Research Paper

Disruption of the Hepcidin/Ferroportin Regulatory System Causes Pulmonary Iron Overload and Restrictive Lung Disease

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1. Introduction

Imbalances of iron homeostasis are implicated in a spectrum of acute and chronic lung diseases (Ghio, 2009; Ghio et al., 2013). In patients with chronic obstructive pulmonary disease (COPD), iron deposits in alveolar macrophages (AM) and the percentage of iron loaded macrophages is associated with increased disease severity (Philippot et al., 2014). On the other hand, thalassemia major, a disease characterized by transfusional iron overload, has been associated with impaired lung function (Carnelli et al., 2003; Guidotti et al., 2016; Kanji et al., 2000). However, whether pulmonary iron accumulation contributes to disease onset and progression is poorly understood.

Tissue iron levels must be maintained in such a way that both iron deficiency and iron overload are prevented. Excess free iron generates oxidative stress causing cell damage and tissue injury (Muckenthaler et al., 2017). In the lung, the risk for oxidative stress is exacerbated by its continuous exposure to an atmosphere with high oxygen levels. The mechanisms maintaining lung iron homeostasis are incompletely understood. Like most other cells, lung cells acquire transferrin-bound iron from the plasma. In addition, lung airway epithelial cells take up iron via the divalent metal transporter 1 (DMT1) and sequester it in epithelial cells lining the conducting airways and lung parenchyma, and in vascular smooth muscle cells. Pulmonary iron overload is associated with oxidative stress, restrictive lung disease with decreased total lung capacity and reduced blood oxygen saturation in homozygous S1c40a1d26/C266 mice compared to wild-type controls. These findings implicate iron in lung pathology, which is so far not considered a classical iron-related disorder.

Emerging evidence suggests that pulmonary iron accumulation is implicated in a spectrum of chronic lung diseases. However, the mechanism(s) involved in pulmonary iron deposition and its role in the in vivo pathogenesis of lung diseases remains unknown. Here we show that a point mutation in the murine ferroportin gene, which causes hereditary hemochromatosis type 4 (S1c40a1d26/C266), increases iron levels in alveolar macrophages, epithelial cells lining the conducting airways and lung parenchyma, and in vascular smooth muscle cells. Pulmonary iron overload is associated with oxidative stress, restrictive lung disease with decreased total lung capacity and reduced blood oxygen saturation in homozygous S1c40a1d26/C266 mice compared to wild-type controls. These findings implicate iron in lung pathology, which is so far not considered a classical iron-related disorder.

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primary iron overload disorder hallmarked by increased systemic iron levels, iron depletion in iron-exporting organs and severe iron deposition in parenchymal tissues (Altamura et al., 2014; Sham et al., 2009; Sham et al., 2005).

Here, we aim to understand whether a disruption in the FPN/hepcidin regulatory system and the subsequent increase in systemic iron levels affects lung iron homeostasis and function. To achieve this goal, we took advantage of a murine disease model of HH type 4, hallmarked by a C326S amino acid substitution in FPN (Slc40a1C326S) (Altamura et al., 2014). We show that resistance of ferroportin to hepcidin binding causes pulmonary iron accumulation in defined lung cell types. Homozygous Slc40a1C326S/C326S mice show increased oxidative stress in the lung, restrictive lung disease and decreased blood oxygen saturation, revealing a role of iron in lung pathology.

2. Material and Methods

2.1. Mice

All mice analyzed were maintained on a pure C57BL/6N genetic background (>99.9% congenic). As controls, age- and gender-matched wild-type C57BL/6N mice born and maintained in the same breeding facility were used. Mice were housed in the Heidelberg University animal facility under a constant light-dark cycle and maintained on a standard mouse diet (LASQCdiet Rod18-A - LASvendi) containing 200 ppm iron with ad libitum access to food and water. All mouse breeding and animal experiments were approved by the Regierungspräsidium Karlsruhe (Projects Nr T-81/14, T-66/13, G-41/16, G-39/16).

2.2. Tissue Iron Quantification

Tissue non-heme iron content was measured using the bathophenanthroline method and calculated against dry weight tissue (Torrance and Bothwell, 1968).

2.3. RNA Extraction, Reverse Transcription and qRT-PCR

Total lung RNA was isolated using Trizol (Life technologies) and was reverse transcribed and used in SYBR-green qPCR, as described in (Altamura et al., 2010). mRNA expression was calculated relative to RPL19 and data were analyzed using the ΔΔCT method (Livak and Schmittgen, 2001). The primers used are listed in Supplementary Information.

2.4. Western Blotting

Protein lysates were obtained by homogenizing snap-frozen tissues in RIPA buffer supplemented with protease inhibitors (Roche Diagnostics), as described in (Galy et al., 2004). Protein concentration was determined using the DC protein assay (BioRad). 50 μg of protein were subjected to western-blot analysis with the antibodies listed in Supplementary Information. Western blot images were quantitatively acquired with the Vilber Lourmat Fusion-FX Chemiluminescence system (Eberhardtz). β-actin was used as loading control.

2.5. Lipid Peroxidation - TBARS Measurements

Thiobarbituric acid reactive substances (TBARS) levels were measured in samples of total lung from 36-week old mice using the QuantiChrom TBARS Assay Kit (BioAssay Systems) following manufacturer’s instructions.

2.6. Bronchoalveolar Lavage and Differential Cell Count

Mice were anesthetized via intraperitoneal injection of a combination of ketamine and xylazine (120 and 16 mg/kg respectively) and sacrificed by exsanguination. A median sternotomy was performed, the trachea cannulated and the left mainstem bronchus ligated, and the right lung was lavaged with Phosphate Buffer Saline (PBS). Bronchoalveolar lavage (BAL) samples were centrifuged and BAL fluid supernatant was harvested and stored at −80 °C. Total cell counts were determined using a haemocytometer and differential cell counts were determined in cytospin preparations stained with May–Grünwald–Giemsa (Merck).

2.7. Iron Quantification in the Bronchoalveolar Lavage Fluid Supernatant

BAL fluid supernatant was concentrated through Speed-Vacuum. Iron measurements were performed using the SFBC kit (Biolabor) following manufacturer’s instructions.

2.8. Measurement of Cytokine Protein Levels in Bronchoalveolar Lavage

Cytokine protein levels (IL6, IL1β and TNFα) were determined in BAL fluid supernatants applying Multiplex bead-array based technology. Measurements were performed on a BioPlex200 System using the Bio-Plex Pro Cytokine Reagent Kit and Bio-Plex Pro Mouse Cytokine sets (Bio-Rad) according to manufacturer’s instructions. Cytokine protein levels are given as picograms in total BAL fluid supernatant.

2.9. Lung Function Measurements

Mice were anesthetized via intraperitoneal injection of Na+-pentobarbital (80 mg/kg), tracheostomized and placed on the flexiVent system (SCIREQ, Montreal, QC, Canada) for measurements of pulmonary function. Mice were then paralyzed with pancuronium bromide injected intraperitoneally (0.8 mg/kg) to avoid breathing artefacts during the measurement. Mice were ventilated at a frequency of 150 breaths/min, with a tidal volume of 11 mL/kg and a positive end expiratory pressure of 3 cm H2O to prevent alveolar collapse. Pressure-volume curves, total lung capacity, pulmonary compliance and elastance were measured as previously described in (Mall et al., 2008) and (Vanoirbeek et al., 2010). All perturbations were performed until at least three acceptable measurements were reached.

2.10. Blood Oxygen Saturation

Oxygen saturation was determined using a noninvasive pulse oximeter for laboratory animals (MouseOx® Plus, Starr life science) following manufacturer’s instructions. Arterial blood oxygen saturation was analyzed in conscious mice exposed to room air with a thigh clip sensor. The oxygen saturation was measured when pulse waves were stable and regular in order to obtain valid values.

2.11. Stereology of Lung Tissue

Lungs from 36 week old mice were instilled via the trachea with 1.5% paraformaldehyde, 1.5% glutaraldehyde in 0.15 M Hepes buffer at hydrostatic pressure of 25 cm and stored in the same solution at 4 °C until further processing. The lungs were embedded in glycol methacrylate according to standard protocol (Muhlfeld et al., 2013) and 1.5 μm thick sections were cut. Light microscopy was performed using a Leica DM6000B microscope (Leica, Wetzlar, Germany) attached to a computer with the newCAST stereology software (Visiopharm, Horsholm, Denmark). Design-based stereology was used to quantify the volume of the lung parenchyma and non-parenchyma as described in (Muhlfeld et al., 2013).

2.12. Erythropoietin (Epo) Measurements

Plasma Epo levels were determined using a mouse Epo ELISA kit (R&D systems) following manufacturer’s instructions.

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