Gene expression data were normalized with the housekeeping ubc1 expression and analysis was performed using the relative expression software tool.

**Mitochondrial Isolation and Measurement of Complex I Activity**

Mitochondria were isolated from control or IDD rat pulmonary arteries (PA, second to sixth division). Briefly, freshly dissected tissues were homogenized in ice-cold isolation buffer (250 mmol/L sucrose, 20 mmol/L HEPES, 1 mmol/L EDTA, and 0.5% BSA) and centrifuged at 2500 g to pellet nuclear material. The supernatant was centrifuged at high-speed (9600 g for 15 minutes at 4°C); the pellet was washed twice in isolation buffer by centrifugation at 9600 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 the 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 evaluate mitochondrial inner membrane peak conductance levels (Online Data Supplement).

**Cell Culture**

Human pulmonary artery smooth muscle cells (PASMCs) were serum-deprived and then stimulated with 50 ng/mL platelet-derived growth factor with or without iron chelator deferoxamine (200 μmol/L, Sigma) for 48 hours. Final cell count or water soluble tetrazolium salt-1–based colorimetric assay was performed (Online Data Supplement).

A separate set of PASMCs was cultured with or without 200 μmol/L deferoxamine for 24 hours and then incubated with fluorescence probes for 30 minutes at 37°C in the dark. Mitochondrial membrane potential was determined using 20 nmol/L tetramethylrhodamine (Life technologies). Reactive oxygen species production was detected using 5 μmol/L 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 means±SEM. Normal distribution was verified using 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 P value of <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 ≤4 weeks and iron status evaluated (Table; Online Figure IA). Both stored and circulating iron levels were reduced after 2 weeks IDD, as reflected in the significantly decreased liver (<30% of control) and serum (<20% of control) iron levels, blood ferritin and transferrin saturation, whereas unsaturated iron binding capacity increased (P <0.05). This was accompanied by reduced hemoglobin (109 versus 147 g/L) and hematocrit (38% versus 48%) in IDD rats, maintained through 4 weeks. Lung and skeletal muscle tissue iron levels were significantly reduced during the 4 weeks (69% and 63% of control, respectively), whereas 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 during the 4-week time course. Mean PAP and RV systolic pressures were elevated significantly by 3 weeks in IDD rats compared with controls, but systemic blood pressure was not affected (Figure 1A and 1B; Online Figure IB). RV hypertrophy

**Table. Iron Markers and Tissue Iron Levels**

<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>Serum iron, μmol/L</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>
</tr>
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<td>Tf sat, %</td>
<td>39±3.1</td>
<td>6±0.7†</td>
<td>5±0.4*</td>
<td>5±0.1*</td>
<td>NA</td>
</tr>
<tr>
<td>Blood ferritin, ng/mL</td>
<td>284±23</td>
<td>104±21*</td>
<td>12±0.5*</td>
<td>115±57*</td>
<td>6398±279†</td>
</tr>
<tr>
<td>Hb, g/L</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>
</tr>
<tr>
<td>Hct, %</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>
</tr>
<tr>
<td>[Fe] lung, μg of tissue</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>
</tr>
<tr>
<td>[Fe] liver, μg of tissue</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>
</tr>
<tr>
<td>[Fe] right ventricle, μg of tissue</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±4.4†</td>
</tr>
<tr>
<td>[Fe] left ventricle, μg of tissue</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±11.3†</td>
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<td>[Fe] skeletal muscle, μg of tissue</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>

Iron-deficient rats were fed with an iron-restricted diet (7 mg iron/kg) for 2, 3, and 4 weeks (n = 3). Iron-replete rats were fed with an iron-replete 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 1-way ANOVA and Bonferroni post hoc test. Hb indicates hemoglobin; Hct, hematocrit; IDD 2WK, iron-deficient diet for 2 weeks; IDD 3WK, iron-deficient diet for 3 weeks; IDD 4WK, iron-deficient diet for 4 weeks; IDD 4WK+Fe, iron-deficient diet for 4 weeks, with ferric carboxymaltose treatment; NA, not available; and Tf sat, transferrin saturation.

* P<0.05 vs control; †P<0.05 vs IDD 4WK; *P<0.05 compared with control; and †P<0.05 compared with IDD 4WK.
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 IC and ID), as previously reported.17 There was a trend of increasing in LV systolic pressure (Online Figure IE). CO was significantly increased at 4 weeks compared with the control group (135%; \( P<0.05 \)) (Figure 1D; Online Figure IF). Pulmonary vascular resistance was significantly increased in 4-week IDD rats, but not systemic vascular resistance (Figure 1E and 1F).

Histological examination of lung sections revealed profound pulmonary vascular remodeling in iron-deficient rats (Figure 2; Online Figure IIA). Smooth muscle actin staining demonstrated tunica media hypertrophy in vessels with diameter <50 or 50 to 100 μm (Figure 2A and 2B; Online Figure IIA), with an increased proportion of peripheral vessels (<50 μm) demonstrating muscularization (Figure 2C). These structural changes were accompanied by a reduction in lumen area, protruding endothelial cells and concertined appearance to the inner elastic lamina (Figure 2A and 2B). Prominent infiltration of macrophages (CD68⁺) with a preferential accumulation surrounding the peripheral remodeled vessels was also evident in iron-deficient rats (Figure 2D and 2E; 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 remodeling, we first investigated HIF1α and HIF2α levels. Iron is required for the optimal activity of prolyl hydroxylases, which act to degrade hypoxia-inducible factors.18,19 Western blots show an increase in both HIF1α and HIF2α levels in the iron-deficient rat lung (Figure 3A). Consistent with HIF1α stabilization under iron-deficient conditions, expression of carbonic anhydrase IX, a downstream target of increased HIF1α, was increased in the remodeled 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,20 we also measured levels of STAT3. This cytoplasmic latent transcription factor is activated by phosphorylation in response to cytokines and recently proposed to act a signaling hub in PAH.21 Levels of phosphorylated STAT3 were increased in IDD rat lungs (Figure 3C–3E), indicating STAT3 activation. The 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.22 Increased NFAT expression was evident in the IDD rat lung with increased expression in remodeled vessels (Figure 3F and 3G).

Both STAT3 and NFAT activation have been associated with altered/suppressed mitochondrial function,22–24 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.

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/left ventricular [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 1-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.
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 tetramethylrhodamine (Figure 4A) and inhibition of reactive oxygen species (mitochondrial ROS) generation (Figure 4B). Accompanying this, iron chelation increased metabolic activity and cell proliferation (Figure 4C and 4D).

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 such as succinate dehydrogenase and aconitase, affecting 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 with control animals (Figure 4E and 4F). Patch clamp measurements on mitochondrial inner membrane vesicles using a recently modified technique25,26 showed a drop in ion channel peak conductance levels in IDD rats (51.2±10.5; n=5) when compared with controls (109.2±10.5; n=5), consistent with altered mitochondrial function (Online Figure IV A and IVB).

**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 analysis14 demonstrated a significant increase in lung $^{18}$FDG uptake in 4-week IDD animals compared with controls (Figure 5A–5C). The calculated glycolysis rate ([glucose]$\times K_i$) was also significantly elevated in IDD rat lungs (Figure 5D). Blood glucose levels in nonfasting 4-week IDD rats were significantly increased compared with controls (IDD for 4 weeks, 10.5±2.1; control, 8.3±0.11 mmol/L; $P<0.05$; Figure 5E), as previously observed.20,21

Consistent with increased $^{18}$FDG uptake, the remodeled pulmonary vessels demonstrated prominent GLUT1 expression by immunofluorescence (Figure 5F). Real-time polymerase chain reaction analysis of IDD rat lungs showed increased expression of $glut1$ (>2-fold) along with $hk1$ 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.7 Iron supplementation for 2 weeks elevated circulating iron indices and tissue iron levels and restored erythropoiesis in IDD rats to control levels (Table). Iron treatment reduced PAP (22±1.6 versus 27±0.6 mm Hg; $P<0.05$), normalized RV mass (Figure 6A and 6B), and reduced pulmonary vascular muscularization (Figures 2A, 2B, and 6C) and perivascular macrophage accumulation (Figure 6E–6G; Online Figure IIA) compared with 4-week IDD rats. Blood glucose concentration was also reduced to control levels (7.8±0.7 mmol/L; Figure 6F).
Dichloroacetate 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 dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, for 2 weeks while remaining on the IDD. Inhibition of pyruvate dehydrogenase kinase facilitates mitochondrial

Figure 4. Effects of iron chelator deferoxamine and ferric carboxymaltose on human pulmonary smooth muscles cells (hPSMCs) in culture. A to D, Representative confocal images and mean data of (A) mitochondrial membrane potential measured by the positively charged dye tetramethylrhodamine (TMRE) and (B) reactive oxygen species (ROS) production (nuclei are stained blue by Hoechst) in live hPSMCs; (C) cell count and (D) metabolic activity in hPSMCs after deferoxamine (DFO, 200 μm) and iron treatment (Fe, 200 μm) compared with untreated controls (CTR). E, Representative mitochondrial complex I activity, from immunocaptured of NAD+ and recorded >30 minutes. F, Summary complex I activity results expressed as mean optical density (OD)/min and presented as fold change compared with CTR (n=4 each group, **P<0.0001 vs CTR).
oxidative phosphorylation and has been reported to attenuate pulmonary hypertension in several experimental models. Dichloroacetate attenuated the rise in PAP (Figure 7A) and the associated RV hypertrophy (Figure 7B) and pulmonary vascular remodeling (Figure 7C; Online Figure IIA and IIB) seen in iron deficiency. CO 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 remodeling in the presence of persistent iron deficiency (Figure 7A–7D).

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 causatively associated with the development of PAH. Survivin expression was increased in the IDD rat lung and tracked pulmonary remodeling severity. Both dichloroacetate and imatinib reduced survivin expression in the lung, consistent with other animal models (Figure 7A–7D).

Discussion

We report for the first time profound vascular remodeling of pulmonary arterioles as a consequence of chronic iron deficiency in rats. The pulmonary vascular changes were associated with raised PAP, pulmonary vascular resistance, 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 remodeling was attenuated in the presence of persistent iron deficiency by dichloroacetate and imatinib, 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 used by other investigators. By 2 weeks, liver iron stores and serum iron and ferritin were substantially reduced (<30% of control level) and remained low. Hemoglobin and hematocrit were also reduced but to a lesser degree (25%). Hemoglobin remained >100 g/L for the duration of the experiment, above the 70 to 90 g/L range regarded as moderate anemia in the rat. Lung tissue iron levels were significantly reduced in IDD rats (69% of control), whereas RV iron levels were reduced to 78% of control level.

The pulmonary vascular remodeling observed in the IDD rats is striking and seems more severe than described in most reports of monocrotaline and hypoxia-induced pulmonary hypertension. There are notable similarities with human PAH, specifically medial hypertrophy coupled with an abnormal intimal endothelium and perivascular inflammatory cell infiltration. In contrast
of PAH.\textsuperscript{12,14,24} We found that iron chelation induces hyperpolarization and postulated to play a central role in the pathogenesis of PAH.\textsuperscript{12,14,24} Support for a direct effect of iron deficiency on pulmonary vascular remodeling comes from the effect of iron chelation on PASMC proliferation in culture. In vivo, vascular remodeling was accompanied by increased expression of HIF1α and HIF2α protein, and downstream targets, carbonic anhydrase IX and GLUT1, biochemical signatures of iron deficiency--induced tissue hypoxia\textsuperscript{39} and integral to the pulmonary vascular remodeling observed in human idiopathic pulmonary arterial hypertension and several experimental models of pulmonary hypertension.\textsuperscript{40–42}

Inflammation is also recognized as a mediator of pulmonary vascular remodeling 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 lungs is relevant here, as is the activation of STAT3 and NFAT22,23; both integrate signaling from cytokines but are also growth and angiogenic factors and effect actions through several downstream targets, including Bcl2 and survivin.

Collectively, the activated HIF, STAT3, and NFAT signaling would be expected to support a proproliferative and antiapoptotic 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 reactive oxygen species generation in PASMCs in culture. There is also clear evidence for mitochondrial dysfunction in vivo. Mitochondria from IDD rat PAs demonstrated decreased mitochondrial complex I activity along with reduced baseline inner membrane conductance levels in comparison with control specimens. Consistent with increased expression of glut1 and the glycolysis enzymes, hkl and pdk1, in IDD rat lungs, parenchymal 18FDG 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{3} Of greater interest, the elevated PAP and vascular remodeling were attenuated by treatment with the metabolic modulator, dichloroacetate,\textsuperscript{12} and the antiangiogenic tyrosine kinase inhibitor, imatinib,\textsuperscript{11} in the presence of persistent iron deficiency. Dichloroacetate acts by inhibition of pyruvate dehydrogenase kinase to restore oxidative phosphorylation\textsuperscript{46} and so reverses the metabolic shift induced by iron deficiency. Imatinib, through inhibition of platelet-derived growth factor, 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{37}

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{1–6} It is at odds with 2 studies in rats, both of which suggest iron deficiency is protective against hypoxia-induced and monocrotaline-induced pulmonary hypertension.\textsuperscript{5,10} However, both these studies have confounding factors. Polycythemia is an important contributor to hypoxia-induced pulmonary hypertension; the lower hemoglobin associated with iron deficiency in that model would be expected to be beneficial, as

Figure 6. Ferric carboxymaltose ameliorates pulmonary arterial hypertension phenotype. A, Mean pulmonary arterial pressure (PAP); (B) right ventricular (RV) hypertrophy; (C) percentage of muscularized vessels (n=25); (D) total macrophages (CD68\textsuperscript{+}) per millimeter squared in lung tissue hypoxia\textsuperscript{39} and integral to the pulmonary vascular remodeling observed in human idiopathic pulmonary arterial hypertension and several experimental models of pulmonary hypertension.\textsuperscript{40–42}

Collectively, the activated HIF, STAT3, and NFAT signaling would be expected to support a proproliferative and antiapoptotic 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
indeed it is in Tibetans acclimatized 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 provides 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.

Figure 7. Effects of dichloroacetate (DCA) and imatinib treatments on hemodynamics, pulmonary vasculature, and survivin expression. A, Mean pulmonary arterial pressure (PAP); B, right ventricular (RV) hypertrophy; C, percentage of muscularized vessels (n≥4); D, cardiac output (n≥6); E, representative pictures of elastic Van Gieson (EVG) and smooth muscle actin (SMA) staining on lung sections; and F, representative bands and summary data of lung survivin protein expression levels in control (CTR; n=5), 2-week iron deficient (IDD 2WK), 4-week iron deficient (IDD 4WK), 4-week iron deficient+DCA, and 4-week iron deficient+imatinib (n=4). Western blot data are normalized to β-actin, and presented as mean±SEM of fold change compared with CTR. Groups were compared by 1-way ANOVA and Bonferroni post hoc test on selected columns, *P<0.05 vs CTR, #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, 50 μm. LV indicates left ventricular.

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 toward aerobic glycolysis, a proinflammatory state and mitochondrial dysfunction. The consequent pulmonary vascular cell apoptosis resistance, hyperproliferation, and inflammatory cell infiltration contribute to the striking pulmonary vascular remodeling 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. ETC indicates electron transport chain; mROS, mitochondrial reactive oxygen species; and PDH, pyruvate dehydrogenase.
In this study, we did not study cardiac function in detail. Both the left ventricle and the right ventricle showed evidence of hypertrophy in the iron-deficient rat. But in keeping with pulmonary hypertension, the RV hypertrophy index (RV/LV+sep) was increased and responded to iron replacement, as well as treatment with dichloroacetate 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 toward aerobic glycolysis. The accompanying mitochondrial dysfunction, apoptosis resistance, and inflammatory cell infiltration contribute to the striking pulmonary vascular remodeling 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.

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Iron deficiency may have a direct effect on pulmonary vascular homeostasis and contribute adversely to the vascular pathology of pulmonary arterial hypertension. Proc Natl Acad Sci U S A. 1998;95:11715–11720.

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 remodeling 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 toward 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.
Iron Homeostasis and Pulmonary Hypertension: Iron Deficiency Leads to Pulmonary Vascular Remodeling in the Rat
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**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), V_m 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 ferri carboxymaltose (75mg/Kg) on cardiac output; rats were fed with an iron deficient diet for 2 weeks, then ferri 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.