

Title- THE INTERPLAY BETWEEN IRON AND OXYGEN HOMEOSTASIS WITH A PARTICULAR FOCUS ON THE HEART.

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Running head- INTERPLAY BETWEEN IRON AND OXYGEN HOMEOSTASIS.

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Abstract- Iron is subject to tight homeostatic control in mammals. At the systemic level, iron homeostasis is controlled by the liver-derived hormone hepcidin acting on its target ferroportin in the gut, spleen and liver, which form the sites of iron uptake, recycling and storage, respectively. At the cellular level, iron homeostasis is dependent on the iron regulatory proteins IRP1/IRP2. Unique chemical properties of iron underpin its importance in biochemical reactions involving oxygen. As such, it is not surprising that there are reciprocal regulatory links between iron and oxygen homeostasis, operating both at the systemic and cellular levels. Hypoxia activates the IRP pathway, and in addition suppresses liver hepcidin through endocrine factors that have yet to be fully elucidated. This review summarises current knowledge on the interplay between oxygen and iron homeostasis, and describes recent insights gained into this interaction in the context of the heart. These include the recognition that the hepcidin/ferroportin axis plays a vital role in the regulation of intracellular iron homeostasis as well as regulating systemic iron availability. As is the case for other aspects of iron homeostasis, hypoxia significantly modulates the function of the hepcidin/ferroportin pathway in the heart. Key areas still to understand are the interactions between cardiac iron and diseases of the heart where hypoxia is a recognised component.

Keywords- iron, oxygen, hepcidin, ferroportin, heart.

INTRODUCTION

Iron is an essential trace element found in all living organisms. It can exist in various oxidation states, but the ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions occur most frequently. The variability of the redox potential of the $\text{Fe}^{2+}/\text{Fe}^{3+}$ pair, according to acidity, ligand and cofactors make iron especially suited as a catalyst for a variety of biochemical reactions in different cellular compartments (23, 44, 45). Most notably, iron-mediated oxidation-reduction reactions are essential to the metabolism of oxygen in living organisms. Oxygen utilisation in oxidative phosphorylation requires electron transfer and oxygen activation by iron in iron-sulfur and heme groups (6, 35, 58), while oxygen activation by dioxygenases requires non-heme iron (57). In addition, the transport of oxygen itself requires reversible binding by heme iron in the haemoglobin molecule (42). Reactivity with oxygen also underpins the ability of free iron to participate in Fenton-type reactions, in which ferrous iron reacts with hydrogen peroxide producing reactive oxygen species (ROS). While required in redox signalling, excessive ROS levels are damaging to proteins and lipids (20,21). Thus, when not in heme or iron-sulfur groups, iron in aerobic organisms is usually chaperoned by proteins for transport (transferrin), and storage (ferritin) (30, 3, 43). The importance of iron for oxygen handling manifests itself when iron is in short supply. At the systemic level, iron deficiency reduces arterial oxygen content by restricting haemoglobin concentration, which when severe may result in global hypoxia (53). At the cellular level, iron deficiency restricts the ability of cells to utilise oxygen in oxidative phosphorylation (11, 14).

MAMMALIAN IRON HOMEOSTASIS

The average amount of iron in a human male is ~4 g, of which ~2 g is present in RBCs and a further ~0.3 g is being continuously recycled from haemoglobin (30). Outside of this, iron is present either intracellularly, or in the plasma complexed to transferrin (Tf-Fe^{3+}), or ferritin (3, 43). Inside cells, 95% of iron is complexed to protein chaperones such as ferritin, or present in heme prosthetic or iron-sulfur groups (6, 35, 58, 3, 43). The remainder, about 5% of intracellular iron, represents the chelatable labile iron pool (LIP) (25).

Cellular iron homeostasis is concerned with the adequate supply of iron for cellular iron-dependent processes, while at the same time restricting the size of LIP to prevent excessive ROS generation from Fenton-type reactions (20, 21). Cellular homeostasis is orchestrated by the iron regulatory proteins, IRP1 and IRP2, which regulate iron uptake by transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT-1); iron storage by ferritin; iron utilisation in heme synthesis by erythroid 5-aminolevulinic acid synthase (ALAS2); and in some cells, iron export by ferroportin (SLC40A1 or FPN) (49,50). The activity of IRPs first involves an iron-sensing step. High intracellular iron reduces the RNA-binding ability of IRP1 and the stability (and hence availability) of IRP2. The second step, which occurs preferentially under conditions of low iron, involves binding of IRP1 and IRP2 at iron regulatory elements IREs. This occurs either at the 5'UTR to cause translational repression (e.g ferroportin, L-ferritin, H-ferritin, Alas2) or at the 3'UTR to increase transcript stability (e.g TfR1, Dmt1) (49,50).

Systemic iron homeostasis is concerned with the adequate supply of iron for key physiological processes such as erythropoiesis, and developmental processes such as bone growth and neuronal development (where iron is required for myelination). Because iron turnover in the body is very low (only ~1.5mg iron absorbed daily from dietary sources, with a similar amount lost from the gastrointestinal tract), systemic iron availability is regulated

primarily at the site of iron recycling from senescent red blood cells in the reticuloendothelial macrophages (16,17,18). Other sites influencing systemic iron availability are enterocytes (the site of dietary iron absorption), and hepatocytes (the site of iron storage) (17,18). Systemic iron fluxes are summarised in Fig.1. Hepcidin is the liver-derived hormone that controls systemic iron availability. It exerts this control through its ability to bind, and internalise ferroportin, the only known mammalian iron export protein at the sites of iron recycling, absorption and storage (15, 40, 47). The hepcidin/ferroportin axis is essential for health. This is illustrated by the consequences of genetic mutations in the hepcidin/ferroportin pathway. Where these mutations impair hepcidin production or activity, they are associated with systemic iron overload (hemochromatosis) (9). Where these mutations are associated with inappropriately high hepcidin, they cause iron refractory iron deficiency anaemia (IRIDA) (13). Hepcidin expression is increased by high transferrin saturation and by inflammation, and decreased by low transferrin saturation, by hypoxia and by a stimulated erythroid compartment through the endocrine action of erythroferrone (24, 27, 52). The stimulation of hepcidin by inflammation explains the anaemia of chronic disease (39, 41), and its suppression by erythroferrone explains the iron overload associated with ineffective erythropoiesis (e.g. β -thalassemia) (26,38). While the hepcidin/ferroportin axis is well recognised for its actions controlling systemic iron availability, a novel role for it in cellular iron homeostasis is considered later in the review.

MAMMALIAN OXYGEN HOMEOSTASIS

Oxygen is the terminal electron acceptor for aerobic metabolism. Thus, systemic oxygen homeostasis is primarily concerned with ensuring a sufficient continuous supply of oxygen for this purpose. Oxygen stores (in myoglobin and haemoglobin) are small relative to the rate of oxygen consumption. Systemic oxygen homeostasis is achieved primarily through the physiological regulation of the cardiopulmonary system. This ensures adequacy of oxygen uptake into the lungs, transfer into the blood and delivery into tissues (7, 12).

At the intracellular level, maintenance of pO_2 is key to the functioning of the cell. When intracellular pO_2 is reduced, cells adapt by switching to anaerobic metabolism, changing mitochondrial turnover and inducing G1 phase cell cycle arrest (48). These adaptations are the result of a genome-wide transcriptional response driven by the binding of hypoxia-inducible factor (HIF) transcription factors to hypoxia-response elements (HREs) in the regulatory region of genes (54). HIF1 α and HIF2 α are synthesized continuously in the cytoplasm then degraded through a pathway involving first hydroxylation by HIF prolyl-hydroxylases (PHDs), then binding of the hydroxylated HIFs by the tumour suppressor protein Von-Hippel-Lindau (VHL), which constitutes the recognition component of the E3-Ubiquitin Ligase complex involved in proteasomal degradation.(32,36). PHDs belong to a family of 2-oxoglutarate-dependent dioxygenases, and require for activity, oxygen as a substrate and ferrous iron as a co-factor. They also have a relatively high K_M for oxygen (particularly PHD2), making them important pO_2 sensors (34). When intracellular pO_2 is low, PHD activity is reduced, allowing HIF proteins to accumulate in the cytoplasm and then translocate to the nucleus prior to binding to HREs.

REGULATORY LINKS BETWEEN OXYGEN AND IRON HOMEOSTATIC PATHWAYS

Given the importance of iron to the biology of oxygen, it is perhaps not surprising that there are reciprocal regulatory links between the pathways for iron and oxygen homeostasis, operating both at the systemic and the cellular levels (Fig. 2).

Hypoxia impinges on iron homeostasis in the following ways. First, hypoxia increases the level of bone-marrow-derived erythroferrone (ERFE), which suppresses hepcidin. The mechanism by which hypoxia affects ERFE is not known, but it has been suggested that it involves elevation of EPO (24, 27, 52). Second, hypoxia increases the expression of the prohormone convertase furin, which is involved both in the transcriptional regulation of hepcidin through hemojuvelin and the bone morphogenetic protein receptor (BMPR) pathway, and in the processing of the hepcidin propeptide to generate the mature secreted peptide (55, 60). Third, hypoxia increases the level of IRP2 by inhibiting its degradation through the F-box and leucine rich repeat protein 5 (FBXL5) pathway (44). This effect could be of particular importance in the heart and muscle, because these tissues have the highest expression of IRP2 (61). Fourth, hypoxia upregulates dietary iron absorption in enterocytes through HIF2 α binding to an HRE in the ferroportin gene (33). Fifth, hypoxia upregulates the expression of a number of genes involved in iron homeostasis including TfR1, Dmt1, ceruloplasmin and heme-oxygenase 1, all of which are HIF-target genes (22).

Iron deficiency impinges on oxygen homeostasis as follows. First, intracellular iron deficiency stabilises HIF because iron is a co-factor required for the activity of PHDs (37). Thus intracellular iron deficiency induces the same transcriptional response as hypoxia. Second, iron deficiency causes translational repression of HIF2 α through an IRE in its 5'UTR, thus linking the expression of HIF2 α -regulated genes to iron availability (51).

In most cases, where there are effects of both iron deficiency and hypoxia on gene expression, these effects are concordant. However, there are exceptions. One example is the IRP1-mediated translational repression of HIF2 α by iron deficiency which has been demonstrated in the kidney (32, 36, 51). Another example is the HIF-mediated upregulation of the ferroportin gene that has been demonstrated in enterocytes (33, 49, 50). These features may allow the formation of important self-limiting feedback loops, which in turn can give rise to different patterns of response, depending on the severity of iron deficiency and hypoxia.

NOVEL MECHANISMS OF LOCAL IRON CONTROL IN THE HEART

Cardiac muscle is a major site of oxygen consumption. An adequate intracellular iron pool is essential to this aerobic activity. This is demonstrated by the finding that deletion of cardiac TfR1 in mice engenders fatal energetic failure in cardiomyocytes, even though systemic iron levels in that setting were maintained (62). This highlights the importance of understanding the mechanisms regulating intracellular iron within the heart. One feature of cardiomyocytes is that they express relatively high levels of ferroportin and hepcidin, despite having no role in systemic iron control (28, 29). Recently, the role of these in the heart has been explored using cardiomyocyte-specific disruption of the hepcidin/ferroportin axis. First, mice carrying a cardiomyocyte-specific deletion of the ferroportin gene were shown to develop fatal left ventricular dysfunction by three months of age. The dysfunction was caused by a three-fold increase in iron levels within cardiomyocytes. Importantly, downregulation of TfR1 was not sufficient to prevent iron overload in ferroportin-deficient hearts, suggesting that ferroportin-mediated iron release is an essential component of iron homeostasis in the heart. The total

quantity of iron in the heart giving rise to fatal cardiac dysfunction was far lower than in a standard mouse model of hemochromatosis that had been generated by ubiquitous deletion of hepcidin. In the ferroportin-deficient heart, iron was preferentially retained within the cardiomyocytes, whereas in the hemochromatosis model, most of the iron was outside of the cardiomyocytes, consistent with the marked upregulation of cardiomyocyte ferroportin in this model. Thus, not only is ferroportin essential for cardiomyocyte iron homeostasis, it also controls the site of deposition of iron in the heart in the setting of systemic iron overload. As such, it determines the severity with which cardiac iron deposition affects cardiac function (28).

The role of the cardiac hepcidin/ferroportin axis has been further investigated using two approaches to increase cardiomyocyte ferroportin (29). Cardiomyocyte-specific deletion of hepcidin resulted in fatal left ventricular dysfunction in mice between three and six months of age, despite the maintenance of normal systemic iron levels. A similar result was obtained in animals with cardiomyocyte-specific knock-in of the ferroportin isoform C326Y, which retains its iron export function but loses its hepcidin binding. In both settings, the cardiomyocytes were found to be iron-deficient due to increased iron export. Intravenous iron supplementation from three months of age prevented the development of cardiac dysfunction in cardiac-hepcidin knockouts, demonstrating the importance of cardiomyocyte iron deficiency in the cardiac dysfunction seen in these models. Taken together, these results demonstrate that the cardiac hepcidin/ferroportin axis is essential for the cell autonomous control of the intracellular iron pool upon which normal cardiac function depends (29).

The cardiac hepcidin/ferroportin axis appears also to protect the heart from the effects of systemic iron deficiency. Cardiac hepcidin protein was upregulated rather than downregulated by dietary iron restriction *in vivo* and by iron chelation *in vitro*. Furthermore, animals with hepcidin-deficient hearts developed a greater hypertrophic response to sustained dietary iron restriction than their littermate controls (29).

Figure 3 summarises the above results and provides a conceptual model of the role of iron export in cardiomyocyte iron homeostasis. One unanswered question is whether these findings in cardiomyocytes extend to other tissues that express both hepcidin and ferroportin, e.g. the brain and the kidney.

REGULATION OF THE CARDIAC HEPCIDIN/FERROPORTIN AXIS BY HYPOXIA

Hypoxia suppresses liver hepcidin, both at the transcript and the protein level, through a mechanism that is not cell-autonomous, i.e. a mechanism that is dependent on the paracrine action of non-hepatocyte derived factors, ERFE in this case. Here, we present previously unpublished results showing that the regulation of hepcidin by hypoxia is distinct in cardiomyocytes compared with hepatocytes in two key respects (Fig. 4). First, while the transcription of hepcidin is suppressed by hypoxia in both cell types, the suppression in cardiomyocytes is cell-autonomous because it can be demonstrated in isolated primary cardiomyocytes. The mechanisms underlying the cell-autonomous suppression of the cardiac-hepcidin transcript remain unclear. Second, in contrast with liver-hepcidin, cardiac-hepcidin protein is upregulated rather than downregulated by hypoxia, indicating that it is subject to

hypoxia-dependent effects at the post-transcriptional level; e.g. effects on the rate of translation or the rate of processing of the hepcidin propeptide. We propose that the differential effects of hypoxia on cardiac and liver-derived hepcidin peptide may relate to differences in the overall action of furin between hepatocytes and cardiomyocytes. Furin is a HIF-target gene, which has been shown to be upregulated by hypoxia in both hepatocytes and cardiomyocytes (29,55). In the liver, as well as being involved in the cleavage of the hepcidin propeptide to release the mature peptide (60), furin has been proposed to have a second action to cleave the BMPR co-receptor hemojuvelin, and so suppress hepcidin transcription (55). In contrast, in cardiomyocytes, we found that furin inhibition did not affect the level of hepcidin transcript. However, furin inhibition blocked the increase in secretion of the hepcidin mature peptide following treatment of cardiomyocytes with the iron chelator desferroxamine (29). Thus, hypoxia, through the upregulation of furin, acts to elevate levels of the mature hepcidin peptide released by the heart.

HYPOXIA, CARDIAC HEPCIDIN AND HEART DISEASE

So far, we have considered the long term consequences of dysregulated cardiac iron homeostasis on heart function. However, it is possible that cardiac hepcidin may induce more rapid changes in intracellular iron availability, as indeed is the case for the systemic regulation of iron availability by hepcidin (2). Rat studies have shown that hepcidin RNA and protein are upregulated in both the ischemic portion of the infarcted heart and in the serum of infarcted animals 24 hrs after myocardial infarction MI (56). In humans, hepcidin has been shown to be elevated in the serum following MI as early as 4 hours, in a manner that is independent of serum iron levels and of inflammatory markers (59). While it is unclear whether the changes in hepcidin are generated by the associated hypoxia or by some other factor, it is nevertheless clear that elevated serum hepcidin levels correlate with poor survival following cardiogenic shock in patients with acute coronary syndrome (31). These observations raise a question as to whether hypoxia and/or hepcidin acutely influence cardiomyocyte iron availability during MI. If so, this could well influence the degree of reperfusion injury and subsequent survival.

As well as its involvement in MI, hypoxia is a common component of chronic heart failure (10). In the setting of CHF, the administration of intravenous iron has been shown in a number of large clinical trials to be of sustained benefit (1, 4, 46). The mechanisms underlying the benefits of iron in this setting have yet to be resolved, but could include the local effects of iron on mitochondrial respiration (through enhanced synthesis and or activity of electron transport chain complexes) and on the HIF pathway (through modulation of PHD activity). The interplay between iron homeostasis and hypoxia in this disease setting could be an important pathological factor. Understanding this interaction may become increasingly important as novel therapeutics for targeting either oxygen or iron homeostatic pathways are developed, for example PHD2 inhibitors for the treatment of anaemia, hepcidin antagonists for the treatment of anaemia and hepcidin agonists for the treatment of iron overload (5,8).

CONCLUSION

Iron and oxygen homeostasis are intimately intertwined, both at the cellular and at the systemic level. In the heart, an organ of high oxygen demand, local iron levels are important for oxygen utilisation and energy production. This review has a particular focus on the importance of regulated iron efflux as a component of cardiomyocyte iron homeostasis.

250 Hypoxia impinges on the regulation of this pathway, and it is already clear that altering iron
251 homeostasis in chronic heart failure may influence outcome. Taken together, these
252 observations raise the question of to what extent may alterations in iron status influence heart
253 disease in which hypoxia is a significant component.

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AUTHOR CONTRIBUTIONS

S.L-L. drafted the manuscript and prepared the figures. S.L-L. and P.A.R. edited and revised the manuscript and approved the final version of manuscript.

DISCLOSURES

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Figure legends

Fig. 1. Systemic iron homeostasis. Most of the iron in the body is present in haemoglobin within mature red blood cells (RBCs, ~2 g) or their precursors in the bone marrow (~300 mg). The liver stores contain ~1 g in the form of ferritin. Other tissues, most notably the cardiac and skeletal muscles contain ~0.5 g, used for the synthesis of heme and iron-sulfur clusters, stored as ferritin, with a small amount free in the labile iron pool (LIP). Up to 25 mg of iron per day is recycled from senescent RBCs by the reticuloendothelial macrophages of the spleen. Approximately 1-2 mg of iron per day is acquired from the diet, with approximately the same amount lost through sloughing of senescent cells of the gastrointestinal tract. Free dietary iron is first reduced to ferrous iron by the ferric reductase duodenal cytochrome b (Dcytb), and then enters duodenal enterocytes via divalent metal transporter (DMT1). Iron in the form of heme can be taken up directly by the heme carrier protein (HCP1) and subsequently broken down by heme oxygenase (HO) to release the iron. The iron exporter ferroportin (FPN) mediates the efflux of ferrous iron from reticuloendothelial macrophages, duodenal enterocytes and hepatocytes. After efflux, ferrous iron is reduced to the ferric form by membrane ferroxidases (FXD), after which it is loaded onto transferrin for transport in the circulation. Approximately ~3 mg of iron is present in the form of Tf-Fe³⁺. A small amount of iron in the serum can also be found as non-transferrin bound (NTBI). Tf-Fe³⁺ is taken up into tissues by transferrin receptor (TfR), while NTBI can be taken up by DMT1 and other transporters, e.g. L-type calcium channels (LTCC) in the heart. Transferrin saturation (Tsat) is sensed in the liver through a receptor complex involving TfR1 and 2, hemojuvelin (HJV), hemochromatosis gene (HFE) and the bone morphogenetic protein receptor (BMPRI/II). When Tsat is elevated, the signalling complex is activated and hepcidin (HAMP) expression is increased. HAMP then binds to and induces the internalisation of FPN, thereby limiting iron release into the circulation.

Fig. 2. Pathways of iron and oxygen homeostasis. Iron-response proteins (IRPs) orchestrate cellular responses to intracellular iron levels. The hypoxia-inducible factor (HIF) pathway orchestrates cellular responses to oxygen levels. Hypoxia inhibits the degradation of IRP2, and upregulates the expression of the HIF-target genes, transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1), which encode proteins of iron uptake. Reduced intracellular iron induces the HIF pathway by inhibiting the HIF prolyl hydroxylase enzymes (PHDs). In addition, HIF2 α is subject to translational repression by IRP1 in response to iron deficiency. HIF2 α controls the transcription of erythropoietin (EPO) in response to hypoxia. In turn, EPO induces erythroferrone (ERFE) production in the bone marrow, which inhibits hepcidin expression in the liver. Hepcidin targets the iron-export protein ferroportin (FPN) in the gut. As well as being regulated by IRPs and hepcidin, FPN in gut enterocytes is upregulated HIF2 α in response to hypoxia. Responses shown in the grey box are ubiquitous to all cell types. Responses shown in yellow boxes are organ specific and relate to systemic iron homeostasis.

Fig. 3. Schematic model of cardiac iron homeostasis in various settings. TfR1 (not shown), DMT1 (not shown) and systemic iron availability determine the rate of uptake of iron into the cardiomyocyte. FPN controls iron export from the cardiomyocyte. In the wild type heart, cardiac hepcidin (HAMP) regulates cardiac FPN in order to maintain intracellular iron homeostasis. Loss of cardiac HAMP (cardiac HAMP knockout (KO)) or loss of cardiac HAMP responsiveness (cardiac FPN C326Y knock-in (KI)) result in cardiomyocyte iron deficiency

because of increased iron export resulting from increased cardiomyocyte FPN. Loss of cardiomyocyte FPN (cardiac FPN KO) causes cardiomyocyte iron overload. Upregulation of cardiac FPN also occurs from loss of systemic HAMP or systemic HAMP responsiveness (systemic FPN C326Y KI). Both cardiomyocyte iron deficiency and cardiomyocyte iron overload cause cardiac dysfunction.

Fig. 4. Hepcidin expression and regulation in the heart. (A) Relative Hamp mRNA expression in heart and liver of adult C57BL/6 mice under control conditions, or after provision of either low or high iron diets from weaning for 6 weeks, or following exposure to 11% O₂ for one week. *p<0.05 relative to control hearts, †p<0.05 relative to control livers. (B) Corresponding immunohistochemical staining for HAMP in heart and liver. (C) Relative Hamp mRNA expression in primary adult mouse cardiomyocytes cultured under control conditions, or in the presence of ferric citrate (FAC, 500uM for 8 hours), or with desferroxamine (DFO, 100uM for 8 hours), or in hypoxia (5% O₂ for 8 hours). *p<0.05 relative to control. (D) Corresponding HAMP protein levels in supernatants of primary cardiomyocytes. *p<0.05 relative to control. Scale bar=20μm. n=3 per group.







