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Regulation of lipocalin 2 (*Lcn2*) gene expression by iron stores, anemia and inflammation.

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Summary

Lipocalin 2 (Lcn2) is a putative iron-trafficking protein. The aim of this study was to determine whether Lcn2 plays a role in conditions associated with altered iron metabolism. Liver Lcn2 mRNA levels were assessed in mouse models of dietary iron overload and iron deficiency. Two models of anemia were analyzed, including acute hemolysis induced by phenylhydrazine treatment and repeated phlebotomies. The Lcn2 response to $CoCl_2$-induced hypoxia and acute and chronic lipopolysaccharide (LPS)-induced inflammation was also studied. Our results show that both types of anemia and hypoxia were associated with a dramatic increase in liver *lipocalin 2* gene expression and serum Lcn2 protein levels. Both acute and chronic inflammation up-regulate lipocalin 2 transcription and secretion. The up-regulation of *Lcn2* gene expression by anemia or hypoxia suggests an important physiological role for Lcn2 in iron metabolism and is in line with its recently proposed role in the transferrin-independent iron delivery pathway. The increase of Lcn2 expression during acute and chronic inflammation is in agreement with the finding that lipocalin 2-mediated iron sequestration constitutes a host-defense mechanism of the innate immune system.

Résumé

La lipocaline 2 (Lcn2) est une protéine active ayant pour fonction de favoriser la circulation du fer dans l'organisme. Le but de cette étude était de déterminer si la Lcn2 contribue ou non aux conditions associées à un métabolisme ayant un niveau de fer altéré. Les niveaux d'ARNm hépatique ont été déterminés dans des modèles de souris ayant soit une surcharge ou une carence en fer. Deux modèles d'anémie ont été analysés, incluant l'hémolyse induite par la phénylhydrazine, ainsi que les phlébotomies répétées. La réponse de la Lcn2 à l'hypoxie induite par le $CoCl_2$, ainsi que l'inflammation élevée et prolongée induite par les lipopolysaccharides (LPS), ont également été étudiées. Nos résultats démontrent que les deux types d'anémie et d'hypoxie sont associés à une augmentation drastique des niveaux d'expression génique de la Lcn2 dans le foie et des niveaux sériques de la protéine Lcn2. La relation entre l'expression du gène Lcn2, l'anémie et l'hypoxie suggère que la Lcn2 joue un rôle important dans le métabolisme du fer et confirme son rôle récemment proposé dans le métabolisme de transport du fer indépendant de la transferrine.

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List of abbreviations

ACD: anemia of chronic disease, anemia of inflammation

β2m: beta-2-microglobulin

Cp: ceruloplasmin

DCT1: divalent metal ion transporter 1

Dcytb: duodenal cytochrome b

DMT1: divalent metal transporter 1

EPO: erythropoietin

Fe^{2+}: ferrous iron

Fe^{3+}: ferric iron

FP1: ferroportin 1

GATA1: GATA binding protein 1

HAMP: hepcidin antimicrobial peptide

Hb: hemoglobin

HCT: hematocrit

Heph: hepheastin

HFE: hemochromatosis (gene)

HH: hereditary hemochromatosis

HIF1: hypoxia inducible factor 1

IFNγ: interferon-γ

IL-1: interleukin-1

IL-6: interleukin-6

IRE: iron responsive element

Ireg1: iron-regulated transporter 1

IRP: iron regulatory protein

Lcn2: lipocalin 2

LIP: labile iron pool

LPS: lipopolysaccharide

MCV: mean corpuscular volume

MHC: major histocompatibility complex

MTP1: metal transporter protein-1

NF-κB: nuclear factor kappa B

NGAL: neutrophil gelatinase-associated lipocalin

NRAMP1: natural resistance-associated macrophage protein 1

NRAMP2: natural resistance-associated macrophage protein 2

PHL: phlebotomy

PHZ: phenylhydrazine

RBC: red blood cell

ROS: reactive oxygen species

SI: serum iron

Tf: transferrin

TfR1: transferrin receptor 1

TfR2: transferrin receptor 2

TIBC: total iron binding capacity

TNFα: tumor necrosis factor α

TS: transferrin saturation

UTR: untranslated region

Acknowledgements

First and foremost, I would like to express my most sincere appreciation to my supervisor Dr. Manuela Santos, for her solid and skillful guidance, as well as her encouragement throughout this dissertation. I feel honored to have been her student, and value greatly the wisdom she has shared with me. Furthermore, I would like to acknowledge two of my colleagues, Hortence Markui and Marco Constante Pereira, both enthusiastic supporters of my efforts. I also thank Christian Dallaire in assistance of my work. Finally I would like to honor my homeland, the great country of China, which gave me this precious opportunity for personal enrichment, and the opportunity to pursue my professional passion of medical research.

Dedication

I dedicate my work to my loving and caring parents, who have always been there to encourage and support me. You both taught me the importance of friendship, respect, and love, for all aspects of life. Your love and strength have sustained my energy to complete this work.

I. INTRODUCTION

I. INTRODUCTION

Iron is an essential element for nearly all living organisms. It is involved in a large number of metabolic processes, including oxygen transport, DNA synthesis and electron transport. However, iron concentrations in body tissues must be closely controlled since too much iron can lead to tissue injury due to the generation of free radicals, while too little iron can lead to anemia. Thus, in light of the multiple biological functions and toxicity of iron, the existence of a variety of human diseases related to disturbed iron homeostasis is not surprising.

1. The significance of iron in biological systems

Iron is an essential element in the normal function of all cells. Many proteins are dependent on iron to perform their functions. These proteins illustrate the indispensable role of iron in biological systems. Proteins that contain iron can be classified into three categories: hemoproteins, iron-sulfur proteins and other iron-containing proteins.

1.1. Hemoproteins

Iron forms the core of heme, the main component of hemoproteins. They carry out three functions: (1) oxygen transport and storage via hemoglobin and myoglobin; (2) electron transport and energy metabolism via cytochromes; and (3) antioxidant functions through catalase and peroxidases, and beneficial pro-oxidant functions by myeloperoxidase. Oxygen transport through hemoglobin is one of the most important functions of iron. Through the ability of hemoglobin to acquire oxygen by contact and release it when

needed, hemoglobin in red blood cells (RBC) transports oxygen from the lungs to the rest of the body [1].

1.2. Iron-sulfur proteins

Iron-sulfur proteins form a second class of iron-containing proteins, in which iron atoms are bound to sulfur, and they also perform a variety of physiological functions. Rubredoxins and ferredoxins, for example, play roles in electron transport, and succinate dehydrogenase, nitrogenase and aconitase participate in redox and non-redox reactions [2].

1.3. Other iron-containing proteins

Other iron-containing proteins refer to a third class of non-heme, non-iron-sulfur iron-containing proteins. These include lipoxygenases, which oxidize fatty acids into leukotrienes, transferrin, which transports iron in the blood, and ferritin, which stores iron in cells.

The diverse range of functions performed by iron-containing proteins provides a sense of the indispensable role of iron in practically all forms of life.

2. Iron toxicity

In human systems, iron is found in two oxidation states: Fe^{2+} and Fe^{3+}. At physiological pH, Fe^{2+} is soluble while Fe^{3+} precipitates as oxyhydroxide polymers. The property of iron to readily participate in one-electron transfer reactions explains its potential to produce extremely toxic free radicals. Fe^{2+} can mediate Fenton reactions to produce hydroxyl

radicals ($OH^{\bullet}$) via the following reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$. Fe^{3+} can generate toxic radicals by catalyzing the Haber-Weiss reaction: $O_2^- + H_2O_2 \rightarrow OH^{\bullet} + O_2 + OH^-$ [3]. Hydroxyl radicals can cause damage to DNA, proteins, polyunsaturated fatty acids, mitochondrial DNA or lysosomes [4, 5].

The severity of iron-mediated toxicity is correlated with the concentration of low molecular weight iron in the labile iron pool within cells. Iron accumulation in tissues can increase the amount of low molecular weight iron within the pool, thus catalyzing the production of more toxic free radicals than are present in healthy individuals, in whom iron is stored as a cofactor for different proteins to prevent its toxic effects.

The disruption of normal iron metabolism has been shown to lead to a variety of iron disorders, ranging from anemia to iron overload to neurodegenerative disorders. Thus, systematic and cellular iron homeostasis must be properly maintained in the body in order to allow iron to be made available for physiological functions, and, at the same time, avoid its toxicity.

3. Disorders of iron metabolism in humans

The importance of maintaining iron homeostasis is clearly demonstrated by the existence of a group of diseases caused by disturbed iron metabolism.

3.1. Iron deficiency disorders

When iron is not available for cells involved in RBC production, hemoglobin synthesis is reduced, resulting in a reduction in the rate of erythropoiesis as well as the production of a microcytic red blood cell population. In the early stage of microcytic anemia, symptoms are often not apparent. In differential diagnosis, four types of microcytic anemia are considered: iron-deficiency, iron-transport deficiency, iron-utilization anemia, and iron-reutilization anemia (anemia of chronic disease).

3.1.1. Iron deficiency anemia

Iron deficiency anemia has had many names: anemia of chronic blood loss, hypochromic-microcytic anemia, chlorosis, hypochromic anemia of pregnancy, infancy, and childhood [6].

The most common cause of anemia is blood loss from the gasterointestinal tract. Women may bleed from menstrual loss. Pregnant women may lose iron to the developing fetus. Increased iron requirement, such as in infants less than two years of age, and reduced iron absorption, as in the case of gastrectomy, may also cause iron deficiency [7].

3.1.2. Iron-transport deficiency anemia

Atransferrinemia.

Iron-transport deficiency anemia is a rare disease caused by reduced levels of transferrin. In these individuals, iron cannot be transported from the intestine and liver to the bone

marrow for erythropoiesis. Hemosiderosis of lymphoid tissue, particularly along the gastrointestinal tract, often occurs in this type of anemia [8].

3.1.3. Iron-utilization anemias

Iron-utilization anemias include diseases such as hemoglobinopathies, primary thalassemia, and sideroblastic or myelodysplastic anemia [9]. Iron-utilization anemias are caused by the abnormal utilization of iron for hemoglobin synthesis, despite the normal presence of iron in the mitochondria of developing RBCs. Dyspoiesis increases the intramedullary death of RBCs, paradoxically leading to erythroid hyperplasia with a relative or absolute reticulocytopenia. In ferrokinetic studies, radiolabeled iron rapidly distributes from blood to the bone marrow, but fails to reappear in developing RBCs at a normal rate. Ineffective erythropoiesis is thus the main characteristic of these anemias.

3.1.4. Iron-reutilization anemia

Anemia of chronic disease (ACD), also called inflammation anemia, is the second most common form of anemia. In ACD, bone marrow erythropoiesis does not respond appropriately to anemic conditions. The early stages of the disorder show symptoms of normocytic anemia whereas microcytic anemia is characteristic of the later stages [10].

ACD results from complications of many chronic disorders such as infection, inflammatory disease, and cancer. Three pathophysiologic mechanisms are believed to be involved: (i) increased iron retention and storage within the reticuloendothelial system can induce hypoferremia, which limits iron availability to erythrons for hemoglobin (Hb) synthesis; (ii)

deficient erythropoiesis resulting from decreased erythropoietin (EPO) production or reduced bone marrow responses to EPO; and (iii) red blood cell survival times are reduced through mechanisms that are not entirely clear. In all three mechanisms, inflammatory cytokines are involved, including interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α).

3.2. Iron overload diseases

Chronic iron overload is characterized by an increase in tissue iron deposition either in a focal site or overall in tissues. The term hemosiderosis is commonly used for tissue iron overload. More severe states of iron overload (greater than five grams of iron present in the body) are referred to as hemochromatosis. Hereditary hemochromatosis (HH), caused by mutations in the hereditary hemochromatosis gene (*HFE*), must be differentiated from other genetic iron overload, non-genetic iron overload, and increased iron storage of undetermined etiology (Table 1).

Table 1. Classification of hemosiderosis and hemochromatosis.

I. Focal hemosiderosis
1. Pulmonary (idiopathic)
2. Renal
3. Hepatic (porphyria cutanea tarda)
II. Primary (genetic) hemochromatosis
1. *HFE* HH [*herediatory hemochromatosis (HFE)*] (HH Type 1)
2. Juvenile hemochromatosis [*Hemojuvelin (HJV)* and *Hepcidin (Hamp)*] (HH Type 2)
3. HH Type 3 [*transferrin receptor 2 (TfR2)*]
4. HH Type 4 [*Ferroportin1 (FP1)*]
5. African iron overload
6. Atransferrinemia [*transferrin (Tf)*]
7. Neonatal hemochromatosis
8. Aceruloplasminemia [*ceruloplasmin (Cp)*]
III. Secondary hemosiderosis or hemochromatosis
1. Congenital hemolytic anemias
2. Defective hemoglobin synthesis (thalassemia)
3. Increased parenteral iron intake, repeated transfusions
4. Iron dextran injections
5. Increased iron absorption (increased iron intake)
i. Ethiopian (teff cereal) hemosiderosis with hemochromatosis
ii. Oral iron therapy with hemosiderosis or hemochromatosis
Iii. Kashin-Bek disease with hemosiderosis

3.2.1. Focal hemosiderosis

Focal hemosiderosis occurs mainly as a result of other diseases. Lungs and kidneys are the most commonly affected sites [11, 12]. Pulmonary hemosiderosis is caused by recurrent pulmonary hemorrhage resulting in iron accumulation. Renal hemosiderosis is caused by various forms of hemolysis resulting in Hb deposits in the glomerulus. The renal parenchyma is able to maintain its normal function, but severe hemosiderinuria causes iron deficiency.

3.2.2. Type 1 HH (HFE-associated)

Type 1 hemochromatosis is an autosomal recessive iron disorder. A candidate gene for hemochromatosis (*HFE*) was found to be localized in the human chromosome 6p21 [13]. C282Y and H63D are two important mutations found in *HFE* [14]. The C282Y homozygote mutation has been found in 60–100% of Type 1 HH patients in the European population, and the H63D mutation is found in approximately 16% of these [13, 14]. *HFE* encodes a protein homologous to major histocompatibility complex (MHC) class I molecules. Although the HFE protein retains many of the structural features of classical class I molecules, its inward displacement of the $\alpha 1$ domain helix prevents peptide binding. Given that the HFE protein interacts with transferrin receptor 1 (TfR1), a molecule that plays a central role in cellular iron uptake [15], *HFE* is thought to influence iron uptake in cells. The body obtains iron through duodenal iron absorption. Duodenal iron absorption efficiency is related to the iron-sensing property of crypt cells, which receive signals about the body's iron requirements, in part through the binding of transferrin to the HFE/TfR1 complex. *HFE* mutations cause signaling of misinformation regarding iron requirements in the body. The consequence of this is excessive intestinal absorption of iron, which in turn leads to a progressive iron loading in the parenchymal cells of important organs such as the liver, pancreas and heart. This can result in various illnesses such as liver disease, diabetes mellitus and cardiomyopathy [16]. Phlebotomy is the most efficient method for removing excess iron in type 1 HH patients.

3.2.3. Non-*HFE* HH

Juvenile hemochromatosis (Type 2 HH)

Type 2 HH is a rare disorder originally identified in a gene heredity study of several Italian families [17]. Juvenile hemochromatosis patients have accelerated iron loading rates as compared with Type 1, HFE-associated HH [17]. Due to the rapid iron loading in Type 2 HH, cardiomyopathies and endocrinopathies occur more commonly than liver dysfunction. Most individuals with juvenile hemochromatosis do not survive beyond thirty years of age due to heart failure. Gene association studies in Type 2 HH have confirmed that the underlying *Hemojuvelin (HJV)* gene is located on human chromosome 1p [18]. *HJV* mutant patients have decreased hepcidin levels, which suggests that *HJV* may modulate hepcidin expression [19]. In addition, juvenile hemochromatosis was also found to be associated with mutations of *Hamp*, located on 19q13, which encodes hepcidin [20].

Type 3 HH

Type 3 HH is caused by mutations in the transferrin receptor 2 *(TfR2)* gene, and is an autosomal recessive disease. From fifteen Italian patients in five different families, four different mutations of *TfR2* have been reported. Patients show a chronic increase in iron absorption resulting in high iron accumulation in the liver, heart and pancreas, suggesting a disruption in intestinal iron absorption due to the *TfR2* mutation [21].

Type 4 HH

Type 4 HH, an autosomal dominant disease, is caused by ferroportin 1 *(FP1)* gene mutations [22, 23]. Iron concentrations increase in the liver, heart and pancreas in type 4 HH patients. Liver macrophages contain high amounts of iron and serum iron is relatively low. Two hypotheses regarding type 4 HH have been put forward at present. The loss-of-function hypothesis suggests that *FP1* mutations impair iron recycling by macrophages, subsequently delivering abnormal signals to the intestine (mechanism as yet not understood), resulting in increased iron absorption and subsequent iron overload [23]. The gain-of-function hypothesis suggests that *FP1* mutations increase iron absorption in the duodenum [22]. In type 4 HH patients, since iron overload is combined with anemia, phlebotomy treatment is not recommended.

African iron overload

In the sub-Saharan region of Africa, approximately 10% of the population has a predisposition for iron overload, also referred to as Bantu siderosis [24]. A risk factor for African iron overload is thought to be dietary iron intake from traditionally brewed beer. Since the prevalence of iron overload in this region is high and not all beer drinkers are affected by iron overload, it is difficult to trace the inheritance pattern. Statistical data, however, suggests that it is an autosomal dominant inherited disease, and the *HFE* gene has been excluded as the causative gene for African iron overload [25]. In African iron overload patients, liver disease occurs more commonly than cardiomyopathy or diabetes. In addition, iron accumulates in hepatic Kupffer cells and spleen in large amounts, suggesting a defect in iron recycling by the reticuloendothelial system [26].

Atransferrinemia

Atransferrinemia is a very rare disease in which transferrin mutations cause impaired erythropoiesis, but iron accumulation is high in the liver due to an increase in non-transferrin bound iron (NTBI) [8, 27].

Neonatal hemochromatosis

Neonatal hemochromatosis is a rare, poorly characterized disease that occurs in newborn infants, with high iron accumulation in the liver, heart and/or pancreas [28]. Liver failure may occur. It is unclear whether iron overload initiates the disease, or if other causes lead to iron overload [29]. Neonatal hemochromatosis is usually jointly present with other congenital diseases with similar liver manifestations, which suggests that it is a heterogenous disorder [30]. Familial cases have not been reported, and *HFE* gene mutations are not found in neonatal hemochromatosis. Liver transplantation is the only efficient therapy and has been found to prolong life in several cases [31].

3.2.4. Secondary iron overload (non-genetic)

Secondary iron overload includes transfusional iron overload and iron overload attributed to defective erythropoiesis (congenital hemolytic anemias or hemoglobinopathies). In thalassemia major patients given transfusions without chelation, iron accumulation in the body is accelerated. Transfusional iron accumulation leads to progressive damage of the heart, liver and endocrine glands [32]. Iron overload-induced heart disease determines the

survival rate of the transfused patients [33]. Liver disease is the second leading cause of death in these cases [34].

3.2.5. Iron overload of undetermined origin

Some liver diseases are associated with elevated iron storage, for example, alcoholic liver disease, non-alcoholic steatohepatitis, and chronic hepatitis C [35]. The mechanisms are unknown. Although the patients do not have a genetic hemochromatosis background, iron-unloading management may improve liver function [36].

3.2.6. Neurodegenerative disorders related to iron metabolism

Neurodegenerative diseases related to iron metabolic disorders can be classified into two categories based on the underlying iron defect: mitochondrial iron disturbance and brain iron disturbance. Mitochondrial iron disturbance is linked to Friedreich's ataxia and X-linked sideroblastic anemia with ataxia. Brain iron disturbance is related to neuroferritinopathy, pantothenate kinase deficiency and acerulosplasminaemia.

Friedreich's ataxia

Friedreich's ataxia is an inherited lethal disease, and is characterized by spinocerebellar degeneration, cardiomyopathy and diabetes mellitus. In gene linkage studies, Friedreich's ataxia was found to be associated with the gene encoding frataxin located on chromosome 9q [37]. Expansions of a trinucleotide repeat in an intron are usually found in patients, but point mutations have are also been identified [37]. Affected patients usually have defects in both alleles of the frataxin gene, ultimately leading to decreased frataxin levels. Frataxin is

a mitochondrial protein whose exact function remains undetermined. Lack of frataxin homologues in yeast and mice leads to increased mitochondria toxicity, due to iron accumulation, elevated sensitivity to oxidative stress and depletion of iron-sulfur proteins [38, 39].

X-linked sideroblastic anemia with ataxia

X-linked sideroblastic anemia with ataxia presents as mild microcytic anemia, bone marrow sideroblastosis and nonprogressive cerebellar ataxia [40]. It is caused by an isoleucine to methionine substitution (I400M) in the ATP-binding cassette transporter 7 (ABC7) gene, located on chromosome Xq. The exact pathogenesis of this disease is unknown. However, since Atm1p, the *S. cerevisiae* homologue of human ABC7, plays a role in iron export in yeast mitochondrion [41], iron accumulation within human mitochondria is thought to result from the loss-of-function mutation in ABC7.

X-linked sideroblastic anemia

X-linked sideroblastic anemia with ataxia is distinct from the non-neurodegenerative classical X-linked sideroblastic anemia, which is an X-linked recessive disease caused by mutations in the erythroid aminolevulinic acid synthase (e-ALAS) gene located on chromosome Xp [42]. The e-ALAS enzyme catalyzes the first step of heme synthesis. Dysfunctions in e-ALAS cause a defect in heme synthesis, leading to iron accumulation in the mitochondria of erythroid precursors. Impaired erythropoiesis induces increased iron absorption from the intestine, resulting in iron overload. The anemia is usually normocytic; microcytic anemia is less common [43].

Neuroferritinopathy

Neuroferritinopathy is a late-onset disease of the basal ganglia, in which patients present with extrapyramidal features similar to those found in Parkinson's disease. Patients with neuroferritinopathy usually have abnormal iron and ferritin aggregates in the brain. Mutations in the ferritin light chain gene leads to a structural change in the encoded protein. The resultant ferritin dysfunction causes iron release from ferritin and iron accumulation in axons or synapses. This iron accumulation enhances oxidative stress in the neuron and results in neurodegeneration [44].

Pantothenate kinase-deficiency

Pantothenate kinase (PANK2)-deficiency is an example of a brain iron-overload disease involving a gene whose function is not related to iron metabolism. PANK2-deficiency usually develops within the first two decades of life, and clinically presents as dystonia, pigmentary retinopathy, optic atrophy, and high concentrations of iron in the globus pallidus [45]. PANK2 gene mutations cause a block in the coenzyme-A biosynthesis pathway. The subsequent increase in cysteine concentrations in the brain leads to iron accumulation due to the iron-chelating properties of cysteine. The increased free radical production and oxidative stress derived from iron accumulation result in neurodegeneration [46].

Acerulosplasminaemia

Aceruloplasminemia is a rare disease, in which decreased ceruloplasimin levels in plasma cause an inability for Fe^{2+} to be oxidized to Fe^{3+}, which is critical for the binding of iron to

transferrin. In acerulosplasminaemia, mutations in the ceruloplasmin gene leads to a decrease in iron released from neurons of the retina and the basal ganglia. Consequently, regional iron accumulation enhances neuronal oxidative stress, resulting in progressive neurodegeneration [47-49].

All the disorders mentioned in this section demonstrate the importance of maintaining iron homeostasis. They emphasize the importance of understanding the principles regulating iron metabolism at the cellular and systemic levels. Within the past 10 years, advances in molecular biology techniques have permitted a dramatic increase in the information available on the various steps of iron metabolism: iron uptake, transport, storage, export and utilization.

4. Cellular iron homeostasis

Cellular iron homeostasis needs to be tightly controlled through iron uptake, storage and export. Thus, the molecular understanding of each step of iron metabolism helps elucidate the principles of normal iron homeostasis and the mechanisms of iron disorders.

4.1. Iron uptake

Iron uptake is necessary for all cells. This section will detail identified pathways of cellular iron uptake: (i) classic transferrin-dependent iron uptake, which is used by almost all cells; (ii) cell-type specific iron uptake in macrophages and enterocytes; and (iii) transferrin-independent iron uptake, which plays a minor role.

4.1.1. Transferrin-dependent iron uptake

Transferrin is the most important iron-transporting protein in the blood, and it binds iron with high affinity. In normal situations, all circulating iron is bound to transferrin. Two functions are served by this transferrin-iron binding: (i) the prevention of free-iron derived toxicity through binding of Fe^{3+} in the blood; and (ii) the facilitation of iron transport in the plasma and iron uptake into cells. Plasma transferrin is mainly synthesized in and secreted from the liver into the blood, where it mediates the exchange of iron among the various sites of absorption, recycling, utilization, and storage.

The delivery of transferrin-bound iron into the cell is mediated by transferrin receptors located on the cell surface. Receptor-mediated endocytosis is the classical pathway used by almost all mammalian cells in order to obtain iron [50-52]. Transferrins and the *transferrin receptor 1* (TfR1) are the components of this classical iron uptake and transferrin-recycling pathway (Figure 1). Cell surface transferrin receptors bind to the iron-transferrin complex with high affinity under neutral pH conditions [53]. Once the transferrin receptor-bound iron-transferrin complex has been endocytosed, it is internalized into endosomes with a neutral pH. ATP-dependent proton pumps subsequently acidify the endosomal interior to pH 5.5 [54-57], which facilitates the dissociation of ferric iron (Fe^{3+}) from transferrin. The ferric iron released within the acidified endosomes is converted to ferrous iron and then transported by the divalent metal transporter 1 (DMT1) into the cytoplasm [58]. Transferrins remain bound to their receptors after the release of iron in the acidified endosomes, and are recycled to the cell surface. In the neutral (pH 7.4) environment at the cell surface, the non-iron bound transferrins lose affinity for their receptors, promoting their

dissociation. Transferrin is thus recycled back into circulation [51, 55]. The recycling process of transferrins enables one transferrin to be re-used up to one hundred times over the course of its eight-day half-life.

Transferrin receptor 2 (TfR2) and cubilin are two alternative receptors for transferrin. TfR2 is highly homologous to TfR1, yet it binds transferrin with a lower affinity [59]. The fact that TfR2 is only expressed in the liver and cannot rescue TfR1-mutant mice from early death suggests that TfR2 and TfR1 do not share the same roles [60, 61]. Cubilin is a novel potential transferrin receptor, which has been shown to mediate endocytosis of the transferrin-iron complex, albeit only in the proximal tubules of the kidney and the yolk sac [62].

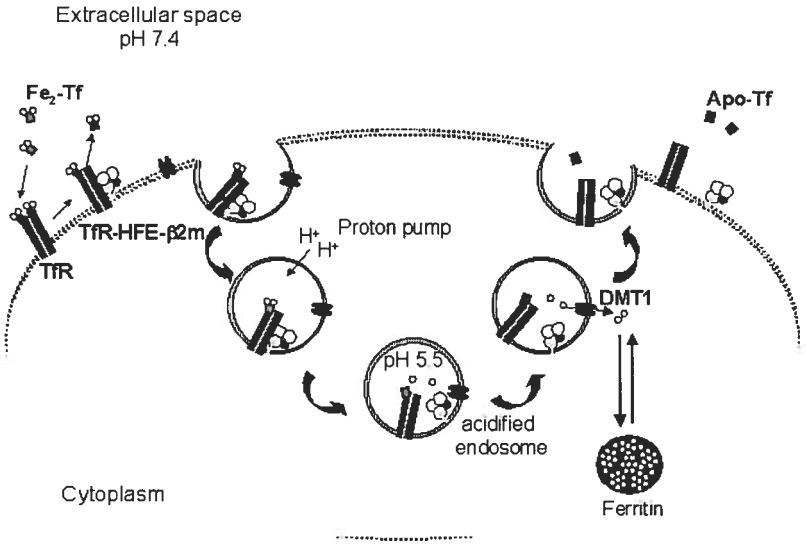

Figure 1. The transferrin (Tf) cycle. Iron-loaded transferrin (Fe_2-Tf) binds to transferrin receptors (TfR), which associate with β2m-HFE molecules on the cell surface. Apo-transferrin (Apo-Tf) and transferrin receptors can be re-used for further cycles of iron binding and iron uptake.

4.1.2. Iron uptake by reticuloendothelial (RE) macrophages

Macrophages can obtain iron in three ways: (i) transferrin receptor-mediated iron uptake; (ii) cell surface receptor CD163-mediated hemoglobin/haptoglobin complexes uptake [63]; and (iii) phagocytosis of senescent or damaged RBC (Figure 2). In the latter two pathways, hemoglobin is broken down by heme oxygenase 1. Iron released from hemoglobin is subsequently transported from the phagosome into the cytoplasm by DMT1. In the cytoplasm, iron will either be stored in ferritin or transported back into the blood by ferroportin 1. Macrophages are the most important cells for iron recycling because they can obtain iron from RBCs and release digested iron back into the circulation for the development of erythron precursors.

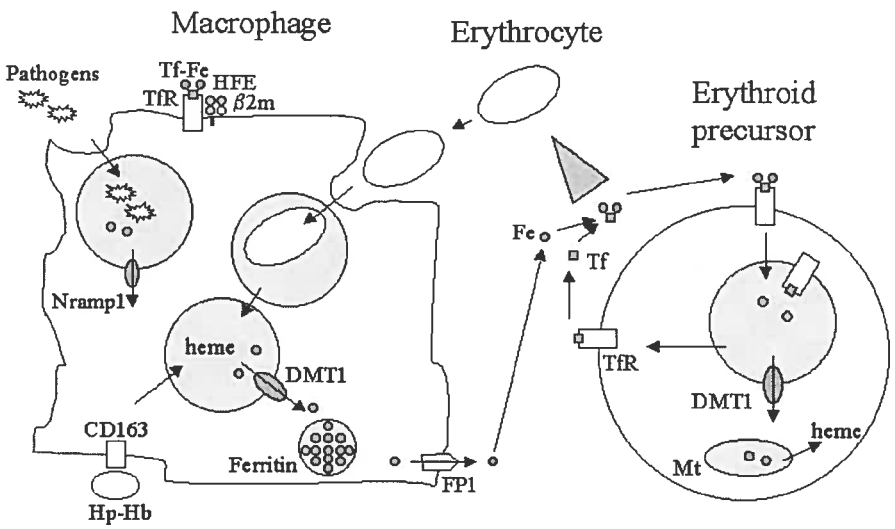

Figure 2. Macrophage iron uptake and recycling. Macrophages can obtain iron by three different mechanisms: (i) TfR-mediated uptake of transferrin-iron complexes; (ii) CD163-mediated acquisition of the haptoglobin/hemoglobin complex; and (iii) phagocytosis of senescent RBCs. In erythroid precursors, iron is taken up for heme biosynthesis through a TfR-mediated mechanism. Nramp1: natural resistance-associated macrophage protein 1, Hp-Hb: hemoglobin/haptoglobin complexes.

4.1.3. Iron uptake by intestinal absorptive cells

Dietary-derived iron is the normal source of exogenous iron. The intestine thus performs an important function in absorbing iron from exogenous sources. The developing enterocyte and mature enterocyte obtain iron through two different pathways. In the duodenum, the apical villus enterocyte matures and migrates from developing crypt cells. The former can absorb exogenous iron, whereas the latter does not. Crypt cells can take up iron only

through the transferrin receptor-mediated pathway. This section will focus on iron absorption by the apical absorptive cells of the duodenum. Low molecular weight iron and heme-iron are the two forms of iron that can be absorbed in this manner.

The process of transporting dietary-derived low molecular weight iron across the intestinal absorptive cells occurs in three steps (Figure 3): (i) uptake of iron through the apical membrane; (ii) distribution of iron in the cell and transport of iron to the basolateral membrane; and (iii) the transfer of iron through the basolateral membrane into the blood [64, 65]. In the first step, dietary ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by the ferric reductase Dcytb, which is located at the apical membrane [66]. Fe^{2+} is then transported into the cell cytoplasm by DMT1. Iron thus joins the intracellular labile iron pool (LIP) [67], from which iron either is directed to ferritin, where iron is stored, or is transported further across the basolateral membrane into the blood by the iron exporter called ferroportin 1 [68].

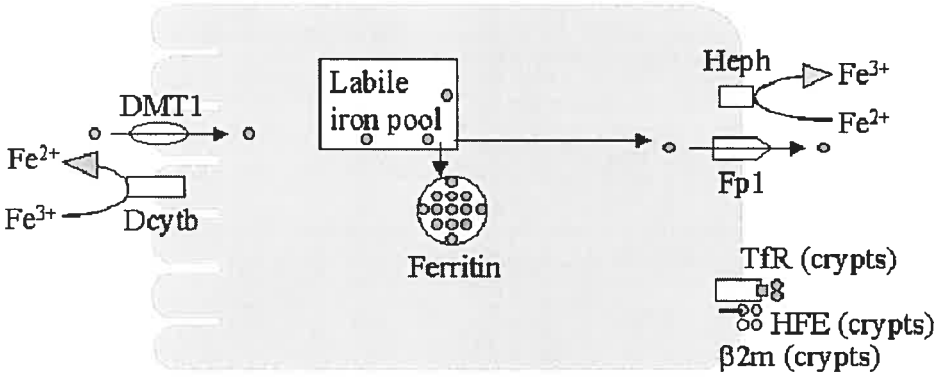

Figure 3. The process of intestinal iron absorption. Dietary iron is taken up through the apical membrane of the intestine into the cytoplasm. Iron is then transferred across the basolateral membrane into the blood. Only crypt cells of the intestine obtain iron via the TfR-mediated pathway. Heph: hepheastin.

It is noteworthy that iron contained in heme derived from meat (containing hemoglobin, myoglobin and other heme proteins) is another important source of iron. Dietary heme iron accounts for approximately 30% of the total dietary iron in North American and Europe [69]. Heme-derived iron can be released by heme oxygenase 1 in the enterocyte, although a heme receptor at the apical membrane has not yet been identified.

An alternative ferric iron uptake mechanism has been identified for dietary iron absorption, referred to as the integrin-mobilferrin pathway (IMP). IMP transports only ferric iron (Fe^{3+}), and no other metals, into enterocytes [70]. Proteins associated with this pathway include mobilferrin, β3-integrin and flavin monooxygenase, whose macromolecular complex (520 kDa) is called paraferritin. The IMP complex is only found in enterocytes, and it is not known to what extent this pathway contributes to dietary iron absorption.

4.1.4. Transferrin-independent iron uptake

The existence of transferrin-independent iron uptake is suggested by two classes of iron disorders: transferrin shortage (atransferrinemia) and iron overload (hemochromatosis,

thalassemia). In hypotransferrinemia the reduction of transferrin levels leads to severe hypochromic microcytic anemia and massive iron loading in all non-hematopoietic tissues, in particular in the liver and pancreas [71-73]. In hereditary hemochromatosis and transfusional thalassemia, two iron overload diseases, non-transferrin bound iron (NTBI) is found in the blood [74-77]. NTBI distributes iron into tissues different from those covered by transferrin-bound iron [74-77]. Furthermore, three pathways have been identified which support the existence of a transferrin-independent iron uptake, mediated by ferritin, L-type Ca^{2+} channels, and lipocalin 2.

Ferritin-mediated iron uptake is supported by several studies. In rats, when ferritin containing radiolabeled iron is injected into the circulation, iron is taken up and stored in the liver [78]. In addition, the liver expresses ferritin receptors, and hepatocytes take up iron-containing ferritin via receptor-mediated endocytosis [79].

The L-type Ca^{2+} channel provides a pathway for iron entry into cardiomyocytes. This pathway is organ specific, because L-type Ca^{2+} channels are specific to cardiomyocytes. This pathway is important in iron overload conditions, as high concentrations of NTBI can utilize this pathway to enter the heart, leading to cardiac iron accumulation, and consequent cardiomyopathy [80].

Lipocalin 2 is a newly identified putative iron transporter. *Lipocalin 2* is the mouse homologue of the human neutrophil gelatinase-associated lipocalin (*NGAL*) gene. Structural analyses have shown that lipocalin 2 can bind siderophores (bacterial-derived

iron binding compounds) and iron [81]. Lipocalin 2-mediated iron transport has been shown to deliver amounts of iron sufficient for the regulation of iron-regulated gene expression in several cell lines [82], including mouse embryonic kidney collecting duct cells, human Wilms tumor kidney cells, dog kidney collecting duct cells, rat embryonic kidney epithelial cells and mouse kidney stroma cells. Lipocalin 2-mediated iron transport can also provide iron for the development of the mesenchyme into epithelia during kidney development [82].

4.2. Iron storage

When iron is present in the cytoplasm, ferrous iron is carried by a carrier molecule to the labile iron pool (LIP). This iron carrier molecule has not been identified, because of the very small amount of iron in the LIP. In the cytoplasm, iron diffuses to various intracellular compartments, such as the mitochondria for heme biosynthesis, or to ferritin for storage.

Ferritin is a heteropolymer complex, composed of twenty-four subunits of H (heavy/heart) and L (light/liver) chains [83]. The central cavity of ferritin can store up to 4500 iron ions [83]. The composition of ferritin varies with the ratio of H and L chains, depending on the organ in which it is found (Figure 4). Ferritin found in the liver and spleen is rich in L-chains, which are associated with high iron storage, whereas ferritin found in the heart is rich in H-chains, which are associated with high iron utilization [83, 84]. Ferritin thus has a variable iron storage capability, with a high H:L ratio when heme synthesis or cell proliferation increases [54, 85]. The degradation of ferritin and the associated release of

iron facilitate the mobilization of iron for cellular utilization, and is associated with lysosomes [86, 87].

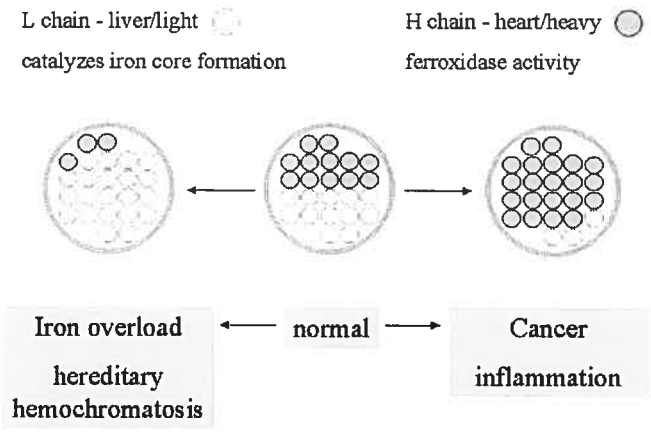

Figure 4. The composition of ferritin. Each ferritin molecule is composed of 24 chains, either L or H chains. The ratio of L and H chains in ferritin varies among organs. Furthermore, the L/H chain ratio in ferritin can be modified in response to iron overload or inflammation.

4.3. Iron export

Duodenal enterocytes, macrophages, hepatocytes and other types of cells have similar mechanisms for releasing iron in order to meet the iron needs of the whole body and also to maintain iron balance within cells. Ferroportin 1 (FP1) is a ubiquitously expressed protein. Ferroportin 1 functions as a cellular iron exporter, by transporting iron from the cytoplasm into the blood [88, 89]. However, ferroportin 1 needs the cooperation of a ferroxidase to perform this function (Figure 5). Two types of copper ferroxidases are found in the body:

ceruloplasmin (Cp) and hephaestin, both of which oxidize Fe^{2+} into Fe^{3+}, for Fe^{3+} binding to transferrin in the blood.

Hephaestin is the homologue of the plasma protein ceruloplasmin. Amino acids involved in copper binding and disulfide bond formation are conserved between ceruloplasmin and hephaestin. Unlike ceruloplasmin, hephaestin is a trans-membrane protein. Since ceruloplasmin circulates in the blood and hephaestin is expressed only in the intestinal villi, ceruloplasmin can facilitate iron export from non-intestinal cells while hephaestin mediates intestinal iron export. Studies in hephaestin knockout mice have shown that ceruloplasmin cannot replace the role of hephaestin in the process of enterocyte iron export [90, 91].

The ceruloplasmin protein is a member of the multicopper oxidase family, which can utilize copper to catalyze biochemical reactions [92]. Since ceruloplasmin contains greater than 95% of the copper found in plasma, it was first thought to be involved in copper metabolism. However, ceruloplasmin knockout mice exhibited normal copper metabolism during copper absorption, transportation, distribution, and excretion steps [93]. These evidence suggest that copper is only required for ceruloplasmin to carry out ferroxidase functions, and that ceruloplasmin does not play an essential role in copper metabolism.

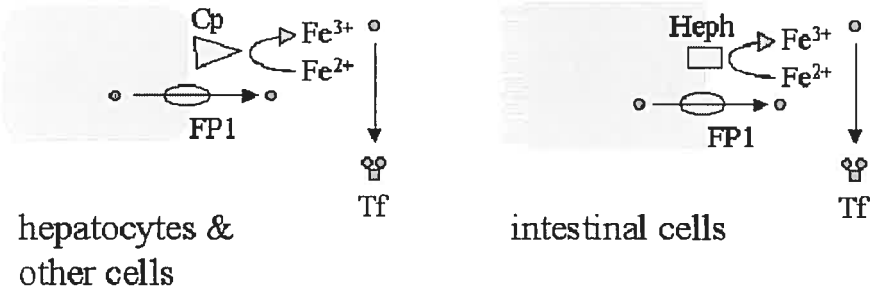

Figure 5. Iron export facilitated by ceruloplasmin (Cp) and hephaestin (heph). Ceruloplasmin and hephaestin are structurally similar, and both have ferroxidase activity, which can oxidize Fe^{2+} into Fe^{3+}.

4.4. Cellular iron homeostasis

Cellular iron homeostasis is regulated by the iron responsive protein/iron responsive element (IRP/IRE) system in the cell (Figure 6). IREs are found in the 3'- or 5'-untranslated mRNA regions of several critical proteins involved in iron metabolism, including ferroportin 1, DMT1, ferritin, and TfR1 [94, 95]. IRPs normally exist in the cytoplasm as cytosolic aconitases. IRPs can bind to IREs to regulate the translation of proteins.

The affinity of IRPs to bind IREs is dependent upon the iron concentration in the labile iron pool (LIP). Under high iron concentrations, IRPs are filled with a 4Fe-4S cluster that prevents the binding of IREs. Under low iron conditions, the 4Fe-4S cluster dissociates from IRPs, which can then bind to IREs.

Through the binding of IRPs to IREs, the IRP/IRE system post-transcriptionally controls protein synthesis. The location of IREs on either the 5'- or 3'-untranslated region of mRNAs determines the consequences of IRP-IRE binding. For example, in ferritin, where 1 IRE is located in the 5'-untranslated region of the mRNA, the IRP/IRE binding blocks the translation pathway, thus decreasing ferritin synthesis. Conversely, in transferrin receptor 1, where IREs are located to the 3'-untranslated region of the mRNA, the IRP/IRE binding stabilizes the mRNA, and thus enhances TfR1 synthesis.

Through the regulating activity of the IRP/IRE system, the up- or down-regulation of iron-responsive genes enables the cell to maintain proper iron homeostasis. For example, when the iron concentration is high in the LIP, ferritin protein synthesis is up-regulated to store more iron, while TfR1 is down-regulated, reducing the uptake of iron.

The IRP/IRE system can be influenced not only by iron concentrations, but also by nitric oxide (NO) levels, oxidants and hypoxia [96, 97]. These factors provide the cell with a greater degree of control over iron levels via a complex self-regulation mechanism.

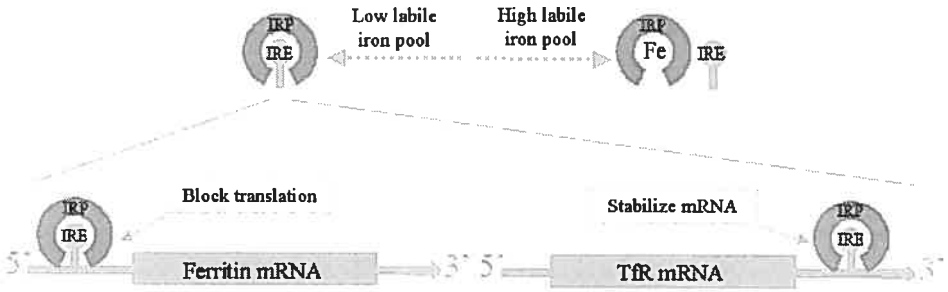

Figure 6. The IRP/IRE regulation system. The affinity of IRPs for IREs depends on the iron level in the labile iron pool. When the iron level is low, the binding of IRPs to IREs at the 5'-untranslated region blocks mRNA translation, while binding of IRPs to IREs at the 3'-untranslated region increases mRNA stability, and thus protein synthesis.

5. Systemic iron homeostasis

5.1. Distribution of iron in adults

The average human has on average 4g of total body iron and approximately 1-2mg of iron are lost daily from the epithelial surfaces of the gut and skin and, in females, through menstruations (Figure 7). These daily losses are replenished through the transfer of ingested dietary iron to the portal circulation. Internal iron requirements, for the production of hemoglobin, are met by recycling iron from the destruction of RBCs by the reticuloendothelial (RE) system and subsequent release of iron into the plasma, where iron is transported while bound to transferrin (Tf) [98]. Excess iron is stored in ferritin in the liver in a non-toxic form that can be mobilized as required. Under normal conditions, liver iron stores are close to 1g, but growing children and menstruating women have reduced liver iron stores due to their increased iron requirements [99]. Another smaller proportion of iron is located in muscle myoglobin and body enzymes.

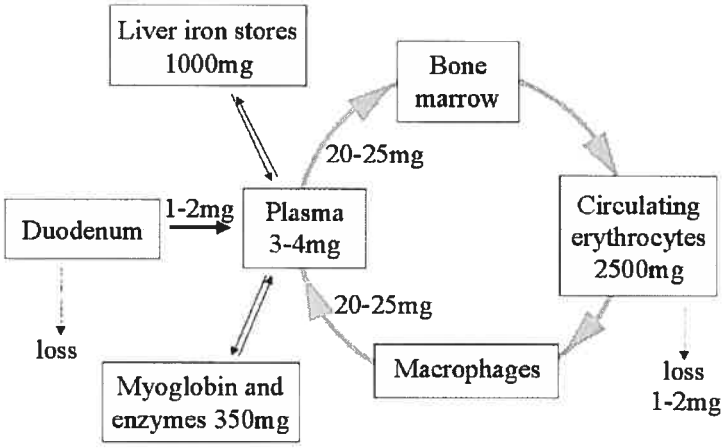

Figure 7. Distribution of iron in adults. Iron is absorbed from the duodenum, transported into the plasma, recycled by macrophages, utilized by erythrocytes and enzymes, and stored in the liver. Iron regulatory factors can change the function of these organs and cells, thus redistributing iron to establish a new iron homeostasis in the body.

5.2. Iron regulators

5.2.1. The four iron regulators

Systemic iron homeostasis is maintained through the control of iron absorption, iron stores and iron recycling to meet the iron demands of erythropoiesis and iron-dependent enzyme activity.

Four iron regulators can change iron mobilization and iron redistribution in the body: (i) The iron stores regulator refers to the amount of iron stored in the body [100]; (ii) the hypoxia regulator refers to oxygen insufficiency in the body; (iii) the erythropoetic

regulator refers to the need for the production of RBCs; and (iv) the inflammatory regulator refers to infection and inflammation stimuli.

In normal adults, acute iron requirements are satisfied by the release of stored iron from the liver and recycled iron from macrophages. When the release of iron from body stores cannot meet the iron demand, as is the case in iron deficiencies, iron absorption from the intestine is accelerated. The erythropoetic regulator can dramatically increase iron absorption from the intestine. It can even override the effect of the stores regulator. For instance, in transferrin-mutant mice, hypotransferrinemia causes insufficient iron delivery into erythroid precursors. These mice develop severe anemia, which requires increased iron absorption. Paradoxically, the mouse also has iron overload in the body, which leads to a decrease in iron absorption. In this case, the erythropoetic regulator will dominate the stores regulator, and thus increased iron absorption from the intestine continues [101].

The activation of the hypoxia regulator can also increase iron absorption from the intestine, which produces an effect similar to that of the erythropoetic regulator. However, the interaction of the hypoxia regulator with the other regulators has not yet been fully investigated.

Infection and inflammation is another strong regulator of iron metabolism. The pathophysiologic response of iron metabolism to inflammation is to withhold iron from invading bacterial pathogens, through reducing iron release from the liver and the macrophages and by suppressing intestinal iron absorption, thus decreasing serum iron

levels. The inflammatory regulator can override the erythropoetic regulator. For example, in anemia of chronic diseases, inflammatory cytokines reduce bone marrow RBC production [102-104]. Such anemia activates the erythropoetic regulator to increase iron absorption from the intestine. Paradoxically, at the same time, the inflammatory regulator produces a decrease in iron absorption. In this contradictory situation, the inflammatory regulator dominates the erythropoetic regulator, ultimately resulting in a decrease in intestinal iron absorption.

This hierarchy between iron regulators predicts the existence of messengers circulating in the blood needed to deliver signals among the sites of iron utilization, storage, absorption, and recycling, to correctly coordinate the efficiency of iron mobilization among these sites.

5.2.2. Hepcidin, a putative iron messenger

Hepcidin is a newly identified iron messenger. It is a small peptide secreted by the liver and excreted through the kidneys [105-107]. Hepcidin can respond to all four known iron regulators. For example, hepcidin expression is increased by iron overload diets in rodents, which represents an iron store regulator [107]. It is also reduced in non-anemic hypoxia, a hypoxia regulator [108], and is elevated in response to anemia, an erythropoetic regulator [109]. Finally, hepcidin expression is increased in different types of inflammation in mice and humans, which represents an inflammatory regulator [108-110].

Hepcidin functions as a regulator of iron absorption in the intestine and iron release from macrophages, as has been demonstrated in hepcidin-knockout mice and hepcidin-transgenic

mice. Hepcidin depletion in knockout mice is associated with increased body iron [111, 112]. Increased hepcidin levels in transgenic mice results in iron deficiency [112]. Thus, the level of serum hepcidin provides a counterbalance to the level of body iron stores. This regulation may be explained by the recent finding that hepcidin regulates iron efflux via its binding to ferroportin 1 (FP1). This binding subsequently leads to FP1 internalization and degradation, resulting in a decrease in FP1 concentration at the cell surface [113].

5.3. Animal models

Studying animal models takes advantage of the similarities between human and rodent iron metabolism regulation. Specific gene disruptions in rodents, including targeted disruptions and spontaneous mutations, have provided crucial information about each gene involved. Studying the function of targeted genes in these rodents has extended our knowledge of the pathways of iron metabolism at the molecular level and the defects that occur in iron metabolism disorders. (Table 2)

Table 2: Useful rodent models for understanding iron metabolism and related diseases.

Mouse model	Gene affected	Iron phenotype	human disease modeling
$Hfe^{-/-}$	Hfe	Increased iron absorption, iron overload, hepatocellular iron deposition, decreased macrophage iron, elevated transferrin saturation	Type 1 HH
$\beta 2m^{-/-}$	β-2 microglobulin	Increased iron absorption, iron overload, hepatocellular iron deposition, decreased macrophage iron, elevated transferrin saturation	Type 1 HH
$Usf2^{-/-}$	Hepcidin	Increased iron absorption, iron overload, hepatocellular iron deposition, decreased macrophage iron, elevated transferrin saturation	Type 2 HH
Hamp (liver transgene)	Hepcidin	Decreased iron absorption, severe iron deficiency and anemia	—
Hpx	Transferrin	Microcytic hypochromic anemia, tissue iron deposition	Atransferrinemia
$TfR^{-/-}$	Transferrin receptor-1	Microcytic hypochromic erythrocytes, decreased iron stores, embryonic lethality	—
$TfRr2^{245x/245x}$	Transferrin receptor-2	Increased iron absorption, iron overload	Type 3 HH
b (Belgrade rat)	DMT1(G185R)	Decreased iron absorption, systemic iron deficiency, impaired iron uptake in duodenum and in erythroid precursors	—
mk	DMT1(G185R)	Decreased iron absorption, systemic iron deficiency, impaired iron uptake in the duodenum and in erythroid precursors	—
Weh (Weissherbst)	Ferroportin 1	Hypochromic anemia, impaired iron transfer from yolk sac to embryo	—
$Cp^{-/-}$	Ceruloplasmin	Iron accumulation in hepatocytes and macrophages	Aceruloplasminemia
sla	Hephaestin	Microcytic hypochromic anemia, impaired intestinal iron transfer	—
$Fth^{-/-}$	H-ferritin	Elevated tissue and serum L-ferritin Embryonic lethality	—
$Frda^{-/-}$ (neuron /heart knockout)	Frataxin	Mitochondrial iron deposits, neurodegeneration and cardiomyopathy	Friedreich ataxia
$Frda^{-/-}$ (muscle knockout)	Frataxin	Mitochondrial iron deposits, cardiomyopathy	Friedreich ataxia
$Hmox1^{-/-}$	Hemeoxygenase 1	Anemia, low serum iron levels, tissue iron deposition	Hmox1 deficiency

6. Iron metabolism and inflammation

One of the most obvious connections between iron metabolism and inflammation is the necessity of iron for the development of the immune system. Iron is required for the normal proliferation and maturation of lymphocytes. Iron deficiency causes a reduction in peripheral T-cell populations and atrophy of the thymus [114, 115]. In addition, the relationship between iron and inflammation is generally recognized in certain baterial infections and inflammations. Since iron is an important nutrient for the proliferation of bacteria, the response of increased iron sequestration from bacteria becomes an important host defense mechanism. This section will illustrate the connection between iron metabolism and the inflammation (anti-bacterial effect) in three aspects: structural similarity of some proteins; regulation of iron metabolism by inflammatory cytokines; and iron sequestration in bacterial infection.

6.1. Structural and functional similarities

The findings that there are structural similarities between some iron metabolism-related proteins and proteins that are particularly important for inflammation suggest that there may be a relationship between iron metabolism and inflammation. Three examples are demonstrated here: (i) hepcidin and defensin; (ii) Natural resistance-associated macrophage protein 1 (*Nramp1*) and DMT1; and (iii) HFE and MHC class I.

6.1.1. Hepcidin and defensin

Hepcidin shares functional similarities with defensins. Antimicrobial peptides are a valuable component of innate immunity in many species [116]. In mammals, antimicrobial

peptides such as defensins [117] represent a substantial part of the immune system for resistance to cellular pathogens. The bactericidal spectrum of hepcidin is similar to that of human defensin-1, which can inhibit the proliferation of a range of pathogens including Gram-negative *Neisseria cinerea* and the yeast *Saccharomyces cerevisiae* [105]. In addition, hepcidin and defensin both appear in human blood [118], and the expression of both can be induced by lipopolysaccharide, matching their roles in host defense.

In addition to these functional similarities, hepcidin's structural features are similar to that of antimicrobial defensins in terms of size and cysteine content [117, 119]. Hepcidin is a small peptide with an approximate molecular mass of 3 kDa; defensins range from 4-6 kDa in size. The hepcidin peptide sequence contains eight cysteine residues in four disulfide bonds; the human β-defensins have three intramolecular cysteine-bonds linking cysteines 1-5, 2-4, 3-6 [120] (Figure 8).

Figure 8. Protein alignment of human β defensins (defen B) and human hepcidin.

6.1.2. NRAMP1 and DMT1

The natural resistance of inbred mice strains to infection by *Salmonella typhimurium*, *Mycobacteria*, and *Leishmania donovani* was documented nearly 30 years ago [121, 122]. A single locus (Bcg), mapped to mouse chromosome 1, has been shown to influence bacterial replication in macrophages [123]. Positional cloning of Bcg identified the gene for natural resistance-associated macrophage protein (*NRAMP1*) as being responsible for the resistance to infection by bacteria [122].

The mammalian *NRAMP* family is divided into two classes, *NRAMP1* and DMT1 (also known as *NRAMP2*). Although the cloning of the DMT1 gene was carried out after that of *NRAMP1* and its identification was based solely on its homology to *NRAMP1* (Figure 9), the function of DMT1 was known before that of *NRAMP1*. A comparative study between *NRAMP1* and DMT1 was aptly used to elucidate the function of *NRAMP1*. Since DMT1 is a divalent cation transporter and can transport Fe^{2+}, Mn^{2+}, and Zn^{2+} in several cell lines, similar studies have been performed on *NRAMP1* that showed that NRAMP1 can also transport Fe^{2+}, Mn^{2+}, and Zn^{2+}, albeit with less efficiency than DMT1 [58, 124, 125]. DMT1 and NRAMP1 are both located within endosomes and lysosomes [126, 127], which further suggests a functional similarity, although some NRAMP1 expression can also be found within phagosomes [128, 129]. However, the ubiquitous expression of DMT1 in the body, in contrast to the exclusive expression of NRAMP1 within macrophage cells, points to a difference of some of the functions of these two proteins [58, 130].

Major controversy exists concerning the direction in which NRAMP1 delivers iron ions. Although DMT1 has been demonstrated to transport Fe^{2+} across the plasma membrane into the cell cytoplasm, the iron transport direction of NRAMP1 remains unclear. Two schools of thought have emerged. One advances the hypothesis that NRAMP1 functions to increase intraphagosomal Fe^{2+} in order to provide a catalyst for the Fenton reaction, which generates the highly toxic hydroxyl radical for bactericidal activity [131]. The other hypothesizes that the function of NRAMP1 is to remove the Fe^{2+} required by intraphagosomal bacteria for growth and the other divalent cations, such as Zn^{2+} and Mn^{2+}, necessary for superoxide dismutase thus providing an effective antioxidant defense [132].

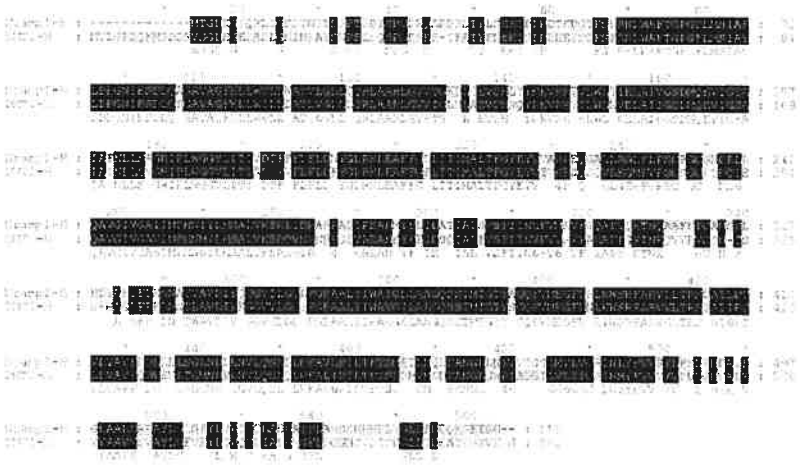

Figure 9. Protein alignment of human NRAMP1 and human DMT1.

6.1.3. HFE and MHC class I

The HFE protein structurally belongs to the class I major histocompatibility complex (MHC) antigen, an important protein family in the immune system (Figure 10). The HFE gene is located on chromosome 6p21, close to the HLA class I gene cluster, and it encodes

a MHC class I protein [133]. As with other MHC class I molecules, the extracellular portion of HFE is composed of α1, α2 and α3 domains, and the α3 domain binds β2-microglobulin [134]. The similarity between HFE and MHC class I molecules has raised questions regarding the function of HFE. MHC class I molecules normally participate in the cellular immune response by binding and presenting antigens to T-cells [135].

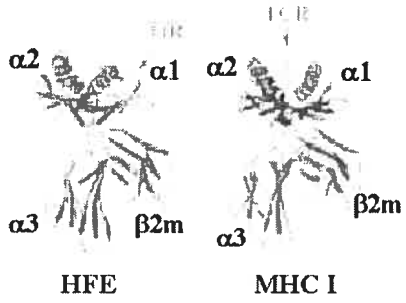

Figure 10. Ribbon diagrams of HFE and MHC class I (MHC I) proteins. α1, α2, and α3 domain helixes of HFE and MHC I proteins are shown. β2m binds to both HFE and MHC I. Transferrin receptor (TfR)-binding site on HFE and T-cell receptor (TCR)-binding site on MHC I are indicated. (from reference: Lebron JA and Bjorkman PJ, 1999 [136])

6.2. Regulation of iron-metabolic proteins by inflammatory stimuli

Many iron-metabolic proteins can be regulated by various inflammatory stimuli. For example, hepcidin synthesis in the liver and its secretion in the serum are known to be increased by lipopolysaccharide (LPS) [107], turpentine [108] and IL-6 [137] administration in mice [138, 139]. Ferritin transcription, translation and secretion are also up-regulated by LPS [140, 141], turpentine [142] and IL-6 [143], as well as by TNFα and interleukin 1α (IL-1α) [144-146] in different tissues and cell types. IFN-γ has been shown

to up-regulate DMT1 expression [147] and to down-regulate that of FP1 in the liver [148]. All of these changes in iron metabolic proteins induced by inflammatory stimuli ultimately lead to hypoferremia and iron accumulation in the liver.

6.3. Iron sequestration from bacteria

It has been shown that animals injected with iron, when infected with dangerous bacteria, demonstrate an increased rate of mortality.

Bacteria are also iron-restricted species, and in multicellular organisms colonized by bacteria, there is no freely available iron for bacterial nutrition [149]. There are two general mechanisms by which bacteria obtain iron. The first is characterized by direct contact between the bacteria and an exogenous iron source. In this mechanism, iron is taken up through bacterial cell surface transporters. The second involves the synthesis and release of iron-chelating compounds from bacteria into the extracellular medium and capture of these compounds by their receptors. One known bacterial iron-chelating compound is called siderophore [150]. Siderophore chelates iron with a very high affinity in order to extract iron from most organic complexes.

In humans, several iron-binding proteins, such as lactoferrin and lipocalin 2, are expressed in an iron-unsaturated form. This iron-unsaturated form enables the sequestering of iron as a defence against invading bacteria.

7. Recent findings inspiring our interest in studying Lcn2

Neutrophil gelatinase-associated lipocalin (NGAL) was first identified as a 25-kDa glycoprotein associated with purified human neutrophil gelatinase [151]. Homologous proteins were also identified in the mouse [lipocalin2(Lcn2)/24p3/uterocalin] and rat (β_2-microglobulin-related protein/neu-related lipocalin) [152]. NGAL/Lcn2 has been shown to be highly expressed in the colonic epithelial areas of inflammation, and its expression was thought to be related to its anti-inflammatory function [153]. The expression of Lcn2 in uterus during the implantation and parturition stages of pregnancy suggests that it may function to induce apoptosis during involution [82, 154, 155]. In addition, lipocalin 2 has also been shown to induce apoptosis of a wide variety of leukocytes [156]. Furthermore, microarray analyses performed in our laboratory two years ago obtained a spectrum of genes, including Lcn2, that could be modulated by different iron regulators.

Two categories of recent findings, combined with the results from microarray analysis, inspired our interest in studying Lcn2.

The finding that NGAL/Lcn2 can function as an iron-binding molecule is supported by the demonstration that it mediates a transferrin-independent iron uptake pathway activated during kidney development [157]. Iron delivered to differentiating epithelial cells by NGAL/Lcn2 is capable of regulating iron-dependent genes, such as ferritin and transferrin receptor 1 (TfR1) [157], that are sensitive to the cellular iron status and are regulated by

iron-responsive elements (IREs) [158, 159]. This finding indicates that such cells can utilize iron provided by NGAL/Lcn2. These observations, demonstrating that NGAL/Lcn2 mediates a transferrin-independent iron uptake pathway *in vitro*, raised the question as to whether this pathway can become activated in response to increased iron demand in *in vivo* rodent models.

It has also been shown that NGAL/Lcn2 binds catecholate-type bacterial ferric siderophores [160], such as enterobactin. Siderophores have a high affinity for ferric iron and are able to acquire iron from mammalian iron-binding proteins, including transferrin and lactoferrin [161]. Since NGAL/Lcn2 binds enterobactin with a higher affinity than the *E. coli* enterobactin transporter, it effectively interferes with bacterial iron uptake in culture conditions, and, in fact, acts as a bacteriostatic agent [160]. We have thus chosen to assess lipocalin 2 expression in two models of inflammation: systemic (LPS) and local (turpentine) inflammation, in evaluating its hypothesized bacteriostatic potential through an iron depletion strategy.

II. OBJECTIVE AND SPECIFIC AIMS

II. OBJECTIVE AND SPECIFIC AIMS

1. Objective

The purpose of this study is to investigate the role of Lcn2 in iron metabolism and inflammation by assessing its expression profile under conditions of altered iron storage, increased iron demand for hemoglobin synthesis, and induced iron redistribution during inflammation.

2. Specific aims

Aim 1: To study lipocalin 2 expression in response to altered iron metabolism. Iron absorption, storage, transport and utilization are regulated in response to altered iron metabolism. Many iron-metabolic proteins can be regulated to perform their needed function and meet the body's iron request. To define the role of Lcn2 in iron metabolism, Lcn2 levels are assessed in different iron storage conditions by using mouse models of iron overload and iron deficiency. The response of Lcn2 to an increased iron demand for enhanced hemoglobin synthesis is assessed in mouse models of anemia and hypoxia. Mouse models of anemia use PHZ (phenylhydrazine)-induced hemolytic anemia, iron deficient anemia, and phlebotomy-induced anemia. The mouse model of hypoxia uses $CoCl_2$-induced hypoxia. Lipocalin 2 mRNA in the liver and its secreted protein in the serum are analyzed.

Aim 2: To investigate lipocalin 2 regulation during inflammation. Since Lcn2 was found to bind bacteria-derived siderophore in vitro, the potential of Lcn2 to prevent bacteria growth in vivo is promising. Thus we are interested in assessing Lcn2 expression in mouse models

of bacterial component (LPS)-induced inflammations. Lcn2 transcription in the liver and its secretion in the serum are analyzed in inflammation. Since the LPS-induced Lcn2 tissue expression pattern may indicate the exact function of Lcn2 during inflammation, we are thus interested in assessing the tissue expression pattern of lipocalin 2 during acute inflammation. Lipocalin 2 mRNA levels are measured in the liver, spleen, duodenum, heart, kidney, thymus, brain, bone marrow, and in isolated hepatocytes and liver mononuclear cells. Lcn 2 expression in response to sterile inflammation is also assessed in a turpentine (TP)-induced acute inflammation model. During inflammation, iron redistribution and hypoferremia commonly occur; this is closely related to the anemia of chronic disease (ACD). To examine the mechanism of hypoferremia and the role of Lcn2 in the pathogenesis of ACD, Lcn2, DMT1, FP1 levels are assessed in the liver of a mouse model of anemia of chronic inflammations. Iron accumulation in hepatocytes and macrophages are also analyzed to investigate their roles in the pathogenesis of hypoferremia and ACD.

III. MATERIALS AND METHODS

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Animals

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of the Centre Hospitalier de l'Université de Montréal (CHUM). C57BL/6 female mice aged 3 or 8 weeks were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All mice were housed under strict specific pathogen-free conditions.

Animal treatments

Control mice were fed a standard commercial diet (Teklad Global 18% protein rodent diet, Harlan Teklad, Madison, WI). Dietary iron overload was produced by giving 8-week-old mice the same commercial diet supplemented with 2.5% (w/w) carbonyl iron (Sigma-Aldrich, St. Louis, MO) for 4 weeks. Iron deficiency was induced by feeding 3-week-old mice an iron deficient commercial diet for 9 weeks. All mice were 12 weeks old at the time of sacrifice.

To induce anemia through phlebotomy (PHL), 0.25 ml of blood was extracted by retro-orbital puncture from anesthetized mice of 9 weeks of age. The procedure was repeated 24 hours later, and the animals were sacrificed by decapitation under sodium pentobarbital anesthesia 16 hours after the last PHL.

Hemolytic anemia was produced by intraperitoneal (i.p.) administration of 40 mg/kg body weight of PHZ (Sigma-Aldrich), once daily for 4 days. The 9-week-old mice were sacrificed the following day as previously described.

Cobalt chloride ($CoCl_2$)-induced hypoxia was elicited by i.p. injection of 60 mg/kg of $CoCl_2$ (Sigma-Aldrich) dissolved in 0.9% saline. The 9-week-old mice were sacrificed after 24 hours as previously described.

Acute infectious inflammation was produced in 9-week old mice by a single dose of lipopolysaccharide (LPS 100μg i.p.) (Sigma Chemical, St Louis,) from *Escherichia coli* serotype 055:B5. To induce sterile inflammation, a single dose of pure nondiluted turpentine oil (100%) [TP 100μl subcutaneous (s.c.)] was injected into the back between the scapulas of anesthetized 9-week old mice. Age-matched control mice were similarly injected with an equivalent volume of sterile saline solution (0.09% NaCl). Animals were sacrificed either 6 or 16 hours after the injection as previously described.

Long-term inflammation was produced by LPS injections over a 4-week period in mice (8-12 weeks old): LPS 100 μg i.p., every 3 days for the first 8 injections, and LPS 50 μg i.p., daily for the final 7 injections. Animals were sacrificed 16 hours after the final injection as previously described.

Hematological measurements and transferrin saturation

EDTA-treated blood samples were obtained by orbital puncture under sodium pentobarbital anesthesia. Red blood cell (RBC) count, hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) were measured in an ABC *vet* counter (ABX hématologie, Montpellier, France). Serum iron, total iron-binding capacity (TIBC) and transferrin saturation were assessed by colorimetric assays [162], using a Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON). TS was calculated from the measured values of TIBC and SI.

Measurement of liver iron concentration

Liver iron concentrations were assessed by acid digestion of tissue samples, followed by quantification of iron using atomic absorption spectroscopy, according to our previously established methods [162].

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total liver RNA was isolated with Trizol reagent (Invitrogen, Burlington, ON) and reverse transcription was performed using a Thermoscript RT-PCR system (Invitrogen). Levels of lipocalin 2, hepcidin, glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and β-actin mRNA were measured by real-time PCR with a Rotor Gene 3000 Real Time DNA Detection System (Montreal Biotech Inc., Kirkland, QC) and a QuantiTect SYBRGreen I PCR kit (Qiagen, Mississauga, ON). All primers were designed by Primer3 software such that a minimum of 1 intron was included in each primer pair. The amplified cDNA

fragments for each primer pair were then verified by agarose gel electrophoresis to confirm the absence of the intron on the amplified fragment, and the absence of non-specific products. The primers were: 5'-CCCATCTCTGCTCACTGTCC-3' and 5'-TTTTTCTGGACCGCATTG-3 for lipocalin 2; 5'-AGAGCTGCAGCCTTTGCAC-3' and 5'-GAAGATGCAGATGGGGAAGT-3' for hepcidin; 5'-TCAAGAAGGTGGTGAAGCAG-3' and 5'-TGGGAGTTGCTGTTGAAGTC-3' for Gapdh; 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTTGAAGGTCTCAAA-3' for β-actin; 5'-TTGCAGGGAGCCATCAGAG-3' and 5'-CAACCTAGTATTTAATAGCAAGATGC-3' for DMT-1; and 5'-CCCATCCCCATAGTCTCTGT-3' and 5'-CTTGCAGCAACTGTGTCACC-3' for FP1. PCR amplifications were performed in triplicate, according to the following program: 15 minutes at 95°C; 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The cycle thresholds, defined as the value at which the amplification curve crosses the threshold line obtained from a standard curve covering a range of four orders of magnitude, were calculated automatically by the Rotor Gene data analysis software. Lipocalin 2 and hepcidin mRNA expressions were then normalized to the Gapdh and β-actin expression levels.

RT-PCR

Samples of cDNA were obtained using the previously described method. Murine cDNA for each sample was subjected to PCR amplification for lipocalin 2 expression (with forward 5'-GAAACCATGGCCCTGAGTGTC-3' and reverse 5'-

AGCCACACTCACCACCCATTC-3' primers) and β-actin expression (with forward 5'-AGCCATGTACGTAGCCATCC-3' and reverse 5'-TTTGATGTCACGCACGATTT-3' primers) in the same tube. PCR was performed using Taq DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA) and a thermal cycler (Hybaid Omnigene; Interscience, Markham, Ontario, Canada). The PCR amplification of lipocalin 2 gave a predicted product size of 682 base pairs (bp), whereas β-actin yielded a product of 250 bp. The PCR reaction mixture consisted of 1x PCR buffer, 200 μM of each dNTP, 1.25 U of *Taq* DNA polymerase, 50 pM of each primer for lipocalin 2 and 10 pM of each primer for β-actin. The PCR amplification was carried out as follows: an initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 45 seconds, 60°C for 1 min, and 72°C for 1 min; and a final extension of 10 min at 72°C. The PCR products were electrophoretically resolved on a 1.5% agarose gel, stained with ethidium bromide, and then photographed. Lipocalin 2 levels are expressed relative to β-actin levels.

Mononuclear cell (MNC) suspensions and hepatocyte isolation

Spleens were mashed through a 40 μm nylon cell strainer, and erythrocytes in splenocyte suspensions were lysed with Puregene RBC Lysis Solution (Gentra Systems, Minneapolis, MN, USA). Mouse hepatocytes were isolated after liver perfusion with liver digest media (GIBCO/BRL Life Technologies, Burlington, ON, Canada), followed by centrifugation on Percoll gradient (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), as described elsewhere [163]. The hepatocytes were then washed with Williams media (GIBCO/BRL

Life Technologies). Liver MNC suspensions were obtained by centrifugation of liver crude cell suspensionson Lympholyte-M (Cedarlane, Hornby, ON, Canada).

Western blotting for serum Lipocalin 2 detection

1.5 µL of serum was boiled for 5 min at 100°C in loading buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and bromophenol blue. Proteins were resolved on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Amersham Biosciences, Baie d'Urfé, QC). The membranes were blocked with 6% nonfat dry milk solution and incubated with anti-sip24/Lcn2 antibody [164] (a gift from Dr. Marit Nilsen-Hamilton, Iowa State University). To detect immunocomplexes, peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Mississauga, ON) was used as secondary antibody. Bands were visualized using an enhanced chemoluminescence system from Amersham Biosciences, Baie d'Urfé, QC.

Histopathology

Liver samples were fixed in buffered 4% formaldehyde and stained with hematoxylin/eosin. Ferric iron (Fe^{3+}) was detected by Prussian blue staining (Sigma diagnostics, St Louis, MO), according to the manufacturer's directions. The Prussian blue color was enhanced with diaminobenzidine (DAB) (Sigma chemical, St Louis, MO).

Statistical analysis

All results are expressed as either group means ± SD (iron concentration, SI, TS, Hb, RBC, HCT, MCV) or group means ± SEM (gene mRNA expression). Student's *t*-test (unpaired,

2-tailed) was used for comparison between treatments and mouse strains. Differences were considered significant where $p < 0.05$.

IV. RESULTS

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Lcn2 mRNA expression in response to increased iron stores

Alterations in body iron stores are accompanied by changes in expression of the iron regulator hepcidin [165], and of genes involved in cellular iron uptake and storage, such as TfR and ferritin [166, 167]. To assess whether *Lcn2* expression is regulated in response to increased body iron stores, mice were fed an iron-enriched diet (2.5% carbonyl iron) for 4 weeks. This treatment led to an elevation of serum iron (22 ± 4 μM for the standard diet vs. 39 ± 1 μM for the iron-enriched diet, $p<0.01$) and in transferrin saturation (45 ± 8% for the standard diet vs. 96 ± 7% for the iron-enriched diet, $p<0.01$; Table 1). Iron loading was further demonstrated by measuring liver iron concentrations that were found to be 4 times higher in mice placed on the iron-enriched diet (286 ± 56 μg iron/g dry weight for the standard diet vs. 1202 ± 209 μg iron/g dry weight for the iron-enriched diet, $p<0.01$; Figure 1a). Iron levels in the spleen were also significantly elevated ($p<0.05$, Figure 1a).

Lcn2 mRNA expression was detected at very low levels in the livers of control mice on the standard diet, and remained unchanged in the liver (Figure 1b) and spleen (data not shown) of mice fed the iron-enriched diet. In contrast, liver mRNA levels of the putative iron regulator hepcidin, used here as a control for the treatments, were significantly enhanced by iron loading ($p<0.05$, Figure 1c).

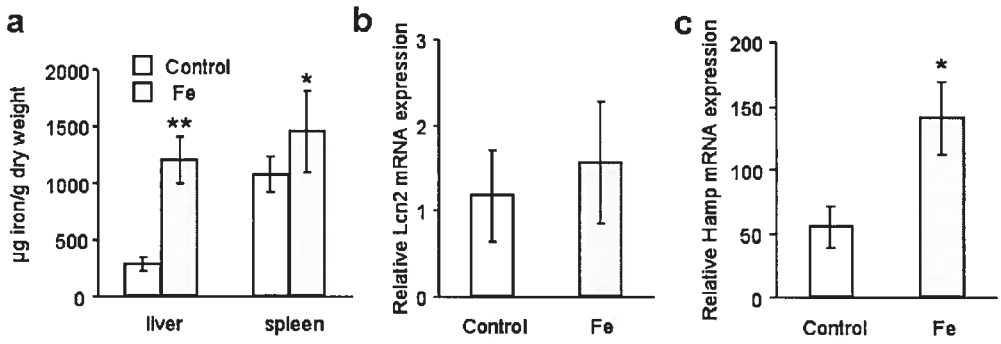

Figure 1. Iron stores, Lcn2 and hepcidin mRNA levels in mice fed a carbonyl iron-supplemented diet (n=6 per group). (a) Iron concentrations in the liver and spleen of control and iron-loaded mice (Fe). The results are expressed as means ± SD. Liver mRNA levels of (b) lipocalin 2 (Lcn2) and (c) hepcidin (Hamp) of mice fed a standard diet (control) and carbonyl iron-supplemented diet (Fe). The results are expressed relative to Gapdh as group means ± SEM. Student's t test: *p < 0.05; **p < 0.01.

Table 1. Hematological indices, serum iron (SI) and transferrin saturation (TS) in iron-deficient and iron-loaded mice.

Treatment	Hb (g/L)	RBC ($x10^{12}$/L)	HCT (%)	MCV (fL)	SI (μM)	TS (%)
Control ($n = 6$)	135 ± 5	9.6 ± 0.4	44 ± 1	46 ± 1	22 ± 4	45 ± 8
Carbonyl iron – Fe ($n = 6$)	144 ± 7	9.3 ± 0.5	45 ± 2	48 ± 2	39 ± 1	96 ± 7
Iron deficiency – IDA ($n = 6$)	124 ± 5	9.0 ± 0.3	38 ± 1	42 ± 1	18 ± 2	35 ± 7
p-value (control versus Fe)	<0.05	NS	NS	<0.01	<0.01	<0.01
p-value (control versus IDA)	<0.01	<0.05	<0.01	<0.01	<0.05	<0.05

Data are presented as means ± SD. NS denotes non-significant differences.

Lcn2 mRNA expression in response to iron deficiency anemia

We proceeded to examine the effects of decreasing body iron levels on the Lcn2 gene expression by feeding mice an iron-deficient diet for 9 weeks. This treatment led to the development of mild anemia and significant depletion of body iron stores, as revealed by modest but statistically significant decreases in erythroid parameters (Hb - 8%, RBC - 6%, HCT - 14%, and MCV - 9%), serum iron and transferrin saturation (Table 1). In addition, iron concentrations in both the liver and spleen were significantly reduced (liver - 286 ± 56 μg iron/g dry weight for the standard diet vs. 159 ± 38 μg iron/g dry weight for the iron-deficient diet, $p<0.01$; Figure 2a). The spleen/body weight ratio in the iron-deficient mice (5.0 ± 0.48 spleen weight x 10^3/body weight) was significantly increased as compared to untreated controls (4.1 ± 0.14 spleen weight x 10^3/body weight, $p<0.05$), which indicated that erythropoiesis had been stimulated [168].

Lcn2 mRNA levels were found to be elevated by 2.6-fold both in the liver (Figure 2b) and spleen (data not shown), while hepcidin levels were reduced to 21% of the control (Figure 2c). Lcn2 protein was clearly detected in the serum of mice with iron deficiency anemia (Figure 2d).

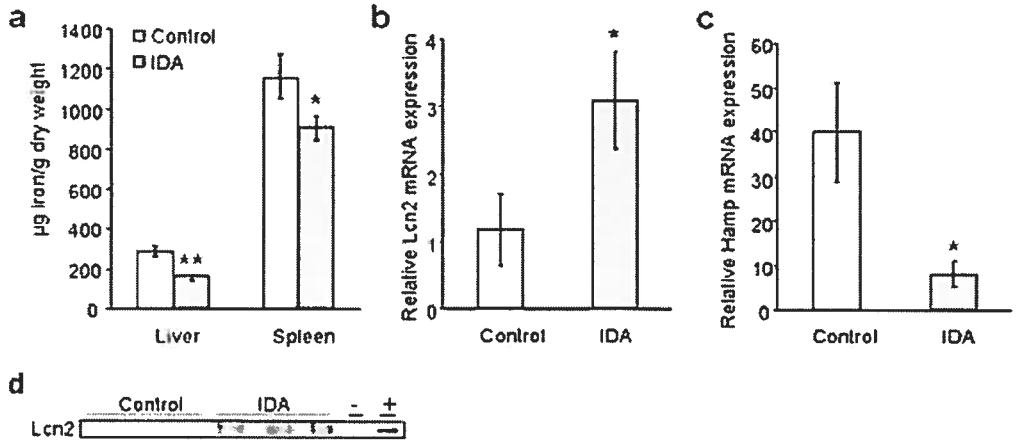

Figure 2. Iron stores, liver Lcn2 and hepcidin mRNA levels and serum Lcn2 protein in control and iron-deficiency anemic mice (n = 6 per group). (a) Iron concentrations in the liver and spleen of control mice and mice with iron-deficiency anemia (IDA). The results are expressed as means ± SD. (b) Lcn2 and (c) hepcidin (Hamp) mRNA levels in the liver of control and iron-deficient anemic mice as determined by real-time PCR. The results are expressed relative to Gapdh as means ± SEM. Student's t test: $*p < 0.05$; $**p < 0.01$. (d) Relative changes in Lcn2 in serum measured by Western blotting. The anti-Lcn2 antibody[164] detected a band of approximately 24 kD.

Lcn2 expression is induced by acute anemia

The lack of Lcn2 mRNA modulation in response to increased tissue iron content and its upregulation after exposure to an iron-deficient diet leading to the development of mild anemia suggest that Lcn2 expression may rise with heightened erythroid demand. Therefore, we examined Lcn2 expression during anemia that was not caused by iron

deficiency. Two murine models of acute anemia were studied, phlebotomy (PHL)-induced and phenylhydrazine (PHZ)-induced anemia.

Hb, RBC, and HCT values in PHL mice were reduced by 63%, 66% and 67%, respectively (Figure 3), while MCV was not affected. In PHZ-treated animals, RBC and HCT values were also markedly decreased (69% vs. control), Hb declined by 25% (Figure 3), and MCV remained unchanged. In contrast to PHL-treated mice, hepatic iron levels, serum iron and transferrin saturation were increased in the PHZ-treated mice (data not shown), as PHZ administration resulted in a large influx of iron into the liver Kupffer cells following massive hemolysis.

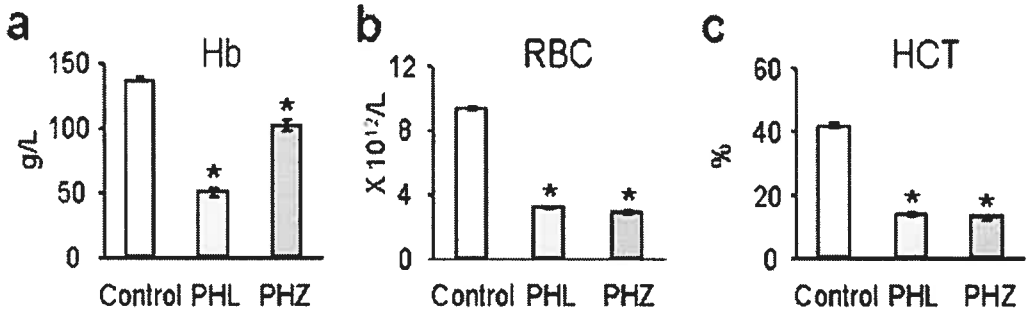

Figure 3. Hematological indices in control, phlebotomized (PHL) and phenylhydrazine (PHZ)- treated mice. (a) Hemoglobin (Hb) concentration, (b) red blood cells (RBC) and (c) hematocrit (HCT). The results are expressed as means ± SD for n=6 animals per group. Student's t test: *$p < 0.01$.

The spleen/body weight ratio of PHL mice (4.9 ± 0.51 spleen weight x 10^3/body weight) was significantly increased as compared to untreated controls (4.0 ± 0.1 spleen weight x 10^3/body weight, $p<0.05$). Compared with control and PHL animals, mice treated with PHZ showed far more severe splenomegaly (4.0 ± 0.1 for controls vs. 19.2 ± 1.2 for the PHZ-treated group, spleen weight x 10^3/body weight, $p<0.01$).

Since we observed significant elevation in expression levels of the housekeeping gene Gapdh by these treatments (particularly by PHZ), which were possibly due to ensuing hypoxia [169], we used β-actin as an internal control to calculate the relative amounts of both Lcn2 and hepcidin mRNA by real-time PCR. We found that Lcn2 mRNA was dramatically increased in the livers of both PHL- ($p<0.01$) and PHZ-treated mice ($p<0.01$; Figure 4a) as compared to control mice. Conversely, hepcidin expression was reduced by 80% in PHL-treated and by 72% in PHZ-treated mice ($p<0.01$; Figure 4b), in agreement with previously-reported data [165]. The Lcn2 protein was readily detected by Western blot analysis in the sera of both PHL- and PHZ-treated mice (Figure 4c), but not in sera of the controls.

These results demonstrate that Lcn2 expression is strongly induced during acute anemia.

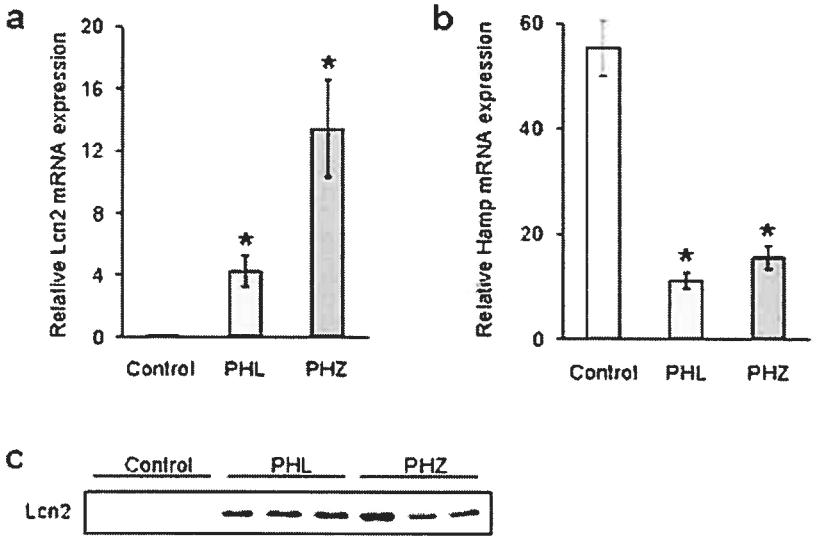

Figure 4. Lipocalin 2 (Lcn2) and hepcidin (Hamp) mRNA and protein levels in mice with acute anemia (n = 6 per group). (a) Lcn2 and (b) Hamp mRNA content in the liver of control, phlebotomized (PHL), and phenylhydrazine (PHZ)-treated mice as determined by real-time PCR. The results are expressed relative to β-actin levels as group means ± SEM. Student's t test. *$p < 0.01$. (c) Relative changes in Lcn2 protein expression in serum as detected by Western blotting.

Lcn2 expression is stimulated during $CoCl_2$-induced hypoxia

Anemia results in tissue hypoxia, which is a known activator of the expression of genes involved in iron homeostasis, such as transferrin [170], TfR [171, 172], ferritin [173], and ceruloplasmin [174]. To evaluate the effect of hypoxia on Lcn2 gene expression, mice were treated with $CoCl_2$, a chemical inducer of hypoxia-like responses. As shown in Figure 5a, a 10-fold increase in liver Lcn2 mRNA expression ($p<0.01$) was observed 24 hours after $CoCl_2$ treatment, and an elevation of plasma Lcn2 protein levels was evident (Figure 5c).

Conversely, hepcidin gene expression was strongly downregulated (Figure 5b, $p<0.01$), a finding that is in agreement with previously reported data on liver hepcidin regulation in mice exposed to low oxygen levels [165]. The down regulation of hepcidin gene expression during iron deficient anemia, PHL-induced anemia, PHZ-induced anemia, and $CoCl_2$-induced hypoxia represents the function of decreased-hepcidin in facilitating iron absorb in the duodenum and iron turnover in macrophages for RBC synthesis.

These results suggest that tissue oxygenation may be a factor in Lcn2 gene regulation by anemia.

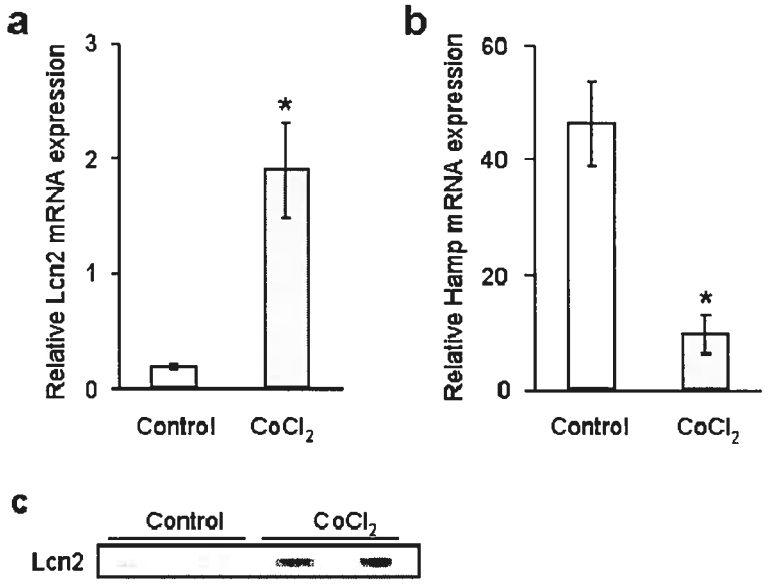

Figure 5. Lipocalin 2 (Lcn2) and hepcidin (Hamp) levels in hypoxic mice. (a) Lcn2 and (b) Hamp mRNA content in the liver of control, and cobalt chloride ($CoCl_2$)-treated mice as determined by real-time PCR. The results are expressed relative to β-actin levels as group

means ± SEM. Student's *t* test. *$p < 0.01$. (c) Relative changes in Lcn2 protein expression in serum as detected by Western blotting.

Lcn 2 expression is up-regulated by LPS and turpentine in the liver.

We treated mice with LPS in order to provide a systemic inflammatory stimulus that mimics bacterial infection. LPS is a membrane glycolipid component of Gram-negative bacteria and exhibits strong immunostimulatory activity in mammals. To generate an acute inflammation model, C57BL/6 mice were administered a single dose of LPS (100 μg, i.p.), and after 16 hours, blood and tissue samples were collected. It has been shown that the liver is a major site of Lcn2 synthesis, and the Lcn2 protein is secreted from the liver into the serum. Thus, we measured Lcn2 mRNA levels in the liver and Lcn2 protein levels in the serum. After a single LPS injection, Lcn2 gene expression in the liver was increased by approximately 124 times during acute inflammation relative to controls (Figure 6a). Lcn2 protein was strongly detected in the serum of LPS-treated mice, but almost undetectable in the serum of control mice (Figure 6c). As seen in most inflammatory conditions, serum iron (SI) and transferrin saturation (TS) were significantly decreased in the serum during acute inflammation (control versus LPS acute inflammatory, SI 16±2 μmol/L vs. 5±1 μmol/L, $p<0.01$, and TS 37±6% vs. 14±3%, $p<0.01$). In the acute inflammation model, hepcidin mRNA was increased (2.1 times, $p<0.01$), in agreement with previous reports (Figure 6b).

To determine whether Lcn2 expression can be regulated in localized inflammation, a turpentine (TP) model was used to induce a sterile local tissue abscess. Mice were

subcutaneously injected in the back with 100μl turpentine. Liver and serum samples were collected 16 hours later. As shown in Figure 6a, Lcn2 expression is 102 times increased ($p<0.01$) in the liver by turpentine treatment, as compared to the control. Increased level of Lcn2 proteins were formed in the serum (Figure 6c) of turpentine-treated mice, whereas Lcn2 levels were not detected in the serum of control mice. Hypoferremia was also observed in turpentine-induced inflammation. Serum iron and transferrin saturation were both significantly decreased by TP treatment (control vs. acute TP inflammation, SI 16±2 μmol/L vs. 10±2 μmol/L, $p<0.01$, and TS 37±6 % vs. 26±3%, $p<0.01$). In this turpentine-induced acute inflammation, hepcidin mRNA was increased (4.8 times, $p<0.01$), which is consistent with previous reports (Figure 6b).

These results suggest that Lcn2 expression can be increased by both systemic (LPS) and local (turpentine) inflammation.

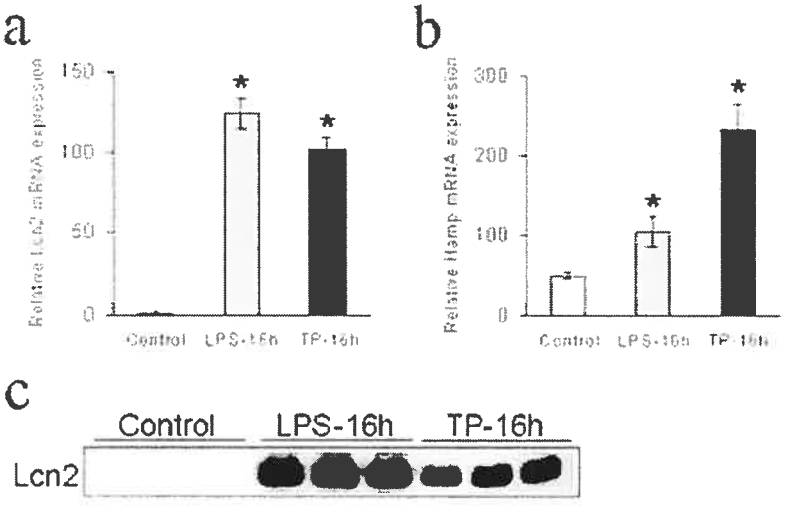

Figure 6. Lipocalin 2 (Lcn2) and hepcidin (Hamp) levels in mice of LPS- and turpentine (TP)-induced acute inflammation. (a) Lcn2 and (b) Hamp mRNA content in the liver of control, and LPS- and turpentine-treated mice as determined by real-time PCR. The results are expressed relative to Gapdh levels as group means ± SEM. Student's *t* test: * $p<0.01$. (c) Relative changes in Lcn2 protein expression in serum as detected by Western blotting.

Tissue expression pattern of Lcn2 during acute inflammation.

It has been shown that liver is the major organ for Lcn2 synthesis during infection [175]. To determine whether Lcn2 can be induced in other organs during infection, we measured the expression of Lcn2 in a variety of tissues during acute inflammation. In this study, different tissues from mice were collected 16 hours following a single injection of LPS (100 μg, i.p.). RT-PCR results of each tissue or cell-type were repeated three times from three different mice. Starting cDNA amounts were well controlled by fine mRNA quantification and dilution. One of the experiment results is shown in Figure 7 from three equal results. In saline-treated controls, basal Lcn2 mRNA expression was observed in the lungs, bone marrow, thymus, brain, spleen, and liver (in order of highest to lowest expression, but not in the duodenum, heart, kidney or muscle (Figure 7). In mice 16 hours post-LPS injection, Lcn2 mRNA levels were up-regulated well beyond the basal expression profile in the lungs, bone marrow, thymus, brain, spleen, and liver. In addition, Lcn2 mRNA was strongly detected in the duodenum, heart, kidney and muscle (Figure 7). In order to investigate the cell type-specific expression of Lcn2 in the liver, hepatocytes and liver mononuclear cells (MNC) were isolated from the livers of LPS- and saline-treated

mice. As shown in Figure 7, Lcn2 mRNA was expressed in hepatocytes, but not in the MNCs, of control mice. Following LPS treatment, Lcn2 expression was up-regulated in both hepatocytes and MNCs (Figure 7).

This result suggests that Lcn2 expression is increased in most tissues during acute inflammation.

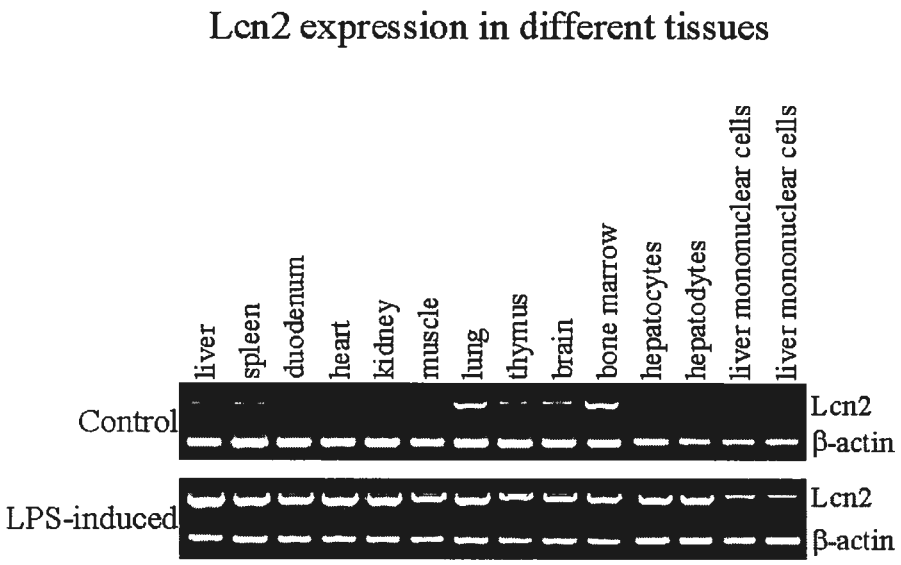

Figure 7. Tissue expression pattern of Lcn2 in control and LPS-induced acute inflammatory mice. Lcn2 and β-actin mRNA are determined by RT-PCR, from (upper panel) control and (lower panel) LPS-treated mice. Lcn2 expression (upper bands) is assessed relative to β-actin expression (lower bands) for each sample.

Lcn2 expression in response to anemia of chronic inflammation

Anemia of chronic disease (ACD) is the second most common form of anemia. ACD develops under chronic inflammatory disorders such as chronic infections, cancer or autoimmune diseases [10]. In this ACD model, LPS was administered every 3 days (100 μg, i.p.) for the first 8 injections, and then daily (50 μg, i.p.) for the 7 final injections over a period of 4 weeks. Samples were collected 16 hours after the last injection. As seen in Figure 8a, Lcn2 transcription was strongly increased in the liver. Lcn2 protein was also markedly increased in the serum during chronic inflammation (Figure 8b). Serum iron and transferrin saturation were both significantly decreased (control vs. chronic inflammation, SI 19±4 μmol/L vs. 15±3 μmol/L, $p<0.05$, TS 40±7% vs. 24±5%, $p<0.01$). In this model, we also observed that mice developed anemia, in which hemoglobin, red blood cell count, and hematocrit decreased by 34%, 39%, 40% relative to controls, respectively (Table 2). Mean corpuscular volume was not significantly affected.

These results demonstrate that Lcn2 expression is up-regulated during anemia of chronic inflammation.

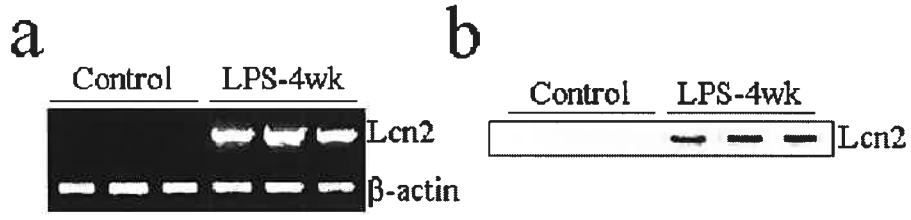

Figure 8. Lipocalin2 (Lcn2) levels in chronic inflammatory mice. (a) Lcn2 mRNA content in the livers of control and 4-week LPS-treated mice as determined by RT-PCR. The results

are expressed relative to β-actin levels. (b) Relative changes in Lcn2 protein expression in serum as detected by Western blotting.

Table 2. Hematological indices in mice injected with LPS for 4 weeks.

Treatment	Hb, g/L	RBC, $x10^{12}$/L	HCT, %	MCV, fL
Control-4wk (*n*=6)	135 ± 5	9.6 ± 0.4	44 ± 2	46 ± 1
LPS-4wk (*n*=5)	89 ± 7	5.8 ± 0.5	27 ± 2	45 ± 1
p-value	<0.01	<0.01	<0.01	>0.05

Data are presented as means ± SD

Liver iron accumulation during inflammation is related to changes in DMT1 and FP1 expression and in macrophage infiltration.

It has been shown that iron can accumulate in macrophages during inflammation [176], which in part explains the mechanism of hypoferremia which occurs upon inflammation. The disturbance in iron distribution contributes to the pathogenesis of ACD, in which iron becomes less available for erythropoeisis. To further investigate the mechanisms of hypoferremia, liver iron concentration was assessed in inflammatory mouse models. Iron was significantly accumulated in the livers of 6-hour acute inflammatory mice (control vs. LPS-6h, iron concentration in the liver 317±30 μg iron/g dry weight vs. 396±41 μg iron/g dry weight, $p<0.01$) (Figure 9a). In chronic inflammatory mice (control vs. chronic

inflammation, iron concentration in the liver was 286±23 μg iron/g dry weight vs. 452±13 μg iron/g dry weight, $p<0.01$) (Figure 9b).

To understand which type of liver cells accumulated iron, we applied an enhanced Prussian blue staining technique to paraffin-embedded slides of the liver. In control mice livers, parenchymal cells were lightly stained, as shown in Figure 9e. In contrast, the livers of chronic inflammatory mice showed heavy iron staining in hepatocytes (Figure 9f). In addition, a strong staining of macrophages was observed (Figure 9g), whereas it was not observed in samples from control mice (Figure 9e).

DMT1 and FP1 are two iron ion transporters located on the cellular membrane of both hepatocytes and macrophages. In cellular homeostasis, iron uptake is mediated by TfR and then delivered by DMT1, and iron export is mediated by FP1. To understand the mechanism by which iron is increasingly accumulated in the liver, we assessed the status of these two iron transporters. DMT1 transcription was increased 2.9 ($p<0.01$) times or 1.6 times ($p<0.01$) in the liver, in 6 hour or 16 hour LPS-induced acute inflammatory models, respectively, which suggests an increased iron uptake by hepatocytes (Figure 9c). FP1 transcription was decreased by 98% or 89% ($p<0.01$) in the same 6 hour or 16 hour paradigms, respectively, which suggest decreased iron export (Figure 9d). It is possible that the changes in DMT1 and FP1 expression co-operate together resulting in increased iron accumulation in the liver.

These results show that iron is accumulated in the liver of acute and chronic inflammation, and iron accumulates in both hepatocytes and macrophages. These results suggest that such iron accumulation is achieved, at least in part, in a co-operative manner by the regulation of two iron transporters, DMT1 and FP1.

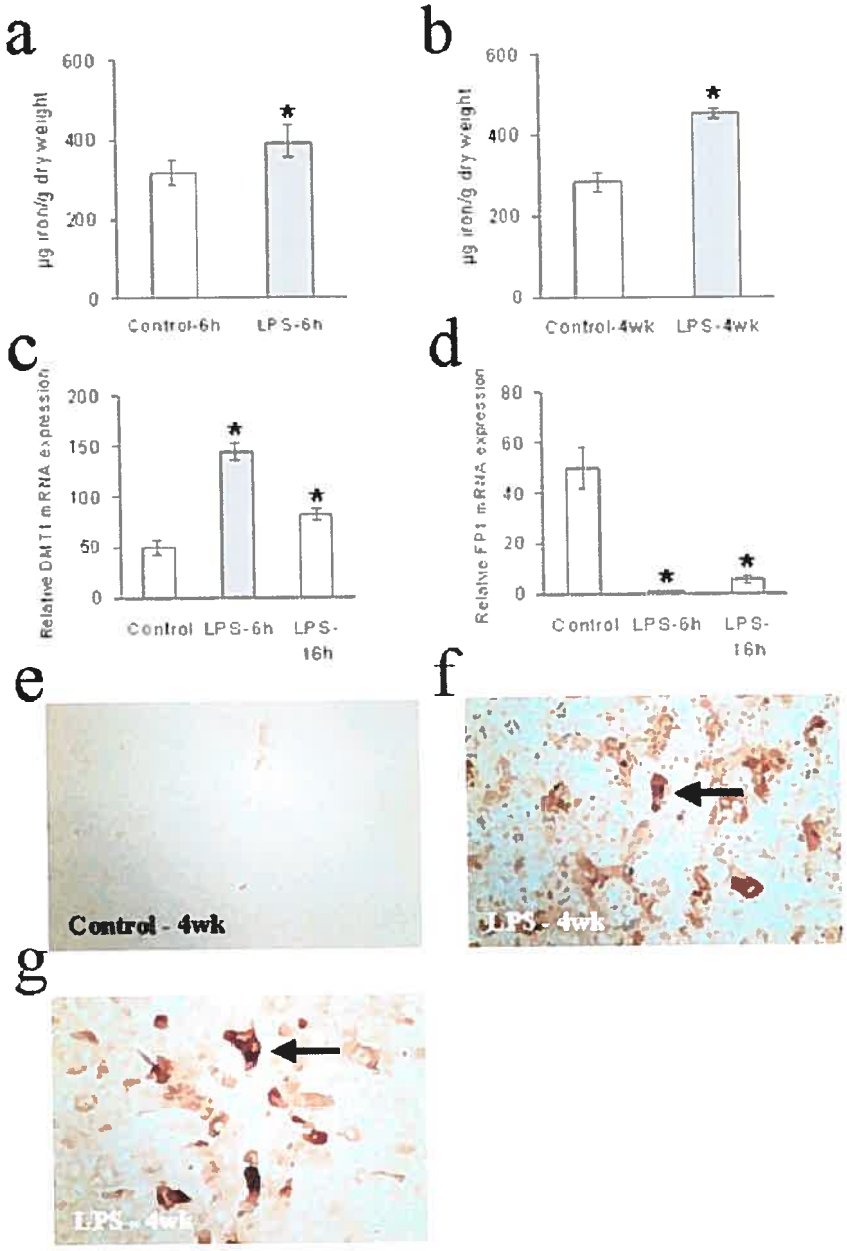

Figure 9. Iron concentrations, DMT1 and FP1 levels, and iron deposition in the liver of control and inflammatory mice. Iron concentrations in the livers of LPS-induced (a) acute and (b) chronic inflammatory mice. (c) DMT1 and (d) FP1 mRNA content in the liver of control and LPS-induced 6 and 16 hour acute inflammatory mice as determined by real-time PCR. The results are expressed relative to Gapdh levels. Columns represent group means ± SEM. Student's *t*-test: * $p<0.01$. Light micrographs of representative liver sections from (e) control and (f-g) chronic inflammatory mice. (g) Macrophages are indicated with arrows. Iron accumulations in hepatocytes and macrophages are shown by enhanced Prussian blue staining of the liver section. Original magnification = 600×.

V. DISCUSSION AND CONCLUSIONS

V. DISCUSSION AND CONCLUSIONS

1. Discussion

The present study investigated the regulation of Lcn2 expression in murine models of altered iron homeostasis. Adaptive responses of Lcn2 to iron stores, anemia and inflammation were examined. Lcn2 expression was increased in mouse models of anemia, hypoxia, and inflammation, but was not altered in dietary iron overload. The observations that both anemia- and hypoxia-induced Lcn2 expression at the mRNA and protein levels suggest that Lcn2 may serve as an iron trafficking protein during periods of enhanced erythroproiesis. In addition, the induction of Lcn2 expression in response to inflammatory stimuli supports a hypothesized role for Lcn2 in the inhibition of bacterial proliferation through an iron depletion strategy.

Lcn2 was initially found to be associated with iron metabolism due to its capacity for iron delivery. The existence of a transferrin-independent iron delivery pathway was provided by the observation that TfR knockout mice are still able to initiate tissue development, and thus presumably obtain iron by an alternative iron-uptake mechanism [177]. This notion is further supported by recent reports of a Lcn2-mediated, transferrin-independent iron delivery pathway believed to be active during early development [157, 160].

The results in mice with anemia induced by either iron-depletion, phlebotomy (PHL) or PHZ-treatment clearly demonstrate that Lcn2 is induced in the liver in response to anemia. This suggests that a Lcn2-mediated iron delivery pathway in the liver can be activated when the iron demands of erythropoiesis increases. The up-regulation of *Lcn2* appears to

be independent of the status of liver iron stores. PHL did not significantly alter liver iron stores in these studies whereas PHZ- treatment led to an increase in liver iron concentrations and serum iron (because of hemoglobin release from lysed RBC); yet both treatments strongly induced *Lcn2* expression. Moreover, *Lcn2* expression was not affected by iron-loading, since mice fed an iron-enriched diet did not show any changes in *Lcn2* levels. In both PHL- and PHZ-induced acute anemia, Lcn2 upreguation might be modulated by four possible mechanisms: increased request of erythropoiesis, increased EPO release in the serum [178, 179], the activation of GATA-1 element on *Lcn2* and/or through hypoxia.

The regulation of *Lcn2/NGAL* during anemia possibly occurs via *cis*-acting regulatory elements, such as the binding site for GATA-1 found in the 5'-flanking region of the *Lcn2/NGAL* gene [180]. GATA-1 is a zinc finger transcription factor that plays a central role in erythroid development, as evidenced by the fact that GATA-1-deficient mice die prenatally from severe anemia [181], with arrest of erythroid maturation at a proerythroblast-like stage [182]. Interestingly, many promoters of genes involved in cellular iron uptake and storage, such as TfR and ferritin, contain *cis* elements recognized by the GATA-1 factor [183]. This transcriptional regulation of iron-related genes likely ensures coordinated regulation of iron availability and hemoglobin synthesis. In this context, activation of the Lcn2-mediated iron transport pathway by anemia could contribute to rapid mobilization of iron from iron-storing cells to supply sufficient amounts of the element for erythropoietic activity.

Our results in anemia/hypoxia models indicate that Lcn2 levels may also be regulated by the level of tissue oxygenation. $CoCl_2$ mimics hypoxic induction of the hypoxia inducible factor (HIF-1), which activates transcription by binding to hypoxia responsive elements. While hypoxia is known to induce the expression of both transferrin and TfR via HIF-1 [170-172], recent evidence indicates that transferrin-dependent iron uptake, however, does not significantly change during hypoxia [173]. Instead, as shown in rat myocardial [184] and human embryonic kidney cells [173], hypoxia induces an as yet to be identified transferrin-independent iron uptake pathway. Stimulation of iron uptake via the Lcn2 pathway by hypoxia could allow cells to sequester iron in amounts sufficient to maintain enzyme function and cellular survival during periods of low oxygen concentration.

In addition to our finding that Lcn2 can respond to anemia and hypoxia, Lcn2 also appears to be capable of responding to inflammatory stimuli.

A wide range of bacteria can use synthesized siderophores to obtain iron from the infected host for proliferation [185]. Siderophores take advantage of their high affinity for iron, and are able to chelate iron not only from iron-containing proteins but also from iron-sequestration proteins including transferrin and lactoferrin. The recent finding that mammalian-derived lipocalin 2 can bind to bacteria-derived siderophores suggests a new host-defence mechanism of the innate immune system.

Our results are in agreement with previous study showing that Lcn2 was strongly induced by a Gram-negative bacterial component, LPS, produced from *E. coli*. This result indicates

that Lcn2 is expressed in response to bacterial infection. This bacteria-specific response is consistent with reports that a human Lcn2 homologue, NGAL, is highly secreted in the serum of bacteria-infected patients [186].

Turpentine-induced tissue injury generates a local inflammatory condition which is characterized by increased IL-1β-dependent IL-6 production [187, 188]. The finding that Lcn2 was induced by turpentine injection indicates that Lcn2 can be up-regulated by local inflammation. Since cancers have been shown to be closely associated with chronic inflammation [189], this aseptic response of Lcn2 may explain the observation that Lcn2/NGAL mRNA is up-regulated in neoplastic tissues, such as colon cancer, lung adenocarcinomas, mucin-producing tumors and pancreatic cancers [190].

In saline-treated controls, basal Lcn2 mRNA expression was observed in the thymus, spleen and lungs. The thymus and spleen are two important organs for the immune system, and the lungs are in direct contact with environmental pathogens in the air. Basal Lcn2 mRNA expression was detected in these tissues, in accordance with the postulated role of Lcn2 in the host-defence mechanism of the innate immune system.

Lcn2 mRNA expression was increased by LPS in almost all of the tissues examined. This broad induction of Lcn2 suggests a new mechanism of tissue-based defence against invading bacteria, distinct from the traditional serum-based defence afforded by the iron-sequestration protein lactoferrin. Iron is a required nutrient for bacterial proliferation, and the appearance of Lcn2 both in the serum and in the tissues suggests that the iron

sequestration potential of Lcn2 can not only limit the survival of bacteria in the circulation, but also inhibit bacteria that have already invaded the cells.

The antimicrobial activity of Lcn2 has been shown to be dependent upon its ability to bind bacteria-derived enterochelin-like siderophores [81]. By using Lcn2 knockout mice, a recent experiment proved that the antibacterial activity of Lcn2 is attributed to its iron sequestration effect through its binding to iron-laden siderophores, and this effect is siderophore-specific to enterochelin-dependent bacteria [191]. The enterochelin-specific prevention of bacterial growth by Lcn2 suggests that Lcn2 can effectively protect against those bacteria that produce the enterochelin-like siderophore, such as *E. coli, Salmonella* spp., *Brucella abortus, Bacillus anthracis, Burkhodria cepacia, Corynebacterium diphtheriae, Paracoccus* spp. and *Vibrio* spp. This characteristic of Lcn2 highlights the potential of artificially synthesised Lcn2 protein or Lcn2 mimetics in future clinical applications.

In the chronic inflammation mouse model, anemia was induced by a 4-week LPS treatment, which mimics ACD. Decreased serum iron levels were seen in both acute and chronic inflammation. The duration of hypoferremia contributes to the pathogenesis of anemia, by which the iron availability for RBC synthesis is decreased. On the other hand, this hypoferremia creates an iron depletion strategy which prevents bacterial proliferation. Increased iron accumulation was observed in both macrophages and hepatocytes. This accumulation highlights the occurrence of iron disturbance during chronic inflammation, and the subsequent generation of hypoferremia. Given that DMT1 and FP1 are both

expressed in macrophages and hepatocytes [192-194], an increased iron uptake, in part, through the up-regulation of DMT1 and a decreased iron export through the down-regulation of FP1 in the liver suggests a mechanism for iron accumulation in both types of cells. It has been shown that increased iron storage through the up-regulation of ferritin synthesis by inflammatory cytokines also facilitates iron accumulation in macrophages [144]. The generation of anemia during chronic inflammation may also involve other mechanisms. For example, cytokines may impair erythrocyte survival [195] and may also stimulate erythrophagocytosis by macrophages [196]. Whether the increased Lcn2 expression during chronic inflammation contributes to the iron accumulation in hepatocytes and macrophages remains to be elucidated.

2. Conclusions

In conclusion, the work presented here indicates that Lcn2 is stimulated by anemia, hypoxia and inflammation. The up-regulation of Lcn2 in anemia and hypoxia suggests that Lcn2 may have an important physiological role in the regulation of iron availability for hemoglobin synthesis and for cellular survival. Lcn2 up-regulation during inflammation is in agreement with an iron sequestration role in the inhibition of bacterial proliferation.

VI. FUTURE PERSPECTIVES

I. FUTURE PERSPECTIVES

In this study, Lcn2 has been shown to be able to respond to anemia, hypoxia and inflammation. Thus, further research should concentrate on investigating the mechanisms of these responses.

Lcn2 expression was induced in two mouse models of anemia: phlebotomy and PHZ-treatment. Since erythropoietin (EPO) is usually up-regulated under anemic conditions, the question of whether Lcn2 is regulated by EPO is of interest. An experiment assessing the expression levels of Lcn2 in EPO-treated mice will answer this question. Since Lcn2 is synthesized in the liver, hepatocyte cultures can be used as an *in vitro* model. To examine the influence of iron levels on Lcn2 expression, iron (ferric citrate) or the iron chelator desferrioxamine (DFO) can be added to the hepatocyte culture. The influence of EPO on Lcn2 expression can also be tested in this hepatocyte culture model. In addition, during enhanced erythropoiesis, GATA1, a zinc finger transcription factor, is required for normal erythroid development by binding to the promoter region of several genes and promoting their transcription. To examine whether Lcn2 can be activated by GATA1, a GATA1 binding assay can be performed on the promoter region of Lcn2 using electrophoretic mobility shift assays (EMSA). If GATA1 can bind to the *Lcn2* gene and promote *Lcn2* transcription, further identification of proteins involved in GATA1-Lcn2 activation is of interest. The activation of the GATA1 pathway should include several other upstream proteins. Different inhibitors for candidate proteins can be added to the hepatocyte culture, thus identifiying putative proteins involved in GATA1 pathway activation and subsequent Lcn2 transcription.

Cobalt chloride-induced hypoxia condition has been shown to increase Lcn2 mRNA expression. $CoCl_2$ mimics hypoxia by increasing the level of hypoxia inducible factor 1 (HIF-1). HIF-1 is a master regulator of the cellular adaptive response to hypoxia. However, to exclude other effects of cobalt chloride on Lcn2 expression, a low oxygen (10% O_2)-induced hypoxia mouse model can be used to assess Lcn2 expression. The low oxygen condition can also be carried out on the hepatocyte culture to assess its influence on Lcn2 expression. To provide molecular evidence of the influence of HIF-1 on Lcn2 expression, an HIF-1 binding assay will be performed on the promoter region of Lcn2 with EMSA techniques. If HIF-1 can bind to the Lcn2 gene and promote its transcription, the further identification of proteins involved in the HIF-1-Lcn2 activation pathway would be of additional interest. Different inhibitors of candidate proteins in the HIF-1 pathway can be added to the hepatocyte culture, and the involvement of these proteins can thus be shown using Lcn2 transcription and HIF-1 binding assay.

LPS has also been shown to increase Lcn2 expression. The toll-like receptor 4 (TLR4) has been found to be the receptor for LPS. To assess whether LPS-induced Lcn2 expression depends on TLR4, a TLR4 spontaneous mutant mouse strain (C3H/HeJ mice [197, 198]) can be used to analyze Lcn2 expression after LPS injection. Upon activation, TLR4 can subsequently activate the NF-κB pathway. To assess whether the LPS-induced Lcn2 expression depends on the NF-κB pathway, an NF-κB pathway inhibitor, pyrrolidine dithiocarbamate (PDTC), can be used to analyze Lcn2 expression during LPS treatment.

Furthermore, since the activation of the NF-κB pathway by LPS involves several proteins, the identification of each of the proteins necessary for Lcn2 expressions is also of interest. Different inhibitors for candidate proteins can be added to LPS-stimulated hepatocyte cultures. Lcn2 transcription and translation can then be assessed and compared to controls. Molecular evidence of the NF-κB-activated Lcn2 transcription can be analyzed by an NF-κB binding assay through EMSA techniques.

Questions addressing the exact functions of Lcn2 up-regulation in anemia, hypoxia and bacterial infection can be further investigated by studying Lcn2 knockout mice to demonstrate the need for Lcn2 in these conditions. The role of Lcn2 in iron sequestration during anemia of chronic inflammation can also be elucidated by studying Lcn2 knockout mice.

VII. REFERENCES

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