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MOLECULAR MECHANISMS OF IRON TRANSPORT ACROSS PLASMA MEMBRANE IN MAMMALIAN CELLS

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PH.D. THESIS

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DECLARATION

This thesis was developed at the Division of Cell and Molecular Biology, Third Faculty of Medicine, Charles University in Prague, Czech Republic within the subject area of Molecular and Cellular Biology, Genetics and Virology of doctoral study programs in biomedicine under supervision of Prof. Jan Kovář, head of the division. The thesis was prepared by myself and all sources and literature are properly cited. This is an original work never presented before to obtain different or the same degree.

All studies involving patient samples were approved by the Ethics Committee and conducted in accordance with the Helsinki Convention. Patient Consent form was obtained from all individuals.

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CONTENTS

LIST OF ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2
ACD	anemia of chronic disease
ACO1	aconitase 1
ALD	alcoholic liver disease
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BIP	binding immunoglobulin protein
BMP	bone morphogenetic protein
BMP6	bone morphogenetic protein 6
BMPR	bone morphogenetic protein receptor
BOX	oxidized form of bilirubin
BvR	biliverdin reductase
C282Y	cysteine substitution by tyrosine on position 282 in protein
CBP	CREB binding protein
CP	ceruloplasmin
CREB/H	cAMP response element-binding protein/H
DCT1	divalent cation transporter 1
Dcytb	duodenal cytochrome b-like
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
EPOR	erythropoietin receptor
ER	endoplasmic reticulum
ERFE	erythroferrone
FIH	factor inhibiting HIF
FLVCR1	feline leukaemia virus subgroup C cellular receptor 1
FPN1	ferroportin 1
GDF15	growth differentiation factor 15

GPI	glycosylphosphatidylinositol
H63D	histidine substitution by aspartic acid on position 63 in protein
HAMP	hepcidin antimicrobial peptide
HCP1	heme carrier protein 1
HFE	hemochromatosis gene (“high-Fe”)
HGF	hepatocyte growth factor
HH	hereditary hemochromatosis
HHC	hereditary hemochromatosis
HIF	hypoxia-inducible factor
HIF-α	hypoxia-inducible factor α
HIF-1α	hypoxia-inducible factor 1α
HIF-2α	hypoxia-inducible factor 2α
HIF-1β	hypoxia-inducible factor 1β
HJV	hemojuvelin
HO	heme oxygenase
HO-1	heme oxygenase 1
HO-2	heme oxygenase 2
HP	hephaestin
HRE	HIF-responsive element
HRG-1	heme responsive gene-1
IDA	iron deficiency anemia
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IRDA	iron deficiency anemia
IRE	iron-responsive element
IRE1α	inositol-requiring ER-to-nucleus signal kinase
IREB2	iron-responsive element binding protein 2
Ireg1	iron regulated transporter 1
IRIDA	iron refractory iron deficiency anemia
IRP	iron regulatory protein
IRP1	iron regulatory protein 1
IRP2	iron regulatory protein 2
JAK	Janus kinase
LC3	light chain 3
LEAP1	liver-expressed antimicrobial protein 1
MTP1	metal transporter protein 1
NRAMP2	natural resistance-associated macrophage protein 2
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NTBI	non-transferrin-bound iron
p-eIF2α	phospho-eukaryotic initiation factor 2α
p-IRE1α	phospho-inositol-requiring ER-to-nucleus signal kinase
p-JNK	phospho-C-Jun N-terminal kinases
PCFT	proton-coupled folate transporter

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGF-BB	platelet-derived growth factor subunit B (homodimer)
PDGFR	platelet-derived growth factor receptor
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PHD	prolyl hydroxylase domain
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RBC	red blood cells
ROS	reactive oxygen species
S65C	serine substitution by cysteine on position 65 in protein
SA	sideroblastic anemia
SCARA5	scavenger receptor class A member 5
SEM	standard error of the mean
SMAD	sma and mothers against decapentaplegic homolog
STAT3	signal transducer and activator of transcription 3
STEAP	six-transmembrane epithelial antigen of prostate
STEAP3	six-transmembrane epithelial antigen of prostate 3
TIM-2	T cell immunoglobulin and mucin-domain 2
Tf	transferrin
TfR	transferrin receptor
TfR1	transferrin receptor 1
TfR2	transferrin receptor 2
TMPRSS6	transmembrane protease, serine 6
TWSG1	twisted gastrulation BMP signaling modulator 1
Ub	ubiquitin
UTR	untranslated region
VDAC2	voltage-dependent anion-selective channel 2
VHL	von Hippel-Lindau tumor supressor
ZIP8	ZRT/IRT-like protein 8
ZIP14	ZRT/IRT-like protein 14

SUMMARY

Iron belongs among the trace elements and its role in humans is irreplaceable. Up to 5 g of iron can be found in adult body distributed among different compounds. Iron ions are therefore essential to all cells of our body and its homeostasis is thoroughly controlled.

Iron uptake into the organism is mediated by enterocyte cells in the small intestine, where heme as well as non-heme forms of iron are absorbed. Non-heme iron is absorbed via Dcytb (duodenal cytochrome b), DMT1 (divalent metal transporter 1), ferroportin, hephaestin, and ceruloplasmin molecules. Although these molecules can also participate in non-transferrin-bound iron transport across plasma membranes within the whole organism, mechanisms of this transport are not yet fully elucidated.

The aim of the present work was to contribute to our understanding of molecular mechanisms that are involved in non-transferrin-bound iron transport across the plasma membrane of mammalian cells. Our project was focused on the description of non-transferrin-bound iron transport in human cells *in vitro* and *in vivo* under conditions of iron deficiency or iron overload. Transformed cell lines, that represent the three main types of cells involved in iron homeostasis, and tissue samples of duodenal biopsies were used as experimental models.

The expression of DMT1, Dcytb, ferroportin, hephaestin and ceruloplasmin molecules was tested in human cell lines Caco-2 (colorectal carcinoma), K562 (erythroleukemia) and HEP-G2 (hepatocellular carcinoma). The Caco-2 cell line represents intestinal cells responsible for iron absorption, the K562 cell line represents erythroid, iron-utilizing cells, and the HEP-G2 cell line is a model of hepatocytes that exhibit a high capacity for iron storage. The expression of the mentioned molecules was also tested in tissue samples from patients with iron deficiency anemia (IDA), hereditary

hemochromatosis (HHC), alcoholic liver disease (ALD) and healthy controls. The level of expression was tested on both mRNA and protein levels. Moreover, HFE (hemochromatosis gene) and TfR1 (transferrin receptor 1) mRNA levels and serum hepcidin levels were analyzed in patient samples.

In *in vitro* experiments we demonstrated that different iron availability affects the expression of tested molecules in cell-type specific manners. In Caco-2 cells, we detected changes that correspond with suggested mechanisms of cellular regulation of iron transport via IRP/IRE interactions. Under iron deprivation, we detected increase iron uptake in K562 cells. Although the increase in iron uptake was dependent on protein synthesis, we detected no changes in the protein expression of tested molecules. Therefore, we assume involvement of others, as yet, unidentified molecules participating in non-transferrin-bound iron transport into these cells.

In *in vivo* studies, we demonstrated decreased serum hepcidin levels in all tested groups of patients compared to controls. However, the change was significant only in the ALD group of patients. Nevertheless, the effect of hepcidin levels on tested iron transport molecules in our patients was not confirmed. Increased mRNA levels of DMT1, ferroportin and TfR1 were detected, however, these changes were not confirmed on the level of proteins. This may be due to the relatively small group of patients, which contributed to the high heterogeneity of results. However, some positive correlations among Dcytb, hephaestin, DMT1, ferroportin, and TFR1 mRNA in all groups of patients indicate coordinated regulation of the expression of these genes.

In our study, we contributed to our understanding of the regulatory mechanisms of iron uptake into the organism via duodenal enterocytes using *in vitro* and *in vivo* experiments. Active transport of non-transferrin-bound iron was also detected in non-enterocyte cells, however, it was most likely with the involvement of other suspected molecules.

SOUHRN

Železo patří mezi stopové prvky a jeho role je u člověka nezastupitelná. V těle dospělého člověka může být přítomno až 5 g železa jako součást nejrůznějších sloučenin. Ionty železa jsou tedy esenciální pro veškeré buňky našeho těla a jejich homeostáza tak musí být důsledně kontrolována.

Železo vstupuje do organismu přes enterocytární buňky tenkého střeva, kde je absorbováno jak v hemové tak nehemové formě. Nehemové železo je přijímáno pomocí molekul Dcytb (duodenal cytochrome b), DMT1 (divalent metal transporter 1), ferroportin, hephaestin a ceruloplasmin. Ačkoli se tyto molekuly mohou podílet i na transportu netransferrinového železa přes plasmatickou membránu v rámci celého organismu, mechanismy tohoto transportu nejsou stále plně prozkoumány.

Cílem předložené práce bylo přispět k pochopení molekulárních mechanizmů, které se podílejí na transportu netransferinového železa přes plasmatickou membránu v savčích buňkách. Náš projekt byl zaměřen na popis transportu netransferinového železa v lidských buňkách *in vitro* a *in vivo* při nedostatku nebo přetížení železem. Jako experimentální modely jsme použili transformované buněčné linie reprezentujících tři základní typy buněk homeostázy železa a vzorky tkání z duodenálních biopsií.

Expresi DMT1, Dcytb, ferroportinu, hephaestinu and ceruloplasminu jsme testovali v lidských buněčných liniích Caco-2 (kolorektální karcinom), K562 (erytroleukemie) a HEP-G2 (hepatocelulární karcinom). Buněčná linie Caco-2 reprezentuje intestinální buňky zodpovědné za absorpci železa, linie K562 představuje erythroidní, železo-využívající buňky, a linie HEP-G2 je modelem hepatocytů s vysokou schopností železo skladovat. Expresi daných molekul jsme dále testovali ve vzorcích duodenální tkáně pacientů s anemií v důsledku nedostatku železa (IDA, iron deficiency anemia), hereditární hemochromatózou (HHC), alkoholovým jaterním postižením (ALD, alcohol

liver disease) a u zdravých kontrol. Míru exprese jsme testovali jak na úrovni mRNA, tak na úrovni proteinů. Ve vzorcích pacientů jsme dále stanovovali hladinu mRNA pro HFE (gen pro hemochromatózu) a TfR1 (transferinový receptor 1) a hladinu sérového hepcidinu.

V rámci *in vitro* experimentů jsme ukázali, že rozdílná dostupnost železa ovlivňuje expresi testovaných molekul v závislosti na buněčném typu. U buněk Caco-2 jsme detekovali změny, které korespondují s předpokládaným mechanismem regulace transportu železa na buněčné úrovni pomocí IRP/IRE interakcí. Za podmínek nedostatku železa jsme zjistili zvýšený příjem železa buňkami K562. Ačkoli byl zvýšený příjmem železa závislý na proteosyntéze, nezaznamenali jsme žádné změny v expresi testovaných molekul na úrovni proteinu. Předpokládáme tedy účast dalších zatím neidentifikovaných molekul podílejících se na transportu netransferinového železa do těchto buněk.

Ve studiích *in vivo* jsme zaznamenali snížené hladiny sérového hepcidinu oproti kontrole u všech testovaných skupin pacientů. Nicméně, pouze v případě pacientů s ALD se jednalo o změnu signifikantní. Vliv hepcidinu na testované molekuly transportu železa u našich pacientů jsme však nepotvrdily. Na úrovni mRNA jsme sice zjistili zvýšení exprese DMT1, ferroportinu a TfR1, ale tyto změny nebyly potvrzeny na úrovni proteinu. Důvodem mohou být relativně malé soubory pacientů přispívající k vysoké heterogenitě výsledků. Přesto, určité pozitivní korelace mezi mRNA hladinou Dcytb, hephaestinu, DMT1, ferroportinu a TfR1 u všech skupin pacientů nasvědčují koordinované regulaci těchto genů.

V rámci naší studie jsme přispěli k porozumění regulačních mechanismů vstupu železa do organismu prostřednictvím duodenálních enterocytů pomocí *in vitro* i *in vivo* experimentů. Aktivní transport netransferrinového železa jsme prokázali i v případě neenterocytárních buněk, avšak velmi pravděpodobně za účasti i jiných suspektních molekul.

1. INTRODUCTION

1.1. IRON AND THE BODY

Iron is an essential element for all organisms due to its involvement in many cellular processes. In biological systems, iron is present in two stable oxidative states, such as ferric ion (Fe^{3+}) and ferrous ion (Fe^{2+}). Under physiological conditions, ferrous iron is subjected to rapid oxidation and ferric iron forms insoluble, biologically unavailable hydroxides. The equilibrium concentration of free ferric iron is under these conditions extremely low, which means an almost complete absence of free ferric iron in biological systems.

Iron is involved in oxygen transfer (hemoglobin, myoglobin) and thanks to its ability to exist in two stable oxidative states it can also function as a donor or acceptor of electrons. Owing to this redox ability, iron is an active part of the electrotransport proteins (iron-sulfur proteins and cytochromes), it participates in the processes of oxidative phosphorylation (mitochondrial aconitase, succinate dehydrogenase), DNA synthesis (ribonucleotide reductase), catalytic oxidation (oxidase) and aides in decomposition of oxygen derivatives (superoxide dismutase). It also participates in hydrogen and nitrogen fixation (nitrogenase, hydroxylase), and many other reactions.

Nevertheless, the very same properties of iron which are beneficial for an organism can also lead to organ damage. Redox reactions of iron contribute to the formation of free radicals, which are often responsible for serious cell injury. As such, free ferrous iron can react with peroxides to produce hydroxyl and hydroperoxyl radicals via the Fenton reaction (Walling *et al.*, 1975; Graf *et al.*, 1984). Hydroxyl radicals are particularly reactive and can cause lipid peroxidation with subsequent damage of cellular membranes,

or damage of DNA and proteins (Gutteridge *et al.*, 1983; Conrad and Umbreit, 1993; Britton *et al.*, 1994; Andrews, 2000a and 2005).

Iron, of all the biogenic trace metal elements, is present in the body in the highest amount, i.e. about 35 mg/kg for females and 45 mg/kg in males. The largest proportion of total iron in the body is incorporated into hemoglobin (65 to 75%). About 10% of iron is present in myoglobin, cytochromes, and other enzymes, about 20-30% is stored in the form of ferritin, and less than 1% is present in the blood.

Under normal conditions, most of the iron in human plasma (15-25 μM) is bound to transferrin (Morgan, 1981). However, about 0.5 μM iron circulates as non-transferrin iron (Batey *et al.*, 1980) which is present in the form of low-molecular-mass complexes or, to a lesser degree, bound to other plasma proteins, such as the previously mentioned hemoglobin, myoglobin, serum ferritin, and others (Hershko, 1975; Muller-Eberhard and Morgan, 1975; Sibille *et al.*, 1988; van Eijk and de Jong, 1992). Although the total body iron content is 3.5 to 4 g, on average, a maximum of 2 mg of iron is absorbed and subsequently lost from the organism each day (Andrews, 1999 and 2000b; Steinbicker and Muckenthaler, 2013) (Fig. 1.1.). Since there is no active mechanism of iron release

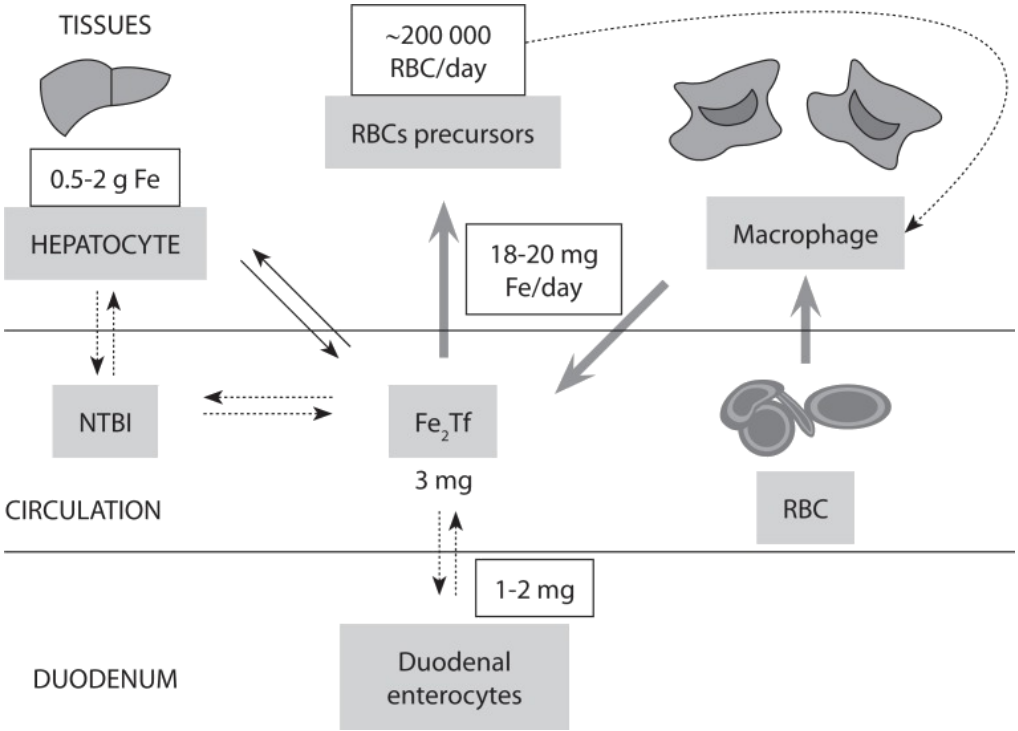

Fig. 1.1. Body iron distribution in healthy state. NTBI – non-transferrin-bound iron, RBC – red blood cells, Tf – transferrin (from Tandara and Salamunic, 2012).

from the body, the regulation of iron content in an organism takes place at the level of iron absorption in intestine.

Generally, iron transport across the plasma membrane fulfils at least three roles in mammals: (1) to absorb iron in the intestine for the purposes of the entire organism, (2) to supply individual cells with required iron, and (3) to clear potentially dangerous iron from the circulation, particularly under the conditions of iron overload (Hentze *et al.*, 2004; Andrews, 2005). For these reasons, not only iron absorption, but also iron transport and storage within the body are very strictly regulated processes (Andrews, 1999; Frazer and Anderson, 2003; Hentze *et al.*, 2004).

1.2. IRON ABSORPTION BY ORGANISM

Dietary iron can be absorbed into the organism in two basic forms. Heme iron, as a source of organic iron, is present predominantly in red meat and creates approximately 20% of total iron in the diet. Up to 80% of dietary iron is therefore consisted of non-heme iron from foods of plant origin. Nevertheless, an efficiency of iron utilization from the diet differs between heme (25 to 50%) and non-heme (1 to 10%) iron, usually resulting in higher absorption of organic iron from animal based foods (Monsen, 1988; Lombard *et al.*, 1997; Anderson *et al.*, 2005; Sharp and Srai, 2007). Both heme and non-heme iron are absorbed into the organism via enterocytes in the duodenum and proximal jejunum (Conrad *et al.*, 1966; Raffin *et al.*, 1974; Muir and Hopfer, 1985; Chowrimootoo *et al.*, 1992).

1.2.1. HEME IRON ABSORPTION

For intestinal heme absorption several mechanisms are considered. Apart from receptor-mediated endocytosis and passive diffusion, active transport is mainly discussed. A potential heme transporter across the intestinal apical membrane, heme

carrier protein 1 (HCP1), was discovered in 2005 (Shayeghi *et al.*, 2005) but later disputed, since the molecule was actually established as a proton-coupled folate transporter (PCFT). However, the role of PCFT/HCP1 as an apical heme transporter has been reestablished with dual functions as a heme/folate importer with high affinity for folate (Le Blanc *et al.*, 2012).

Imported heme is metabolized in intestinal cells by heme oxygenase-1 (HO-1) and -2 (HO-2), which produces carbon monoxide, biliverdin, and free ferrous iron (Raffin *et al.*, 1974; Maines *et al.*, 1986; Maines, 1988). Inducible HO-1 is expressed in macrophages, microglia, and other cells whereas constitutive HO-2 can be mainly found in testicular, liver, renal, neuronal, and vascular cells (Ryter *et al.*, 2006). Biliverdin is then metabolized to bilirubin by biliverdin reductase. Free iron becomes part of the labile iron pool, is stored in a ferritin, or transported from the enterocyte into the bloodstream (Fig. 1.2.).

Besides, some parts of imported heme molecules can remain intact and active mechanisms of heme export from enterocytes are involved. Intracellular heme can be transported across the basolateral membrane into the circulation as well as across the apical membrane back into the lumen. The feline leukaemia virus subgroup C cellular

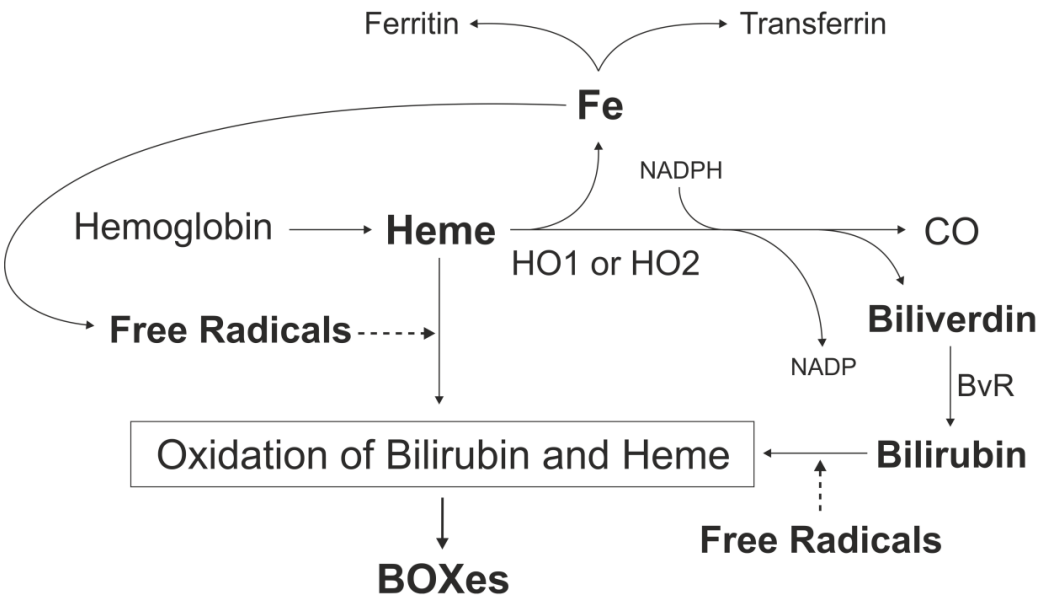

Fig. 1.2. Heme metabolism. BOX – oxidized form of bilirubin, BvR – biliverdin reductase, HO – hemoxygenase (from Clark and Sharp, 2006).

receptor 1 (FLVCR1) and ATP-binding cassette sub-family G member 2 protein (ABCG2) are supposedly responsible for this transport (Quigley *et al.*, 2004; Krishnamurthy *et al.*, 2007; Keel *et al.*, 2008; Vlaming *et al.*, 2009) (summarized in Fig. 1.3.).

1.2.2. NON-HEME IRON ABSORPTION

Non-heme iron transport, highly important for intestinal iron absorption, is mediated by divalent metal transporter 1 (DMT1) and ferroportin molecules. DMT1 (NRAMP2, DCT1) is a transmembrane iron importer with a high affinity for divalent iron. DMT1 is a proton symporter and therefore it is active only in specific low-pH environments (Fleming *et al.*, 1997; Gunshin *et al.*, 1997; Canonne-Hergaux *et al.*, 1999; Tandy *et al.*, 2000). Ferroportin (Ireg1, MTP1) is the only known protein mediating iron export from cells. As well as DMT1, ferroportin is a transmembrane transporter with affinity for ferrous iron (Abboud and Haile, 2000; Donovan *et al.*, 2000; McKie *et al.*, 2000). The expression of both molecules is regulated on least two levels: via cellular iron levels or the amount of iron in the diet using the IRE/IRP post-transcriptional regulatory mechanism (see 1.5.2.), or post-translationally via hepcidin (see 1.5.1.). Moreover, regulation on the transcription level involving the hypoxia and HIF/HRE mechanism can participate here as well (see 1.5.2.).

Dietary iron is mostly present in its ferric form, therefore, prior to intake into enterocytes the ferric iron is reduced, likely by duodenal cytochrome b ferrireductase (Dcytb) (McKie *et al.*, 2001; Latunde-Dada *et al.*, 2008; Wyman *et al.*, 2008). However, some other non-enzymatic mechanisms can participate in the reduction of dietary iron prior to its uptake into cells (Pollack *et al.*, 1963; May *et al.*, 1999; Ghio *et al.*, 2003; Lane and Lawen, 2008). Ferrous iron is then transported across the apical membrane of the enterocytes by the DMT1 importer or other possible metal transporters such as ZRT/IRT-like protein 8 and 14 (ZIP8 and ZIP14) (Jenkitkasemwong *et al.*, 2012; Wang *et al.*, 2012). Free iron is utilized within the cell or transported by ferroportin through the basolateral membrane out of the enterocytes. Once iron is exported, it is oxidized by

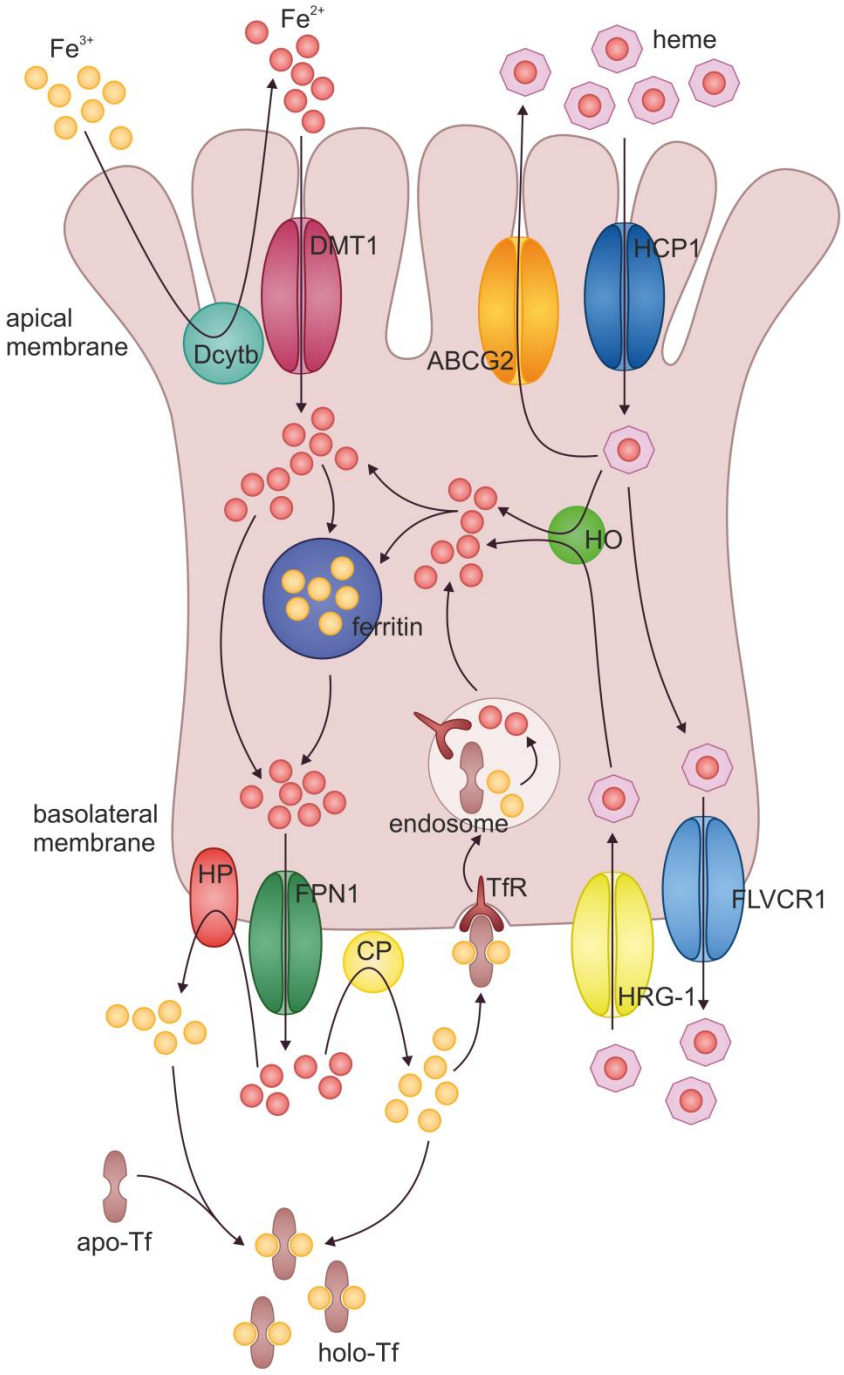

Fig. 1.3. Absorption of iron by enterocyte. ABCG2 – ATP-binding cassette sub-family G member 2 protein, CP – ceruloplasmin, Dcytb – duodenal cytochrome b, DMT1 – divalent metal transporter 1, FLVCR1 – feline leukaemia virus subgroup C cellular receptor 1, FPN1 – ferroportin 1, HCP1 – heme carrier protein 1, HO – hemoxygenase, HP – hephaestin, HRG-1 - heme responsive gene-1, Tf – transferrin, TfR – transferrin receptor.

transmembrane ferroxidase hephaestin or plasma ceruloplasmin (Vulpe *et al.*, 1999; Hellman and Gitlin, 2002). In circulation, ferric iron is bound by plasma transferrin and delivered within the organism (summarized in Fig. 1.3.).

Moreover, another non-heme iron absorption process was recently recognized. It is generally accepted that clathrin-dependent endocytosis of ferritin (see 1.4.) is responsible for iron uptake into cell. However, the process itself has not been sufficiently clarified. T cell immunoglobulin and mucin-domain 2 (TIM-2) and scavenger receptor class A member 5 (SCARA5) molecules were identified as ferritin receptors in mice but not in humans (Chen *et al.*, 2005; Li *et al.*, 2009). In human cells, though, transferrin receptor 1 (TfR1) was described as a ferritin receptor responsible for ferritin internalization into endosomes and lysosomes (Li *et al.*, 2010).

1.3. IRON TRANSPORT WITHIN ORGANISM

Iron release into the circulation for its utilization in the body is mediated via enterocytes and macrophages. While enterocytes are responsible for iron uptake from the diet, macrophages release iron that is recycled from senescent or damaged erythrocytes.

Mammalian cells usually acquire iron from the bloodstream by transferrin (Tf) uptake via transferrin receptor (TfR) mediated endocytosis (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). However, the physiological relevance of non-transferrin low-molecular-mass iron uptake has also been clearly demonstrated especially in iron overload states (Qian and Tang, 1995). The uptake of non-transferrin iron by cultured cells as well as by tissues such as liver, pancreas, heart, and brain in *in vivo* studies has been shown by many authors (Brissot *et al.*, 1985; Basset *et al.*, 1986; Wright *et al.*, 1986; Sturrock *et al.*, 1990; Kaplan *et al.*, 1991; Seligman *et al.*, 1991; Inman and Wessling-Resnick, 1993; Ueda *et al.*, 1993; Baker *et al.*, 1998; Musilkova *et al.*, 1998; Kovar *et al.*, 2006; Balusikova *et al.*, 2009). Moreover, heme iron can be transported within the organism too (Latunde-Dada *et al.*, 2006).

1.3.1. TRANSPORT OF TRANSFERRIN-BOUND IRON

Iron transport in mammals and uptake by cells of the organism is mainly realized via iron binding to plasma transferrin and through endocytosis mediated by transferrin receptors (Qian *et al.*, 1997).

Free iron is transported from cells into the serum in its ferrous form. Prior to its binding to transferrin it has to be oxidized into the ferric form by ferroxidase hephaestin or ceruloplasmin (see 1.2.2.). When present in serum, two ferric ions are bound to the iron-free transferrin molecule (apo-transferrin) with relatively high affinity (Holmberg and Laurell, 1947; Laurell and Ingelman, 1947; Aisen and Listowsky, 1980). Transferrin with ferric iron (holo-transferrin) is subsequently bound to the transferrin receptor 1 (TfR1) under extracellular pH of 7.4, in order to be transported into the cells (Jandl *et al.*, 1959; Dautry-Varsat *et al.*, 1983). Only one ferric iron can be bound to transferrin, but transferrin affinity for the transferrin receptor afterwards is very low. Transferrin receptor 2 (TfR2) is present in the serum too, but its affinity toward transferrin is significantly lower compared to TfR1 and therefore TfR2 mainly serves as a regulatory molecule (Kawabata *et al.*, 1999 and 2000).

Complexes of holo-Tf/TfR1 are accumulated on the membrane and internalized in endosomes. After internalization, ferric iron is released from Tf/TfR1 complexes in endosomes due to decreased endosomal pH. It is reduced into ferrous iron by six-transmembrane epithelial antigen of prostate 3 (STEAP3) molecules and transported to the cytoplasm via DMT1 transporter (Fleming *et al.*, 1998; Gruenheid *et al.*, 1999; Tabuchi *et al.*, 2000; Canonne-Hergaux *et al.*, 2001; Knutson, 2007). Lately, ZIP14 was shown to participate in endosomal iron transport, too (Zhao *et al.*, 2010). Iron-free transferrin is released from Tf/TfR1 complex after endosome recycling onto the cell surface (Fig. 1.4.).

Transferrin iron uptake is regulated by TfR1 expression changes which inversely reflect intracellular iron levels using the IRP/IRE mechanism and also by hypoxia via the HIF/HRE regulatory system (see 1.5.2.).

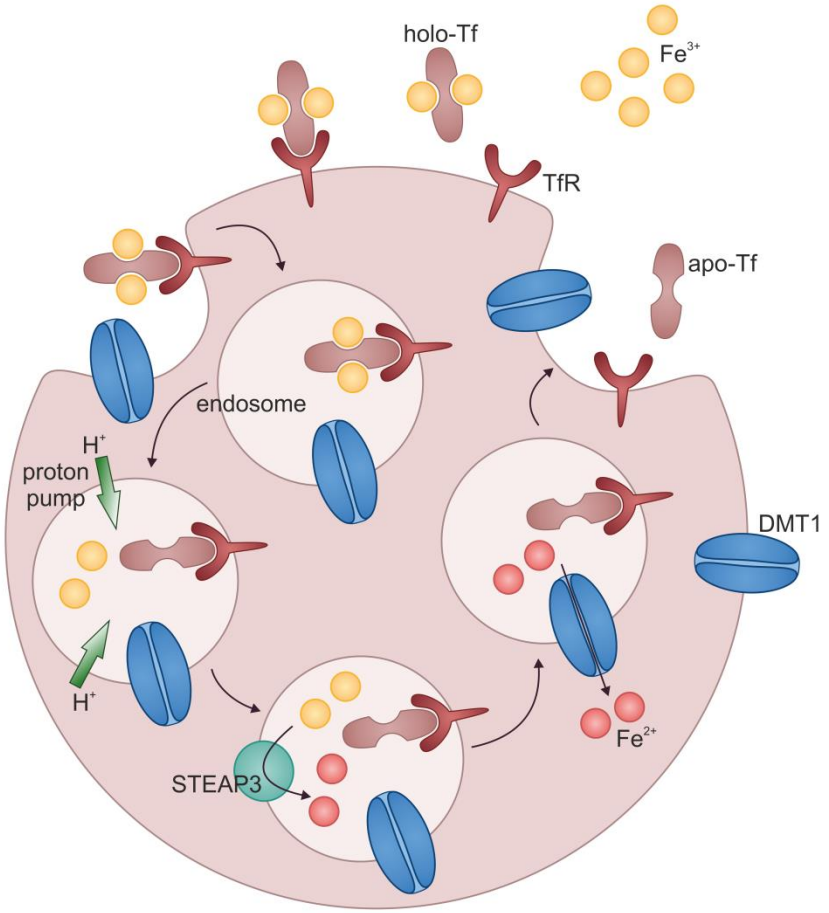

Fig. 1.4. Cellular iron uptake via transferrin cycle. DMT1 – divalent metal transporter 1, STEAP3 – six-transmembrane epithelial antigen of prostate 3, Tf – transferrin, TfR – transferrin receptor.

1.3.2. TRANSPORT OF NON-TRANSFERRIN-BOUND IRON

Within the organism, mammalian cells can take up iron from low-molecular-mass sources via an alternative transferrin-independent mechanism that is actually the same as in intestine (see 1.2.2.). DMT1 probably together with Dcytb ferrireductase are involved in iron absorption, while ferroportin, together with the plasma ferroxidase ceruloplasmin, are involved in the iron export from cells into the plasma (Fleming *et al.*, 1997; Andrews,

2002; Canonne-Hergaux and Gros, 2002; Hentze *et al.*, 2004; Andrews, 2005; Richardson, 2005; Dunn *et al.*, 2007).

Although Dcytb was shown to play an important role in the reduction of ferric iron, some other molecules with ferroxidase activity, such as proteins of the STEAP family, have been detected. As mentioned before (1.3.1.), STEAP3 is also responsible for iron reduction in endosomes (Knutson, 2007). Currently, a prion protein has been demonstrated as possible ferrireductase in mice and HEP-G2 cells (Tripathi *et al.*, 2015). The role of the ferrous iron transporter DMT1 is well established especially in enterocytes, but also other metal transporters, such as ZIP8 and ZIP14, have been identified for non-transferrin-bound iron import into the cells. Both, ZIP14 and DMT1 have been found to be abundantly expressed in liver, pancreas, and heart tissue whereas ZIP8 is highly expressed in lung and placenta. DMT1 is also increasingly produced by brain, prostate, and thymus (Liuzzi *et al.*, 2006; Jenkitkasemwong *et al.*, 2012; Wang *et al.*, 2012; Nam *et al.*, 2013). Other molecules suspected of iron import are for example β3 integrin, lipocalin 2 and voltage-dependent anion-selective channel 2 (VDAC2) (Yang *et al.*, 2002; Devireddy *et al.*, 2005; Valis *et al.*, 2008). Though ferroxidase ceruloplasmin can be found as a free plasma molecule, a transcription variant of ceruloplasmin with glycosylphosphatidylinositol-anchor (GPI-ceruloplasmin) was described as an essential molecule for ferroportin stabilization in plasma membrane. GPI-ceruloplasmin expression has been detected in hepatocytes, glioma cells, astrocytes, and macrophages (De Domenico *et al.*, 2007a; Marques *et al.*, 2012).

Similarly, heme transport within organism also corresponds with heme absorption via enterocytes (see 1.2.1.). But in contrast to enterocytes, the transmembrane heme importer responsible for heme uptake from serum is well established. Heme responsive gene-1 (HRG-1) has been found to be involved in heme import into the brain, heart, kidneys, and small intestines. In intestinal cells, however, HRG-1 is localized in the basolateral membrane only and thus is not responsible for heme uptake from the diet but only from the circulation. Moreover, HRG-1 is also responsible for heme transport from lysosomes (Yanatori *et al.*, 2010). Heme cellular exporters FLVCR1 and ABCG2 have been found, in addition to duodenum, in liver, kidneys, lungs, spleen, brain, placenta and others tissues (Quigley *et al.*, 2004; Keel *et al.*, 2008; Krishnamurthy and Schuetz, 2006; Krishnamurthy *et al.*, 2007; Vlaming *et al.*, 2009) (Fig. 1.3. and 1.5.).

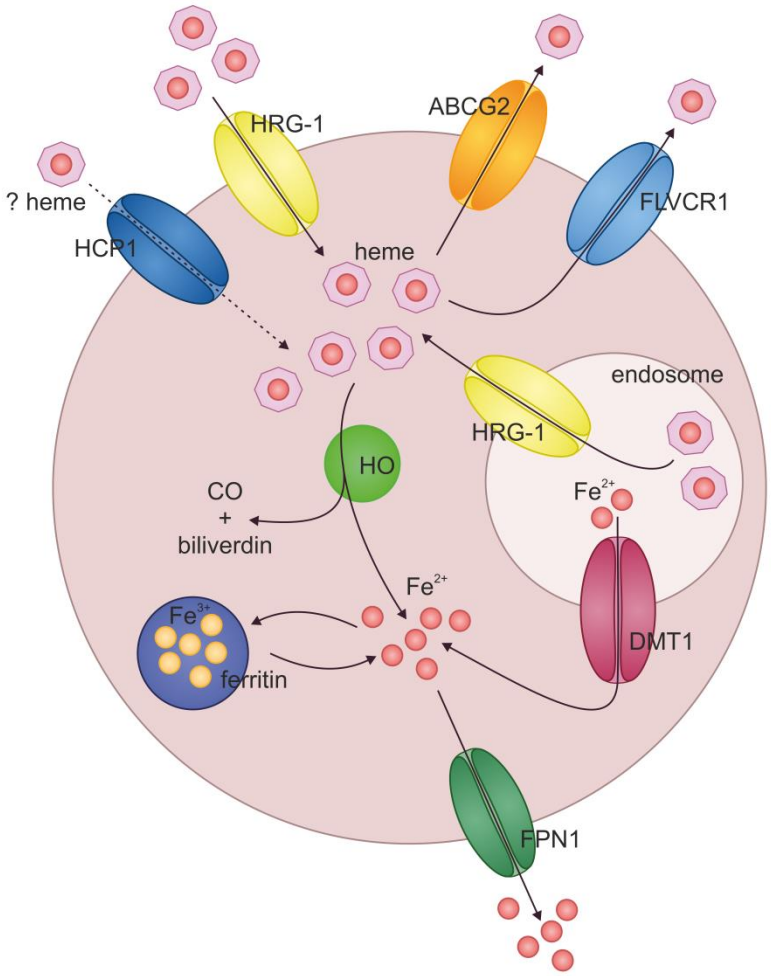

Fig. 1.5. Heme transport in non-intestinal cells. ABCG2 – ATP-binding cassette sub-family G member 2 protein, DMT1 – divalent metal transporter 1, FLVCR1 – feline leukaemia virus subgroup C cellular receptor 1, FPN1 – ferroportin 1, HCP1 – heme carrier protein 1, HO – hemoxygenase, HRG-1 – heme responsive gene-1.

A very special mechanism of iron uptake is involved in macrophages, since reticuloendothelial macrophages are responsible for iron recycling from red blood cells. Senescent red blood cells are phagocytized and lysed in phagolysosomal compartments, hemoglobin is degraded, heme is transported from phagolysosomes by HRG-1 and free iron is released after heme oxidation (see 1.2.1.). Once in macrophages, iron can be stored in ferritin deposits or returned into the circulation by ferroportin (Andrews, 2000b) (Fig. 1.6.).

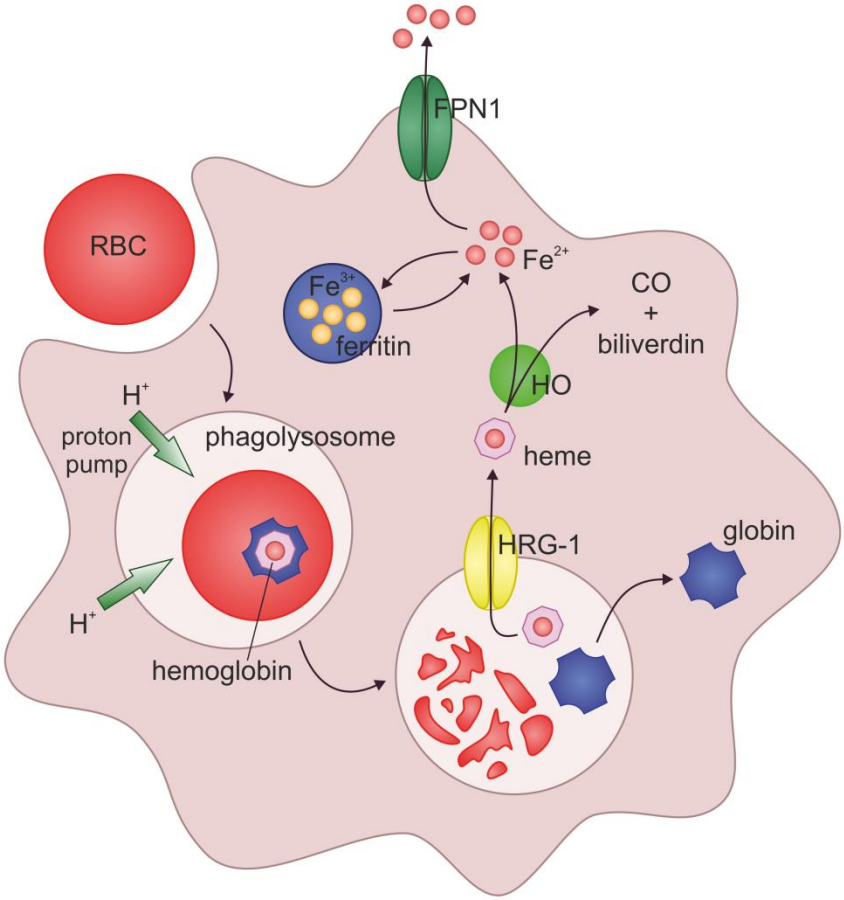

Fig. 1.6. Iron recycling by macrophage. FPN1 – ferroportin 1, HO – hemoxygenase, HRG-1 – heme responsive gene-1, RBC – red blood cell.

1.4. IRON STORAGE IN ORGANISM

Since free iron cations transported into the cytoplasm are potentially toxic, the cells eliminate this toxicity basically in the three ways: free iron is (1) distributed to different compartments, where it is further used for the synthesis of important proteins; (2) exported from the cell into the circulation, or (3) stored in deposits. However, the distribution of iron storage in various organs and tissues is not equal. The highest storage

capacity for the iron is in the cells of parenchymatic tissues such as liver, pancreas, heart, and endocrine organs (Hower *et al.*, 2009).

The two main iron-storage molecules within the cells are ferritin and hemosiderin. For most vertebrates, ferritin is composed of two subunits designated as L- (light) and H- (heavy) ferritin. These subunits are organized into higher structures each of which consists of 24 molecules of L- or H-ferritin (Fig. 1.7.). Hence, each ferritin cluster is able to enclose up to several thousand ferric ions (Munro and Linder, 1978; Harrison and Arosio, 1996). Ferritins differ in amounts of L- and H subunits and are referred to as isoferritins, which are present in individual tissues (Theil, 2003). H-ferritin is a ferroxidase and its activity facilitates rapid incorporation of iron into ferritin complexes as well as its rapid release. On the other hand, isoferritins rich in L- subunits accept iron more slowly and therefore store it for longer periods. Small amounts of ferritin can also be found in plasma. Serum ferritin is proportional to the total body iron level and is used as a clinical indicator of iron quantity in individuals (Walters *et al.*, 1973; Lipschitz *et al.*, 1974).

Another storage molecule for iron in the cell is hemosiderin. It is a macromolecular complex of ferritin and lipid structures. Hemosiderin is primarily present in Kupffer cells in the liver, but during chronic iron overload it can also be found in the cells of damaged organs (Richardson, 2005).

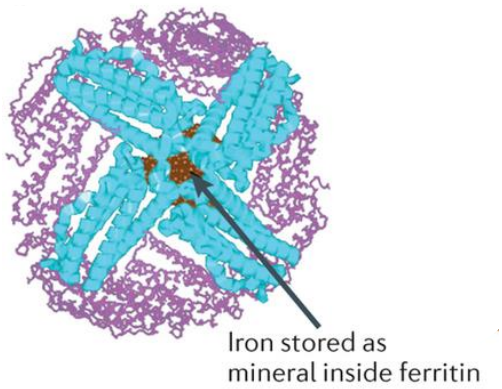

Fig. 1.7. 3D model of ferritin structure. The heavy subunit and the light subunit are shown in blue and purple, respectively (from Rouault, 2013).

1.5. REGULATION OF IRON HOMEOSTASIS

Iron homeostasis is essential to maintain sufficient iron levels to enable appropriate cell functions as well as to avoid the risk of cell injury due to the iron overload. Since there is no active mechanism for removing iron from an organism, iron intake into the body and its distribution within the body needs to be very strictly regulated.

1.5.1. HOMEOSTASIS OF BODY IRON LEVEL

Whole-body iron homeostasis is primarily mediated by the active form of the regulatory peptide hepcidin (Nicolas *et al.*, 2002a; Ganz, 2004; Dunn *et al.*, 2007). Active hepcidin negatively regulates iron uptake into the organism and its release into the serum from hepatocytes and macrophages. This regulation is based on hepcidin/ferroportin interaction that leads to ferroportin internalization and degradation with a subsequent decrease of iron transport into the bloodstream (Nemeth *et al.*, 2004; De Domenico *et al.*, 2007b) (Fig. 1.8.). Besides ferroportin, other iron transport molecules such as DMT1 and TfR1 have been suggested as hepcidin targets (Brasse-Lagnel *et al.*, 2011; Du *et al.*, 2011 and 2012).

The *HAMP* gene, which encodes hepcidin (originally identified as LEAP1, liver-expressed antimicrobial peptide) is predominantly expressed in hepatocytes (Krause *et al.*, 2000; Park *et al.*, 2001). Its expression responds to body iron levels via tissue iron stores and transferrin saturation; however it also responds to erythropoiesis, hypoxia, inflammation, and others (Nicolas *et al.*, 2002b; Ganz, 2003 and 2004; Rishi *et al.*, 2015) (Fig. 1.9.).

Iron-mediated hepcidin regulation involves activation of hepcidin transcription via the BMP-SMAD pathway. Within this pathway, the response to acute changes in extracellular iron level involves HFE (hemochromatosis gene), TfR1, and TfR2 molecules. Chronic iron overload regulation seems to involve HFE and TfR2, bone morphogenetic protein 6 (BMP6), hemojuvelin (HJV), and matriptase-2 (Ramos *et al.*,

2011). Membrane serine protease matriptase-2 is responsible for HJV cleavage and its inactivation as an agonist of BMP signaling (Silvestri *et al.*, 2008b).

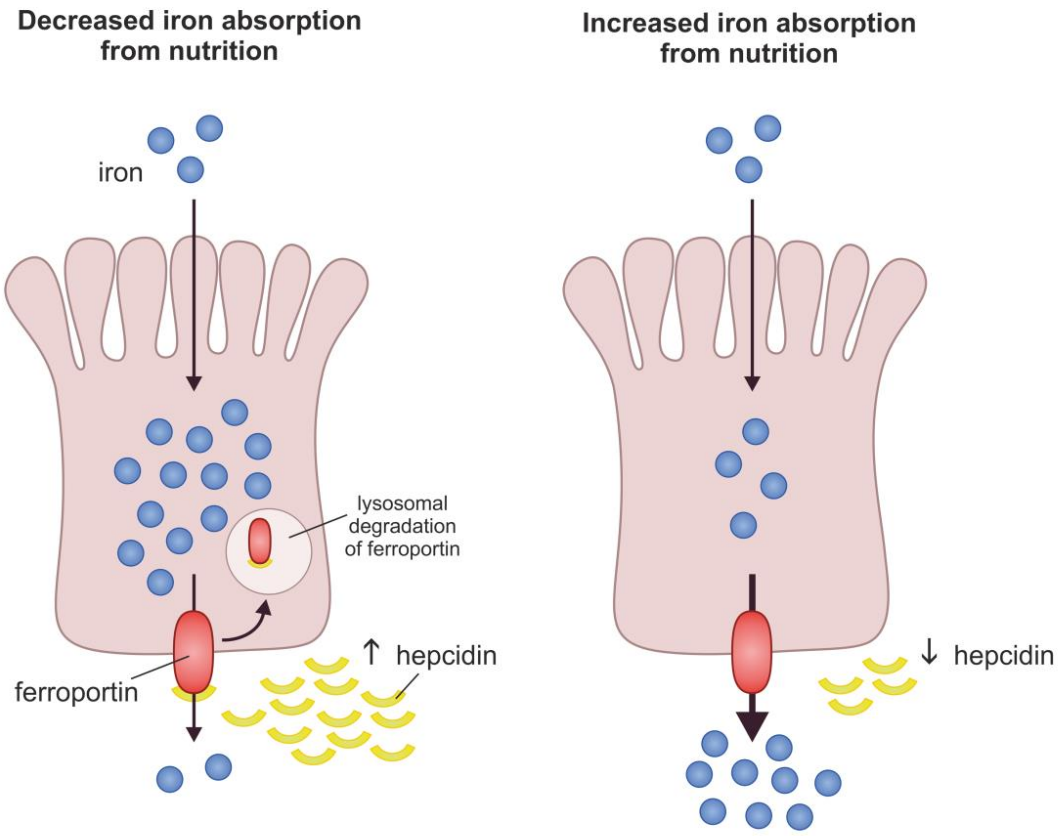

Fig. 1.8. Hepcidin effect on iron absorption via enterocytes. When hepcidin serum levels are high, hepcidin is bound to iron exporter ferroportin resulting in ferroportin internalization and degradation. Subsequently, iron release from enterocytes into the bloodstream is decreased. On the other hand, low hepcidin serum levels result in increased iron absorption via enterocytes since ferroportin stays present in basolateral membrane of the cells.

Inflammatory regulation of hepcidin is mediated by inflammatory cytokines via the JAK-STAT3 signaling pathway. Lipopolysaccharide and IL-6 were shown to increase hepcidin transcription via phosphorylation of STAT3 (Sakamori *et al.*, 2010). Moreover, it is suggested that iron-mediated and inflammatory pathways can interact and therefore HFE molecule can be involved in JAK-STAT3 mediated hepcidin regulation.

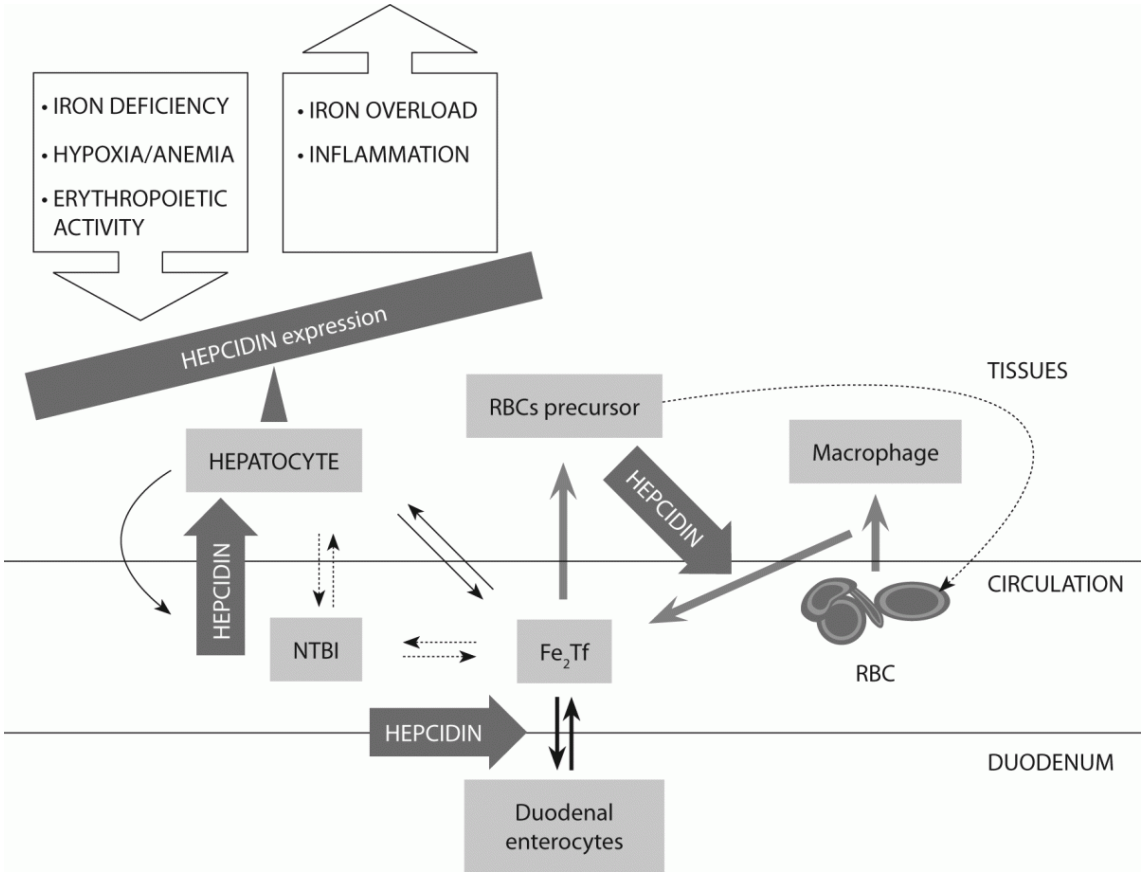

Fig. 1.9. Systemic regulation of iron homeostasis by hepcidin. NTBI – non-transferrin-bound iron, RBC – red blood cells, Tf – transferrin (from Tandara and Salamunic, 2012).

Initially, erythropoietin (EPO) was regarded as the main regulatory molecule for erythroid control of hepcidin expression. However, it has been shown that EPO can regulate hepcidin expression only indirectly (Pak *et al.*, 2006; Ashby *et al.*, 2010; Sasaki *et al.*, 2012). Subsequently, the role of other erythroid regulators of hepcidin has to be considered. Growth differentiation factor 15 (GDF15), twisted gastrulation BMP signaling modulator 1 (TWSG1) and erythroferrone (ERFE) have all been identified as potential hepcidin regulators, though their role needs to be verified. (Tanno *et al.*, 2007 and 2009; Casanovas *et al.*, 2013; Kautz *et al.*, 2014a and 2014b).

Since erythropoiesis increases under hypoxic conditions, like erythroid control, hypoxia decreases hepcidin level too. Because the hepcidin promotor sequence includes HRE regions, HIF involvement seems obvious (see 1.5.2.). Moreover, hypoxia modulates

expression of some iron sensing molecules such as TfR2, HJV and matriptase-2 that have been shown to affect hepcidin expression via the BMP-SMAD pathway, as mentioned above (Silvestri *et al.*, 2008a; Lakhal *et al.*, 2011). Besides, IL-6 and platelet-derived growth factor (PDGF) levels also increase under hypoxia (Sonnweber *et al.*, 2014).

In addition to the described regulatory mechanisms, other factors could also affect hepcidin expression. Additional regulators of hepcidin synthesis include hepatocyte growth factor (HGF), epidermal growth factor (EGF), hormones like estrogen and testosterone, and other potential regulatory pathways such as the Ras-Raf and mTOR pathway. (Goodnough *et al.*, 2012; Yang *et al.*, 2012; Guo *et al.*, 2013; Latour *et al.*, 2014; Mleczko-Sanecka *et al.*, 2014) (summarized in Fig. 1.10.).

1.5.2. HOMEOSTASIS OF CELLULAR IRON LEVEL

Cellular iron homeostasis is controlled by regulatory system based on iron regulatory protein (IRP) and iron-responsive element (IRE) interactions. IRP/IRE system regulates post-transcriptionally the expression of various iron homeostasis-related molecules (Eisenstein, 2000; Cairo and Recalcati, 2007). IREs are stem-loop regulatory sequences present in untranslated regions (UTRs) of mRNA. They bind two known cytoplasmic regulatory molecules IRP1 (ACO1, aconitase 1) and IRP2 (IREB2, iron-responsive element-binding protein 2) after their activation associated with iron deprivation (Muckenthaler *et al.*, 2008). IRP binding to IRE, located in the 5'UTR, causes translational inhibition and thus decreased levels of the target protein (Muckenthaler *et al.*, 1998). On the other hand, IRP/IRE interaction in 3'UTR mediates mRNA stabilization and thus increases protein levels (Binder *et al.*, 1994) (Fig. 1.11.).

The molecules, the expression of which is regulated by IRP/IRE mechanism, e.g. TfR1 (3'UTR), DMT1 (3'UTR), ferroportin (5'UTR), ferritin L and ferritin H (5'UTR), HIF-2α (5'UTR) etc., are responsible for iron import and export from cells as well as iron utilization and storage (Hentze *et al.*, 1987; Koeller *et al.*, 1989; Gunshin *et al.*, 1997; McKie *et al.*, 2000; Tchernitchko *et al.*, 2002; Sanchez *et al.*, 2007).

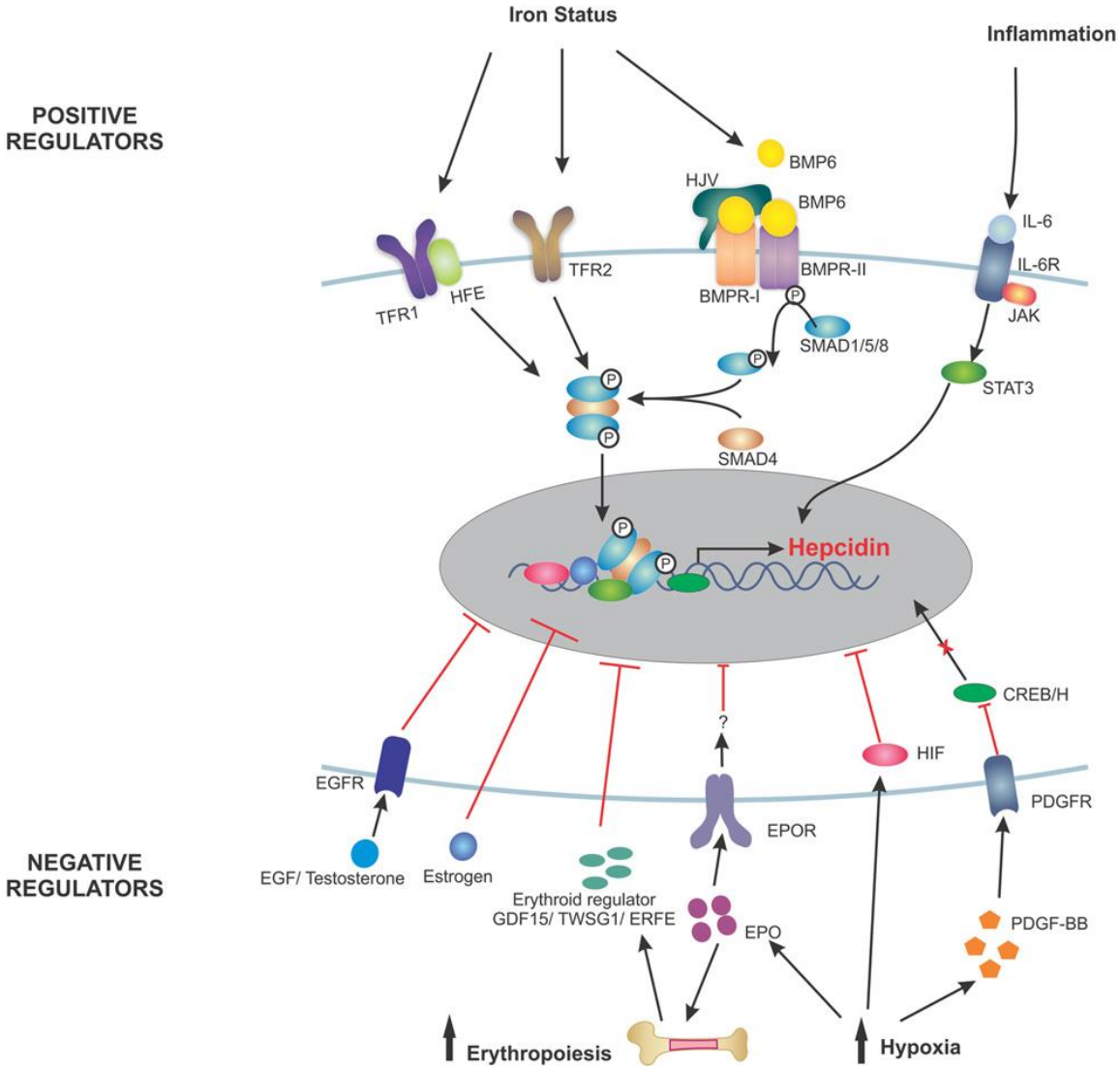

Fig. 1.10. Positive and negative regulators of hepcidin expression. BMP6 – bone morphogenetic protein 6, BMPR – bone morphogenetic protein receptor, CREB/H – cAMP response-element binding protein/H, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor, EPO – erythropoietin, EPOR – erythropoietin receptor, ERFE – erythroferrone, GDF15 – growth differentiation factor 15, HFE – hemochromatosis protein, HIF – hypoxia-inducible factor, HJV – hemojuvelin, IL-6 – interleukin 6, IL-6R – interleukin 6 receptor, JAK – Janus kinase, PDGF-BB – platelet-derived growth factor-BB, PDGFR – platelet-derived growth factor receptor, SMAD – sma and mothers against decapentaplegic homologue, STAT3 – signal transducer and activator of transcription 3, TFR – transferrin receptor, TWSG1 – twisted gastrulation BMP signaling modulator 1 (from Rishi *et al.*, 2015).

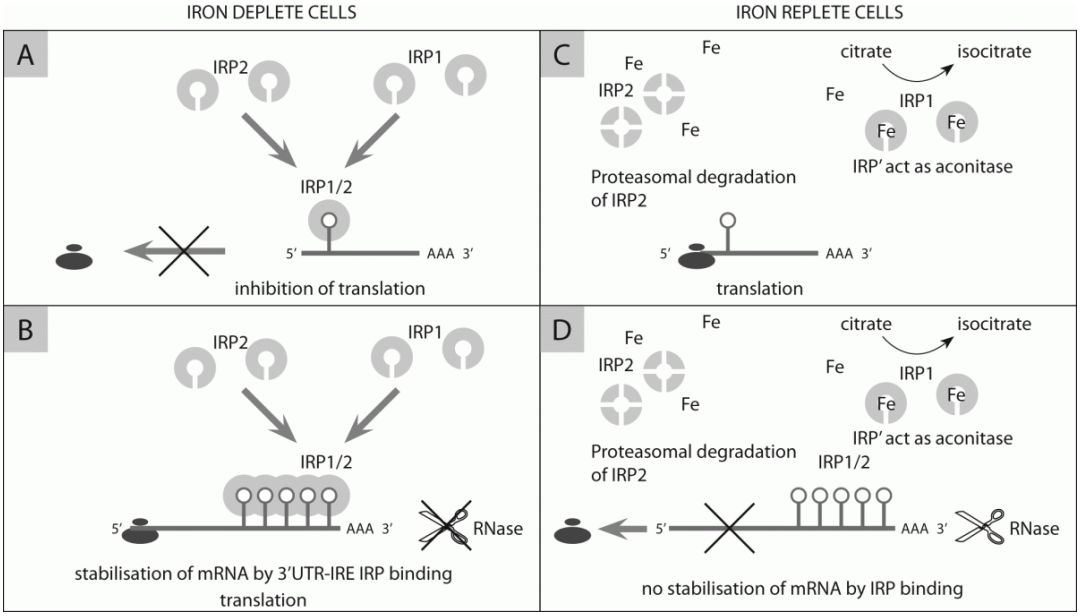

Fig. 1.11. Maintenance of cellular iron homeostasis by IRE/IRP system. Under iron deprivation, the IRP molecules bind to IRE sequences. Translation of iron storage molecules (5'UTR) is inhibited (A) when translation of iron transport molecules (3'UTR) is increased (B). Under iron overload, molecules of IRP1 function as aconitases while IRP2 molecules are degraded in proteasomes. Therefore, translation of iron storage molecules is not affected by IRPs binding and can be processed (C). On the other hand, mRNA of iron transport molecules is not stabilized and thus can be cleaved by RNases (D). IRE – iron-responsive element, IRP – iron regulatory protein, UTR – untranslated region (from Tandara and Salamunic, 2012).

Similarly another local regulatory mechanism involving HIF/HRE interaction can participate in cellular iron homeostasis (Wang and Semenza, 1993). The hypoxia-inducible factor (HIF) has been shown in previous studies to regulate intracellular iron by binding to HIF-responsive elements (HRE) within the genes coding for Tf, TfR, hepcidin, DMT1, Dcytb, ceruloplasmin, HO-1, and others (Bianchi *et al.*, 1999; Lok and Ponka, 1999; Tacchini *et al.*, 1999; Christova and Templeton, 2007; Lakhal *et al.*, 2011; Latunde-Dada *et al.*, 2011). As the HRE sequences are usually located in enhancer or promotor regions, HIF/HRE regulation affects gene expression at transcriptional level. There are three subunits of hypoxia-inducible factor that are differentially expressed in different tissues. The most important and ubiquitously expressed is subunit HIF-1α (Semenza, 1998; Ke and Costa, 2006).

During normoxia, the HIF-α subunits are hydroxylated by prolyl hydroxylase domain (PHD), bound to von Hippel-Lindau factor (VHL), and degraded in proteasomes (Srinivas *et al.*, 1999; Masson *et al.*, 2001; Masson and Ratcliffe, 2003). In hypoxia, PHD is inactivated and HIF-α is stabilized. Stabilized subunits translocate into the nucleus where they are bound to HIF-1β (ARNT, aryl hydrocarbon receptor nuclear translocator) (Wang *et al.*, 1995). Heterodimers of HIF-α and HIF-1β then bind to HRE and regulate expression of target genes (Crews, 1998) (Fig. 1.12.).

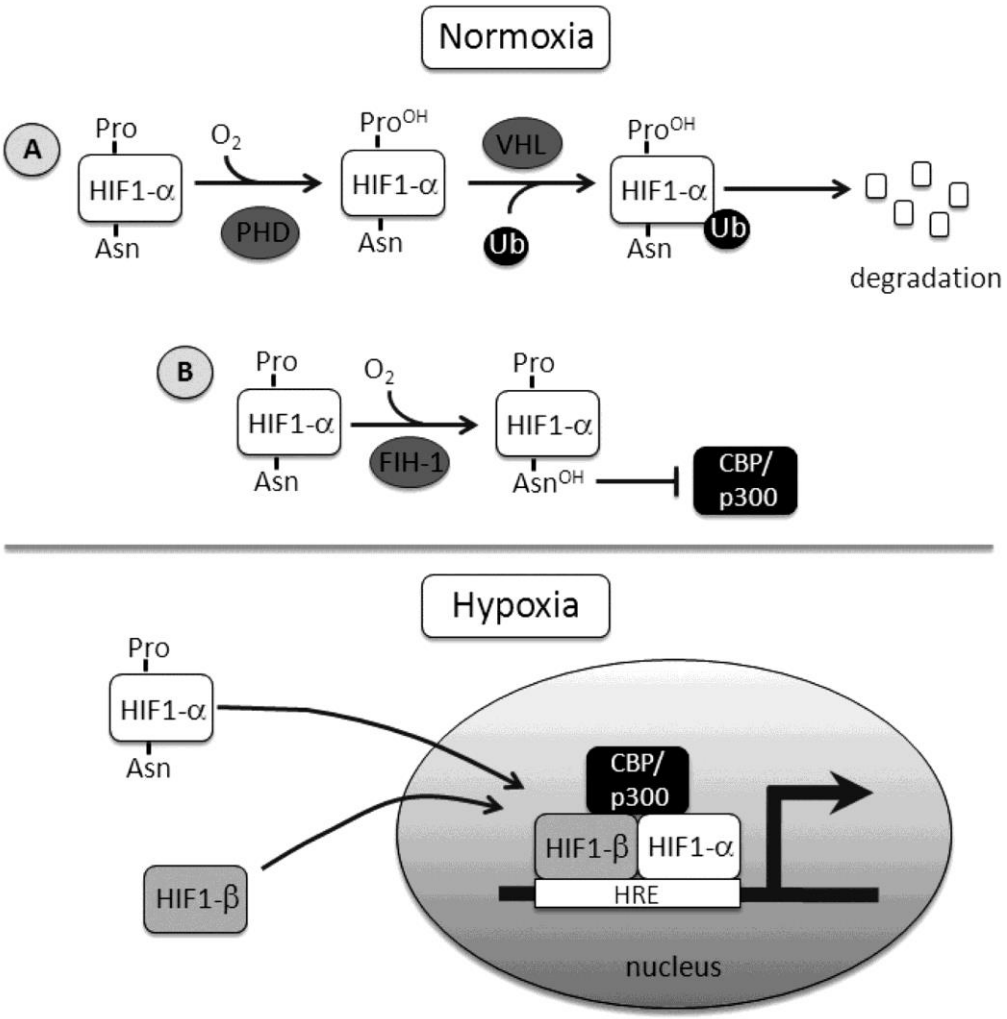

Fig. 1.12. Regulation of transcription via HIF/HRE mechanism. CBP – CREB binding protein, FIH - factor inhibiting HIF-1, HIF – hypoxia-inducible factor, HRE – HIF-responsive element, PHD – prolyl hydroxylase domain, VHL – von Hippel-Lindau tumor supressor, Ub – ubiquitin (from Shimoda and Laurie, 2014).

1.6. IRON METABOLISM-RELATED DISORDERS

Since deregulation of iron homeostasis may lead to both iron deprivation and iron excess, a variety of diseases resulting from both states can develop. We can distinguish primary and secondary iron disorders based on their different origins. Primary disorders develop as a result of a genetic abnormality whereas secondary disorders usually results from non-genetic causes. Some of primary iron disorders mentioned in following chapters are listed in Tab. 1.1.

1.6.1. IRON DEPRIVATION ASSOCIATED DISORDERS

Iron deprivation is the most frequent cause of microcytic anemia. Patients are usually asymptomatic in the early stages of the disease, but general symptoms of anemia such as fatigue, lightheadedness, and weakness can be manifested later (DeLoughery, 2014).

The common causes of low iron levels include chronic bleeding, increased physiological requirements, inadequate absorption due to chronic infection or genetic mutation, and insufficient or poor nutrition. Typical disorders resulting in microcytic anemia can therefore include iron deficiency anemia, anemia of chronic disease, thalassemia, iron refractory iron deficiency anemia, sideroblastic anemia, and others.

1.6.1.1. Primary cause of iron deficiency

Primary iron deficiency can result from DNA mutations in several genes coding for various proteins involved in iron transport and metabolism. Nevertheless, iron homeostasis is very complex process and many molecules are involved not only in iron import into the organism but also in cellular iron transport within the organism. Therefore, iron deficiencies can be associated with tissue iron overload at the same time.

Tab. 1.1. Selected iron disorders of genetic origin (from Tandara and Salamunic, 2012).

Disease	Protein involved	Phenotype/inheritance
HH type 1	HFE	Iron overload (autosomal recesive)
HH type 2A	Hepcidin	Iron overload (autosomal recesive)
HH type 2B	Hemojuvelin	Iron overload (autosomal recesive)
HH type 3	TfR2	Iron overload (autosomal recesive)
HH type 4 (Ferroportin disease)	Ferroportin	Iron overload (autosomal dominant)
Hypotransferrinemia	Transferrin	Iron overload, anemia (autosomal recesive)
Aceruloplasminemia	Ceruloplasmin	Iron overload, anemia (autosomal recesive)
DMT1-iron overload	DMT1	Iron overload, anemia (autosomal recesive)
IRDA	Matriptase-2	Iron deficiency anemia (autosomal recesive)

DMT1 – divalent metal transporter 1, HFE – hemochromatosis gene, HH – hereditary hemochromatosis, IRDA – iron deficiency anemia, TfR2 – transferrin receptor 2.

Thalassemia is autosomal recessive disorder caused by abnormal formation of hemoglobin and therefore of red blood cells. The source mutation is present in genes coding for α, β, or δ globin chains forming the hemoglobin hetero-tetramer. Patients suffering of thalassemia therefore synthetize lower amounts of hemoglobin resulting in lower numbers of red blood cells in circulation and anemia. As was mentioned before, the side effect of thalassemia can be organ iron overload especially due to repeated blood transfusions (Martin and Thompson, 2013).

Iron refractory iron deficiency anemia (IRIDA) (autosomal recessive) is caused by mutations in *TMPRSS6* (transmembrane protease, serine 6) gene encoding for the matriptase-2 protein. Matriptase-2 is responsible for hemojuvelin cleavage and thus negative regulation of hepcidin expression (see 1.5.1.). Malfunction of matriptase-2 results in increased hepcidin production and subsequent degradation of ferroportin. Since

ferroportin is responsible for cellular iron efflux, insufficient amounts of iron are absorbed into the body (Finberg, 2009; De Falco *et al.*, 2013).

The origin of sideroblastic anemia (SA) can be a congenital genetic disorder as well as have non-genetic causes. Anyway, sideroblasts are produced instead of healthy red blood cells during SA. Therefore, iron is available in the body but it is not incorporated into hemoglobin. For this reason, patients with SA very often suffer simultaneously from iron overload (Caudill *et al.*, 2008).

Aceruloplasminemia is autosomal recessive disease manifested by lack of ceruloplasmin, which is partly responsible for plasma iron oxidation into its ferric form. Ceruloplasmin deficit results in limited production of red blood cells together with iron storage in the liver cells, macrophages, neuronal tissue, and others (Harris *et al.*, 1998).

Hypotransferrinemia is characterized by partial deficiency in plasma transferrin caused by a mutation in gene coding for transferrin. Iron is therefore unavailable for erythropoiesis, in contrast to its abnormal storage in parenchymal tissues with the result of severe anemia together with a serious iron overload (Hamill *et al.*, 1991; Beutler *et al.*, 2000).

1.6.1.2. Secondary cause of iron deficiency

Disorders with secondary iron deficiency are mainly accounted for by iron deficiency anemia (IDA) and anemia of chronic disease (ACD). Both diseases are also the most prevalent form of anemias (Theurl *et al.*, 2006). Especially, since iron deficiency due to inadequate nutrition is the most common cause of microcytic anemia, IDA is one of the major health problems worldwide.

In IDA, the amount of hemoglobin and red blood cells is decreased as a result of inadequate diet with insufficient iron absorption, or excessive bleeding. On the other hand, ACD is usually observed in patients with infection, inflammation, or malignancy. The main cause of ACD involves elevated production of cytokines with a consequent up-regulation of hepcidin production and decreased iron release into the circulation (Weiss and Goodnough, 2005; Poggiali *et al.*, 2014) (see 1.5.1.).

1.6.2. IRON OVERLOAD ASSOCIATED DISORDERS

Iron accumulation in organism, production of reactive oxygen species (ROS) and subsequent oxidative stress leads to a limitation of cell functions that can affect many different tissues in the body (McCord, 1998). Iron-induced oxidative stress and subsequent apoptosis particularly damages parenchymatic organs that contain cells with numerous mitochondria and have high respiratory activity such as hepatocytes, cardiomyocytes and pancreatic β-cells. Liver cells are especially affected due to their high capacity for iron storage (Hower *et al.*, 2009), mainly in the form of ferritin and hemosiderin (Morgan and Walters, 1963; Munro and Linder, 1978). Therefore organ damage resulting in fibrosis, cirrhosis, hepatocellular carcinoma, heart failure, glucose intolerance or diabetes mellitus can be seen in patients with iron overload.

1.6.2.1. Primary cause of iron overload

Primary iron accumulation, as in the case of iron deprivation, develops as a result of a genetic abnormality and is mostly represented by hemochromatosis (Swinkels *et al.*, 2006). Hemochromatosis leads to an increase in the absorption of iron by enterocytes and subsequently, the excess iron is stored primarily in the liver, pancreas, skin, and heart tissue.

Hereditary hemochromatosis (HHC) or hemochromatosis type I is the most common type of hemochromatosis. It is an autosomal recessive disease with incomplete penetrance caused by a mutation in the *HFE* gene. *HFE* gene protein products are involved in the regulation of iron transport by hepcidin but the exact mechanism is not yet completely known (see 1.5.1.). The main cause is the C282Y (p.Cys282Tyr) mutation in the *HFE* gene that represents 80 – 90% of patients with HHC. Less severe but still frequent mutations are also H63D (p.His63Asp) and S65C (p.Ser65Cys) mutations with clinical significance, but only when combined with C282Y. Either way, symptoms are usually developed after the third decade of patient's life and can be treated by repeated

phlebotomy (Feder *et al.*, 1996; Jazwinska *et al.*, 1996; Jouanolle *et al.*, 1996; Hanson *et al.*, 2001).

Hemochromatosis type II or juvenile hemochromatosis (autosomal recessive) leads to a severe form of iron overload that manifests in early age. The disease is caused by a mutation in the gene for hemojuvelin that is involved in hepcidin regulation, or directly in gene for hepcidin. In both cases, hepcidin levels are decreased and iron is abnormally absorbed (Hentze *et al.*, 2004) (see 1.5.1.).

Other types of hemochromatosis are less common and are represented by hemochromatosis type III (an autosomal recessive mutation in *TfR2* gene) (Camaschella *et al.*, 2000), ferroportin disease (an autosomal dominant mutation in *ferroportin* gene) (Montosi, 2001; Njajou *et al.*, 2001), hemochromatosis type V (an autosomal dominant mutation in *H-ferritin* gene) (Kato *et al.*, 2001), aceruloplasminemia (see 1.6.1.1.), hypotransferrinemia (see 1.6.1.1.), and others.

1.6.2.2. Secondary cause of iron overload

Secondary liver iron overload can be developed as a consequence of ethanol liver damage, porphyria cutanea tarda, non-alcoholic steatohepatitis, chronic hepatitis C, frequent blood transfusions, etc. (Andrews, 2005). However, the most common cause of secondary iron accumulation in the liver is alcohol abuse (Siegmund and Brenner, 2005; Sebastiani and Walker, 2007; Cubero *et al.*, 2009).

Ethanol oxidation to acetaldehyde as well as oxidation of acetaldehyde within the body can affect the redox status of the cell and affect iron metabolism via changes in the expression of iron homeostasis-related molecules, especially hepcidin (e.g. Suzuki *et al.*, 2002; Bridle *et al.*, 2006; Harrison-Findik *et al.*, 2006; Harrison-Findik, 2007). Hepcidin levels decrease in response to ethanol exposure what increases protein expression of DMT1 and ferroportin (Nemeth *et al.*, 2004; Brasse-Lagnel *et al.*, 2011) resulting in increased iron uptake into the body (see 1.5.1.). Moreover, ethanol exposure can increase the expression of iron transport related molecules, such as TfR1, via hypoxia-responsive elements (Suzuki *et al.*, 2002; Kohgo *et al.*, 2005) (see 1.5.2.). Increased TfR1 levels in plasma membrane of hepatocytes increase iron uptake into these cells and thus can play

an important role in iron accumulation in the liver due to ethanol abuse (Tuoi Do *et al.*, 2011).

2. AIMS

The aim of the presented work was to contribute to our understanding of the molecular mechanisms involved in iron transport across plasma membranes and its regulation in mammalian cells. The project is mainly focused on non-heme and non-transferrin iron (NTBI) transport in human cells *in vitro* and *in vivo* under conditions of iron deficiency or iron overload.

As an experimental model, we employed human cells including the Caco-2 cell line (colorectal carcinoma representing intestinal cells involved in iron absorption), the K562 cell line (erythroleukemia representing erythroid cells involved in iron utilization), and the HEP-G2 cell line (hepatocellular carcinoma representing hepatocytes involved in iron storage). Furthermore, human duodenal tissue samples from patients with hereditary hemochromatosis, iron deficiency anemia, and alcoholic liver disease, and healthy controls were also studied.

In particular, our studies were focused on:

1. NTBI uptake in iron-utilizing cells under iron deprivation and the involvement of known NTBI transport molecules in this process (paper 1).
2. Expression of known NTBI transport molecules in various types of human cells under iron deficiency and excess (paper 2).
3. Expression of iron transport related molecules in duodenum of patients with hereditary hemochromatosis, iron deficiency anemia, and alcoholic liver disease (paper 3 and 4).

3. RESULTS AND COMMENTS

3.1. COMMENTS ON PAPERS

Iron uptake into the organism takes place in the small intestine, specifically in the duodenum and proximal jejunum. Heme as well as non-heme iron is absorbed from the diet via the apical membrane of enterocytes and subsequently transported into the bloodstream through the basolateral membrane of the cell (Conrad *et al.*, 1966; Raffin *et al.*, 1974; Muir and Hopfer, 1985; Chowrimootoo *et al.*, 1992).

Heme is thought to be imported into enterocytes via the heme carrier protein 1 (HCP1) transporter (Le Blanc *et al.*, 2012). Within the cell, heme is metabolized by heme oxygenase molecules while carbon monoxide, biliverdin, and free ferrous iron are produced (Raffin *et al.*, 1974; Maines *et al.*, 1986; Maines, 1988). Non-heme iron, usually present in nutrients in its ferric form, is reduced to ferrous iron by duodenal cytochrome b (Dcytb) and transported into the cell by divalent metal transporter 1 (DMT1) (Fleming *et al.*, 1997; Gunshin *et al.*, 1997; Canonne-Hergaux *et al.*, 1999; Tandy *et al.*, 2000; McKie *et al.*; 2001, Latunde-Dada *et al.*, 2008; Wyman *et al.*, 2008). Once inside the enterocyte the iron can be utilized, stored in ferritins, or exported into the bloodstream by ferroportin (Abboud and Haile, 2000; Donovan *et al.*, 2000; McKie *et al.*, 2000). In the circulation, ferrous iron is re-oxidized by hephaestin or ceruloplasmin and bound to transferrin as the main transport molecule for iron within the organism (Aisen and Listowsky, 1980; Vulpe *et al.*, 1999; Hellman and Gitlin, 2002). Transferrin-bound iron is absorbed into individual cells via transferrin/transferrin receptor complex-mediated endocytosis (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Additionally,

non-transferrin-bound iron (NTBI) transport across plasma membrane also takes place within the organism, but its significance increases especially during pathological states (Qian and Tang, 1995; Andrews, 2002 and 2005). NTBI transport is therefore more effective in states of iron overload or iron deficiency depending on cell type. In other words, different iron transport mechanisms are suggested in different cell types in accordance with cell function relative to iron handling.

Since there is no active mechanism responsible for iron elimination from the body, iron homeostasis usually depends on proper regulation of iron absorption. Systemic iron homeostasis is therefore strictly controlled by hepatic peptide hepcidin (Nicolas *et al.*, 2002a; Ganz, 2004; Dunn *et al.*, 2007). The negative regulatory mechanism involves hepcidin binding to ferroportin, the only known iron exporter, and subsequent ferroportin internalization and degradation (Nemeth *et al.*, 2004). The local regulation of iron homeostasis involves post-transcriptional regulatory mechanisms mainly involving iron regulatory protein (IRP)/iron-responsive element (IRE) interactions (Eisenstein, 2000; Cairo and Recalcati, 2007). Lately, hypoxia-inducible factor (HIF)/HIF-responsive element (HRE) regulation has also been considered (Wang and Semenza, 1993; Lee and Andersen, 2006).

In our studies we focused on NTBI iron transport mechanisms across the plasma membrane of human cells *in vitro* and *in vivo*. We tested molecules participating in the transport of NTBI in enterocytes as well as non-intestinal cells. The level of mRNA and protein expression of DMT1, Dcytb, ferroportin, hephaestin and ceruloplasmin molecules were tested under conditions of iron deficiency or excess using real-time PCR and western blot analysis. Moreover, some of the regulatory molecules such as hepcidin, HFE and TfR1 were also tested. In *in vivo* studies, hepcidin serum levels were assessed using commercially produced ELISA kits and all patients were tested for HFE gene mutations together with serum iron parameters.

3.1.1. IRON TRANSPORT ACROSS PLASMA MEMBRANE: *IN VITRO* STUDIES

Transferrin-bound iron transport is considered as the main iron transport mechanism within the body, while the NTBI transport mechanism is not yet fully elucidated.

Therefore, we decided to measure NTBI uptake as well as expression of molecules involved in NTBI transport in highly relevant cell types and under strict iron-defined conditions to elucidate NTBI transport molecules significance within the organism (paper 1 and 2).

During *in vitro* analyzes we tested changes in the expression of NTBI transport molecules under iron deprivation and overload in three main types of cells involved in iron handling. Caco-2 (colorectal carcinoma) human cell line represented intestinal cells responsible for iron absorption into the body, K562 (erythroleukemia) human cell line represented erythroid cells as major iron-utilizing cells, and HEP-G2 (hepatocellular carcinoma) human cells modeled hepatocytes as major storage cells (paper 2, p. 53). Moreover in K562 cells, NTBI uptake under conditions of iron deficiency was measured prior to the assessment of the expression of iron transport-related molecules (paper 1, p. 47).

Thanks to the identical defined conditions of cells cultivation we can demonstrate that different iron availability affects the expression of tested molecules in cell-type specific manners. However, due to the lack of systemic iron regulation by hepcidin, the influence of cellular regulation on iron homeostasis can be considered only. Although we detected some changes in the mRNA level of tested molecules, it did not always correlate with the expression on the level of proteins.

In Caco-2 cells, the proteins level of DMT1 and ferroportin were increased under conditions of iron deficiency and excess, respectively (Fig. 4 and 5, paper 2, p. 60). Without hepcidin involvement in the regulation, these changes correspond with the assumption of increased iron absorption into cells during iron deficiency and increased iron efflux under iron overload. Since the expression of DMT1 and ferroportin proteins reflects increase on mRNA levels (Fig. 1, paper 2, p. 58), we suggest involvement of IRP/IRE regulatory mechanism. Our model was therefore in agreement with the suggestions concerning mechanism of cellular regulation of iron uptake (Muckenthaler *et al.*, 2008).

Despite some changes in the expression of tested molecules on mRNA level in HEP-G2 and K562 cells (Fig. 2 and 3, paper 2, p. 59), slight or no changes at all were observed on the level of proteins. This is in agreement with our findings considering testing of K562 cells under iron deprivation conditions, in paper 1 (Fig. 3 and 4, paper 1, p. 50 and 51). We detected significant increase in iron uptake under iron deprivation in K562 cells (Fig. 1, paper 1, p. 50). Together with the involvement of protein synthesis

(Fig. 2, paper 1, p. 50), we expected participation of some others NTBI transport molecules in this system (Valis *et al.*, 2008).

For NTBI iron uptake into the cells, ferric iron needs to be reduced to its ferrous form. Although Dcytb was shown to play an important role, other molecules responsible for iron reduction have also been detected. At least, six-transmembrane epithelial antigen of prostate (STEAP) proteins were presented as reductases involved in iron metabolism. Among other, a family member STEAP3 has been confirmed as a ferrireductase involved in iron reduction in endosomes (Knutson, 2007). Moreover, other mechanisms can participate in reduction of iron prior to its uptake into the cells since there is also evidence of non-enzymatic iron reduction. Although the ferrous iron transporter DMT1 function has been well demonstrated, other metal transporters, such as ZRT/IRT-like protein 8 and 14 (ZIP8 and ZIP14), have also been linked to iron import (Liuzzi *et al.*, 2006; Jenkitkasemwong *et al.*, 2012; Wang *et al.*, 2012; Nam *et al.*, 2013). Molecules such as lipocalin 2 or voltage-dependent anion-selective channel 2 (VDAC2) should be also discussed (Yang *et al.*, 2002; Devireddy *et al.*, 2005; Valis *et al.*, 2008). On the other hand, the iron exporter ferroportin was found to be essential for iron efflux from cells and no new molecules have been identified for iron export. Until now, two molecules responsible for iron oxidation after iron release from the cell were discovered. A transmembrane molecule of hephaestin was shown to co-localize with ferroportin whereas ceruloplasmin is a plasma molecule. Nevertheless, a transcription variant of ceruloplasmin was described as a glycosylphosphatidylinositol-anchored isoform essential for ferroportin stabilization in membranes (De Domenico *et al.*, 2007a; Marques *et al.*, 2012).

3.1.2. IRON TRANSPORT ACROSS PLASMA MEMBRANE: *IN VIVO* STUDIES

Studies *in vivo* (paper 3 and 4) were focused on NTBI uptake into the human organism. Since there were just animal or patient studies focused predominantly on mRNA level of NTBI transport molecules, we assessed the expression of DMT1, Dcytb, ferroportin, and hephaestin in duodenal biopsies of defined group of patients on both mRNA and protein level.

The group suffering from iron deficiency was represented by patients with iron deficiency anemia (IDA) and the group with primary iron overload was represented by individuals diagnosed with hereditary hemochromatosis (HHC), who were divided into HHC treated and HHC untreated groups of patients (paper 3, p. 65). Since alcohol consumption is considered a high risk factor for iron metabolism disorders, a group of patients with alcoholic liver disease (ALD) represented patients with secondary iron overload or deficiency (paper 4, p. 77). According to body iron levels, the ALD group was divided into patients with iron deprivation, iron overload, and normal iron status. Subjects with no evidence of disturbed iron homeostasis were used as healthy controls.

All patients were tested for the presence of mutations in the HFE gene in order to confirm or exclude the effect of the genotype on iron metabolism. Each individual was also tested for serum iron parameters and consequently classified as iron deficiency, iron overload, or controls.

Serum hepcidin of IDA patients was slightly decreased with no statistical significance when compared to controls (paper 3, p. 72). Nevertheless, decreased hepcidin serum levels were expected in IDA and increased iron absorption should follow due to an effort of the organism to elevate iron levels. Hepcidin is supposed to interact with ferroportin and DMT1 transporters, but no changes in protein levels of these molecules were detected (Fig. 1, paper 3, p. 70). Simultaneously, increased mRNA levels of DMT1 and ferroportin in IDA patients suggest that IRP/IRE interactions should take part in the regulation of gene expression in association of iron deprivation, however, changes in protein levels were not observed, as mentioned above (Fig. 1, paper 3, p. 70).

Increased mRNA levels of DMT1 and ferroportin were also found in HHC patients after treatment (Fig. 2, paper 3, p. 71). This is not surprising since systemic iron regulation of post-phlebotomized patients can react to massive blood loss just like with anemia. Still, the regulatory mechanism was not confirmed at the protein level. No changes at all were observed in HHC patients without treatment (Fig. 2, paper 3, p. 71). As expected, since there were no changes in DMT1 and ferroportin protein levels, serum hepcidin was not changed in any group of the HHC patients compared to controls (paper 3, p. 72). We also tested the expression of hemochromatosis (HFE) and transferrin receptor 1 (TfR1) genes (Fig. 1 and 2, paper 3, p. 70 and 71). According to the crypt cell hypothesis, the HFE/TfR1 complex on the basolateral membrane of enterocytes acts as a regulator of iron homeostasis by sensing the amount of body iron (Philpott, 2002). However, this theory has recently been doubted (Goswami and Andrews, 2006; Gao *et*

al., 2009). We detected increased mRNA levels of HFE and TfR1 in IDA and HHC treated patients compared to controls, but this was most likely due to the IRE/IRP regulation of TfR1 gene expression. Unfortunately, due to an insufficient amount of samples we had no chance to test protein levels of these molecules.

Very similar results were found for individuals with ALD after classification of patients into the individual ALD group (Fig. 2, paper 4, p. 82). Increased mRNA levels for DMT1, ferroportin and TfR1 were observed in ALD patients with normal iron status and ALD patients with anemia, increased mRNA for HFE was found in ALD patients with iron overload. As before, no changes were confirmed in protein level. Therefore, it is surprising that we detected a significant decrease in serum hepcidin in both iron deficient and iron overload ALD groups (Fig. 3, paper 4, p. 85). Hepcidin suppression can be caused directly by ethanol or indirectly via regulatory molecules (Suzuki *et al.*, 2002; Harrison-Findik, 2007; Vecchi *et al.*, 2009). As mentioned above, with these changes in serum hepcidin levels, we would expect subsequent effects on protein levels, at least for ferroportin.

However, some positive correlations among Dcytb, hephaestin, DMT1, ferroportin and TFR1 mRNA in all group of patients indicate coordinated regulation of these genes (Table 2, paper 3, p. 72; Table 2, paper 4, p. 83).

3.2. LIST OF PAPERS

1. Kovar J., Neubauerova J., Cimburova M., Truksa J., **Balusikova K.**, Horak J.
   Stimulation of non-transferrin iron uptake by iron deprivation in K562 cells.
   Blood Cells Mol Dis. 37(2): 95-99, 2006. doi:10.1016/j.bcmd.2006.06.004.
   IF 2006: 2.678

2. **Balusikova K.**, Neubauerova J., Dostalikova-Cimburova M., Horak J., Kovar J.
   Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess.
   Mol Cell Biochem. 321(1-2): 123-133, 2009. doi: 10.1007/s11010-008-9926-y.
   IF 2009: 1.896

3. Dostalikova-Cimburova M., Kratka K., **Balusikova K.**, Chmelikova J., Hejda V., Hnanicek J., Neubauerova J., Vranova J., Kovar J., Horak J.
   Duodenal expression of iron transport molecules in patients with hereditary hemochromatosis or iron deficiency.
   J Cell Mol Med. 16 (8): 1816-1826, 2012. doi: 10.1111/j.1582-4934.2011.01458.x.
   IF 2012: 4.753

4. Dostalikova-Cimburova M., **Balusikova K.**, Kratka K., Chmelikova J., Hejda V., Hnanicek J., Neubauerova J., Vranova J., Kovar J., Horak J.
   Role of duodenal iron transporters and hepcidin in patients with alcoholic liver disease.
   J Cell Mol Med. 18(9): 1840-1850, 2014. doi: 10.1111/jcmm.12310
   IF 2014: 4.014

3.3. PAPER 1

STIMULATION OF NON-TRANSFERRIN IRON UPTAKE BY IRON DEPRIVATION IN K562 CELLS

Kovar J., Neubauerova J., Cimburova M., Truksa J., Balusikova K., Horak J.

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Stimulation of non-transferrin iron uptake by iron deprivation in K562 cells

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Abstract

We tested the effect of iron deprivation on the uptake of iron from ferric citrate by human erythroleukemia K562 cells. The iron uptake after 24-h preincubation in defined iron-free medium was approximately 2–3× higher than after the preincubation in control transferrin-containing medium. The preincubation of K562 cells in iron-free medium together with the inhibitor of protein synthesis cycloheximide completely abrogated the stimulation of the iron uptake. The preincubation in iron-free medium resulted in a slight decrease (20%) of DMT1 mRNA level. The level of Dcytb, ferroportin and hephaestin mRNA did not exert any significant change. We also did not find any significant effect on the protein level of DMT1, Dcytb, ferroportin and hephaestin. We conclude that iron deprivation stimulates the uptake of non-transferrin iron in K562 cells and that this stimulation depends on protein synthesis. It seems that the expression of an unknown or seemingly unrelated protein(s) is involved.

Keywords: Non-transferrin iron uptake; Iron deprivation; K562 cells

Introduction

The plasma level of the non-transferrin low-molecular-mass form of iron is usually below 1 μM. However, sometimes it may rise to values over 20 μM [1]. Most of this form of iron is represented by ferric citrate [2]. Mammalian cells can take up iron from low-molecular-mass sources via an alternative transferrin-independent mechanism(s). The process of non-transferrin iron uptake has been studied extensively, including human K562 and HeLa cells, and shown to be of physiological significance [3–11].

Progress has been made in describing some molecules involved in transport of free iron across biological membranes. DMT1 (divalent metal transporter 1) and ferroportin (also known as Ireg1 or MTP1) are the only known plasma membrane transport proteins which were proved to be involved in the transport of free iron in mammals. Ferroportin, probably together with a ferroxidase hephaestin or ceruloplasmin, are involved in the iron export from enterocytes into the plasma. DMT1 together with Dcytb (duodenal cytochrome b-like) ferrireductase are involved in iron absorption by intestinal enterocytes. DMT1 is also involved in the iron transport across the endosomal membrane. However, DMT1 is a proton symporter active only in specific low-pH environment [12–15]. There are good reasons to believe that other molecules involved in the iron uptake and differing from mentioned molecules exist [16–18]. Several candidate molecules can be considered. Plasma membrane iron-binding protein melanotransferrin (p97) was considered. However, later findings showed that melanotransferrin is not involved in the iron uptake, including K562 cells [19,20]. The role of molecules involved in iron redox reactions (ferrireductases and ferroxidases), such as mentioned Dcytb, hephaestin and ceruloplasmin, is seriously considered [14,15,21]. The role of β_3 integrin as a putative membrane iron transporter was introduced by Conrad and his coworkers [22]. Newly the involvement of mouse lipocalin 24p3/human NGAL (neutrophil gelatinase-

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associated lipocalin) and their receptors in the iron uptake is considered [17,18]. Thus despite this progress the character of non-transferrin ferric iron uptake by mammalian cells, with the exception of enterocytes, still remains obscure.

In this study we tested the effect of iron deprivation on the uptake of iron from ferric citrate by erythroleukemia K562 cells employing chemically defined culture media. So far the effect of decreased iron availability on the uptake of non-transferrin iron by mammalian cells has been tested rarely. We found that iron deprivation significantly stimulates the uptake of iron by K562 cells. However, we did not detect the involvement of any tested gene (DMT1, Dcytb, ferroportin, hephaestin, ceruloplasmin).

Materials and methods

Materials

Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO) and $^{55}FeCl_3$ for [^{55}Fe]ferric citrate preparation was from DuPont NEN (Boston, MA). Rabbit polyclonal antibodies NRAMP22-A, DCYTB11-A, MTP11-A and HEPH11-A against human DMT1, Dcytb, ferroportin and hephaestin from Alpha Diagnostic International (San Antonio, TX), rabbit polyclonal antibody A0031 against human ceruloplasmin from DakoCytomation (Glostrup, Denmark), and mouse monoclonal antibody Ab-4 against human α,β-tubulin from Neomarkers (Fremont, CA) were used.

Cells and culture conditions

The human cell line K562 (erythroleukemia) was obtained from M. Wessling-Resnick (Harvard School of Public Health, Boston, MA) and the human cell line HeLa (cervical carcinoma) from J. Kaplan (University of Utah School of Medicine, Salt Lake City, UT). Cells were maintained in the FBS medium [11], based on the RPMI 1640 medium and supplemented with 10% fetal bovine serum, at 37°C in a humidified atmosphere of 5% CO_2 in air.

In the experiments, defined media were employed. Iron-free medium was a defined serum-free medium, based on RPMI 1640 and containing supplements substituting serum, without addition of any iron source as described previously [11]. Transferrin medium contained in addition 5 μg/ml of iron-saturated human transferrin as a source of iron [10].

Iron uptake measurement

Measurement of non-transferrin iron uptake by the cells was carried out in iron-free medium containing 1 μM [^{55}Fe]ferric citrate as described in detail previously [11].

Real-time PCR

Total RNA was prepared from K562 cells using the RNAeasy MiniKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA was reverse transcribed in a 20 μl reaction using the reverse transcription kit TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) with random primers according to the manufacturer's instructions. Transcribed cDNA was subjected to real-time quantitative PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using a commercially available kit Sybr green PCR Master Mix (Applied Biosystems). Amplification of DMT1 cDNA was performed with forward primer 5′-TTGGGTTGGCAATGTTT-GATT-3′ and reverse primer 5′-CGGGTGGCTTCTTCTGTCA-3′. For the quantification of hephaestin cDNA, forward primer 5′-GGGAATGGCACAACCAGTCT-3′ and reverse primer 5′-CCAGGAGCCCATCCTTGTT-3′ were used. For the quantification of ferroportin, Dcytb, and GAPDH cDNA, previously described primers were used [23–25]. All data were normalized to the amount of GAPDH cDNA in the sample. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression employing the ABI Prism 7000 SDS Software Version 1.1 (Applied Biosystems).

Western blot analysis

Western blot analysis of the levels of DMT1, Dcytb, ferroportin, hephaestin, ceruloplasmin, and α,β-tubulin (loading control) was carried out with some modifications as described in detail previously [26]. Proteins separated by SDS-PAGE were blotted onto 0.2 μm nitrocellulose membrane for 2 h at 0.25 A using MiniProtean II blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% BSA in TBS (100 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 10 min (DMT1) or 40 min (ferroportin), with 5% fish gelatine in TBS for 1 h (Dcytb, hephaestin) or with 5% non-fat milk in TBS for 20 min (ceruloplasmin, α,β-tubulin). Washed membrane was incubated with primary antibodies. After the incubation, the washed membrane was incubated with corresponding horseradish peroxidase-conjugated secondary antibody. Both primary and secondary antibodies were diluted in 5% BSA (fish gelatine, non-fat milk)/0.1% Tween-20/TBS and 0.1% Tween-20/TBS was used for washing. The horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using the Supersignal reagent from Pierce (Rockford, IL) and LAS 1000 CCD device (Fuji).

Statistical analysis

Statistical significance of differences was determined using Student's *t* test.

Results and discussion

Iron deprivation stimulates iron uptake

We tested the effect of changed availability of iron on the uptake of iron from [^{55}Fe]ferric citrate (1 μM) by human erythroleukemia K562 cells employing defined culture media with defined sources of iron. Preincubation with a high level of non-transferrin iron (500 μM ferric citrate) did not result in any significant change of iron uptake by K562 cells but it resulted in a significant increase of the uptake by HeLa cells (data not

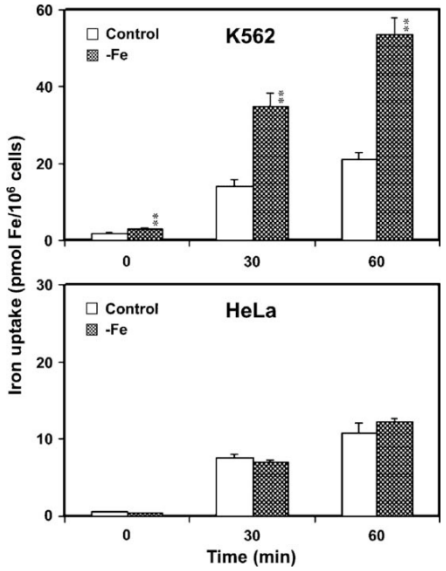

Fig. 1. Effect of preincubation in iron-free medium on non-transferrin iron uptake. The effect of 24-h preincubation in iron-free medium (-Fe) and in control transferrin medium (Control), containing 5 μg/ml of transferrin as a source of iron, on the iron uptake from [^{55}Fe]ferric citrate (1 μM) by K562 and HeLa cells was measured during 60 min of incubation. Each column represents the mean±standard error of the mean (SEM) of three experimental values obtained in one representative experiment of three independent experiments. **Statistical significant increase ($P<0.01$) when compared with the control.

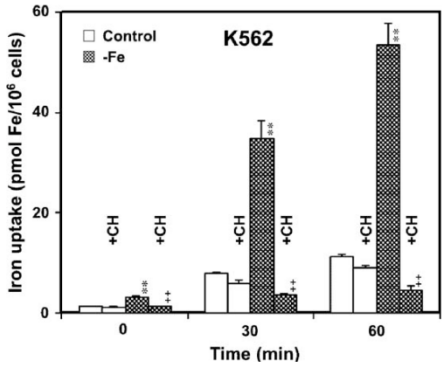

Fig. 2. Effect of cycloheximide on the stimulation of non-transferrin iron uptake by preincubation in iron free medium. The effect of 24-h preincubation in iron-free medium (-Fe) with or without 15 μg/ml of cycloheximide (CH) on the iron uptake from [^{55}Fe]ferric citrate (1 μM) by K562 cells was measured during 60 min of incubation. Control cells were preincubated in FBS medium (Control) containing 10% fetal bovine serum. Each column represents the mean±standard error of the mean (SEM) of three experimental values obtained in one representative experiment of two independent experiments. **Statistical significant increase ($P<0.01$) when compared the preincubation in iron-free medium (without cycloheximide) with the control (without cycloheximide). $^{++}$Statistical significant decrease ($P<0.01$) when compared the preincubation in iron-free medium (with cycloheximide) with the preincubation in iron-free medium (without cycloheximide).

shown). The stimulation of non-transferrin iron uptake in cells incubated with high levels of non-transferrin iron has been reported previously [6,7,27].

Surprisingly, we found that 24-h preincubation in defined iron-free medium (see Materials and methods) significantly stimulated the iron uptake (2–3×) by K562 cells when compared with the uptake after the preincubation in control transferrin medium (see Materials and methods) containing 5 μg/ml of iron-saturated human transferrin as a source of iron (Fig. 1). To our knowledge, this is the first case where a significant stimulation of non-

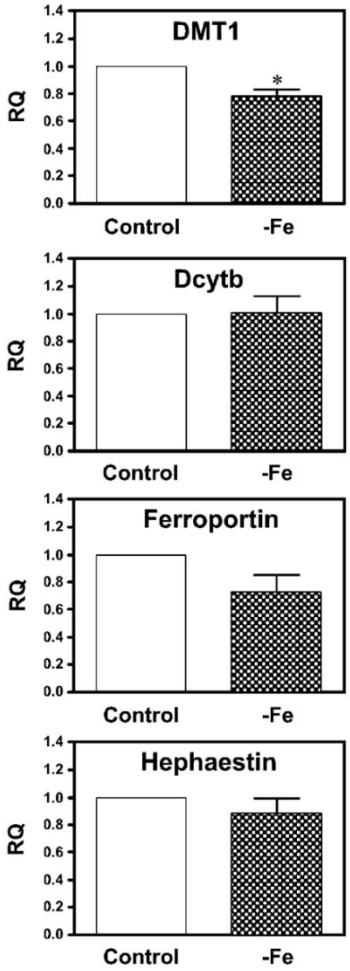

Fig. 3. Effect of preincubation in iron-free medium on the mRNA levels of DMT1, Dcytb, ferroportin and hephaestin. The effect of 24-h preincubation in iron-free medium (-Fe) and in control transferrin medium (Control), containing 5 μg/ml of transferrin as a source of iron, on the mRNA level was determined by quantitative real-time PCR in K562 cells. Relative quantity of mRNA (RQ) in the cells preincubated in iron-free medium is compared with RQ in the control cells as a calibrator. The mean±standard error of the mean (SEM) of four independent experiments is shown. *Statistical significant decrease ($P<0.05$) when compared with the control.

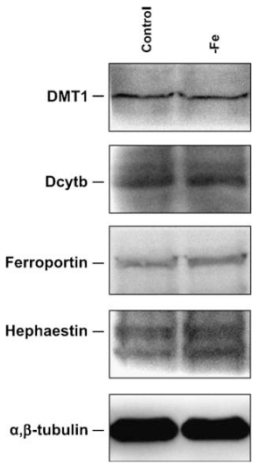

Fig. 4. Effect of preincubation in iron-free medium on the level of DMT1, Dcytb, ferroportin and hephaestin. The effect of 24-h preincubation in iron-free medium (-Fe) and in control transferrin medium (Control), containing 5 μg/ml of transferrin as a source of iron, on the protein level was determined by western blot analysis in K562 cells employing polyclonal antibodies against human DMT1, Dcytb, ferroportin and hephaestin. Monoclonal antibody against human α,β-tubulin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

transferrin iron uptake by iron deprivation has been clearly documented.

On the contrary, the preincubation in iron-free medium had no significant effect on the iron uptake by HeLa cells (Fig. 1). The data demonstrate that the uptake of non-transferrin iron in K562 and in HeLa cells is regulated differently in spite of the fact that the parameters of the iron uptake from ferric citrate in K562 and HeLa cells seem to be similar [11,28]. In K562 cells, the iron uptake is stimulated by the iron deprivation but it is not affected by an excess of extracellular non-transferrin iron. The stimulation of the iron uptake by K562 cells could be related to an extra requirement for iron by erythroid cells. On the other hand, the stimulation of non-transferrin iron uptake in HeLa cells by the excess of extracellular non-transferrin iron could rather reflect the capacity of cells to clear potentially dangerous non-transferrin iron from the environment [27,29].

Iron uptake stimulation depends on protein synthesis

In order to elucidate whether the stimulation of non-transferrin iron uptake by K562 under iron deprivation is related to a new protein synthesis, we tested the effect of the inhibitor of protein synthesis cycloheximide on the stimulation. The iron uptake after 24-h preincubation in iron-free medium was approximately 4–5× higher than the uptake after the preincubation in control FBS medium (see Materials and methods) containing 10% fetal bovine serum. The preincubation in iron-free medium together with cycloheximide (15 μg/ml) resulted in a total abrogation of the stimulation of the iron uptake. On the other hand, the effect of cycloheximide on non-stimulated iron uptake after the preincubation in the control medium was relatively negligible (Fig. 2).

The data demonstrate that the stimulation of non-transferrin iron uptake by iron deprivation in K562 cells requires protein synthesis and that the induction of expression of a relevant gene(s) can be involved. An increased expression of the transferrin receptor via well-known mechanism comprising the activation of the iron regulatory protein (IRP) [30,31] is not involved here because it was shown previously [28] that K562 cells do not produce autocrine transferrin for binding the iron.

Expression of suspected genes is not involved

In order to assess whether the expression of the genes with known or suspected role in non-transferrin iron uptake is involved here, we tested the effect of iron deprivation on their expression. We have found previously that there is no detectable level of β_3 integrin (putative membrane iron transporter) [22] and β_3 integrin mRNA in K562 cells under control conditions as well as under iron deprivation (our unpublished data). Similarly, we have shown previously that plasma membrane iron-binding protein melanotransferrin (p97) is not involved in non-transferrin iron uptake by K562 cells [19].

We tested the expression of DMT1, Dcytb, ferroportin, hephaestin and ceruloplasmin employing quantitative real-time PCR (mRNA level) and western blot analysis (protein level). However, we did not detect any ceruloplasmin as well as ceruloplasmin mRNA in K562 cells but we detected both ceruloplasmin and ceruloplasmin mRNA in CaCo2 and HeLa cells used as a positive control (data not shown). The preincubation in iron-free medium did not significantly affect the mRNA level of the other tested genes with the exception of DMT1. In the case of DMT1, we demonstrated certain statistical significant decrease (about 20%) of mRNA level after iron deprivation (Fig. 3). We did not find any significant effect of the preincubation in iron-free medium on the protein level of DMT1, Dcytb, ferroportin and hephaestin (Fig. 4).

Taken together, we conclude that iron deprivation significantly stimulates the uptake of non-transferrin ferric iron by human erythroleukemia K562 cells. This iron uptake stimulation depends on protein synthesis and thus probably involves the induction of gene expression. However, we did not detect the involvement of any tested relevant gene. It seems that the expression of an unknown or seemingly unrelated protein(s) is involved in the stimulation. Good candidates could be here proteins of newly discussed system NGAL/NGAL receptor [17,18].

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3.4. PAPER 2

DIFFERING EXPRESSION OF GENES INVOLVED IN NON-TRANSFERRIN IRON TRANSPORT ACROSS PLASMA MEMBRANE IN VARIOUS CELL TYPES UNDER IRON DEFICIENCY AND EXCESS

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Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess

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Abstract We studied the effect of iron deficiency, i.e., 24-h preincubation in iron-free medium, and the effect of high level of non-transferrin iron, i.e., the preincubation in ferric citrate medium containing 500 μM ferric citrate, on the expression of DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin in various functional types of human cells. The expression of these proteins potentially involved in non-transferrin iron transport across cell membranes was tested on mRNA level by quantitative real-time PCR as well as on protein level by western blot analysis in Caco-2 (colorectal carcinoma), K562 (erythroleukemia), and HEP-G2 (hepatocellular carcinoma) cells. We found that changes in non-transferrin iron availability, i.e., iron deficiency and high level of non-transferrin iron, affect the expression of tested proteins in a cell type-specific manner. We also demonstrated that changes in the expression on mRNA level do not often correlate with relevant changes on protein level.

Keywords Non-transferrin iron transport · DMT1 · Dcytb · Ferroportin · Hephaestin · Ceruloplasmin

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Introduction

Iron, due to its ability to accept or donate electrons, plays a key role in essential biological functions in organisms, such as oxygen transport, mitochondrial energy metabolism, or deoxynucleotide synthesis. On the other hand, free iron is supposed to catalyze the formation of highly reactive free radicals that can cause cell damage. Therefore, it is of crucial importance for both the cells and the organism to maintain systemic iron homeostasis to ensure adequate iron supply but to prevent accumulation of excess iron [1, 2].

Intestinal iron absorption is a rigorously regulated process, consisting of two steps involving iron uptake across the apical membrane and iron export across the basolateral membrane of duodenal enterocytes. Prior to absorption, dietary ferric iron (Fe^{3+}) has to be reduced to ferrous form (Fe^{2+}) at the apical surface in order to be transported by divalent metal transporter 1 (DMT1) into enterocytes [3, 4]. This reducing role is attributed to membrane-bound duodenal cytochrome *b* ferrireductase (Dcytb) [5–7]. However, absence of the murine Dcytb homolog (Cybrd1) results in no iron-deficient phenotype, suggesting that Cybrd1 is not perhaps an essential component of intestinal iron absorption mechanism in mice [8]. Inside enterocytes, ferrous iron can be stored in the protein ferritin or transported across the basolateral membrane by ferroportin (MTP1) [9–11] and then oxidized by the ferroxidase hephaestin, membrane-bound homolog of the plasma ferroxidase ceruloplasmin, before being bound by plasma transferrin [12].

In erythroid cells and hepatocytes, transferrin/transferrin receptor-mediated endocytosis is the main pathway of cellular iron uptake. Iron-loaded serum transferrin is bound by the membrane transferrin receptor and internalized via receptor-mediated endocytosis into endosomes, where an

acidic pH causes conformational changes and iron dissociation. Released iron is then reduced by endosomal ferrireductase and transported into the cytosol by DMT1 [13, 14]. In erythroid cells, six-transmembrane epithelial antigen of the prostate-3 (Steap3) was recently identified as the ferrireductase responsible for reduction of iron in the endosomes. Steap3 was also shown to be highly expressed in the liver [15, 16]. Ferrous iron is exported across plasma membrane of erythroid cells and hepatocytes through ferroportin and oxidized by the plasma ferroxidase ceruloplasmin [17]. Ferroportin is also responsible for iron export in macrophages which recycle iron from senescent red blood cells to the plasma [18]. The presence of ferroportin reported in erythroid cells provides an evidence that these cells also possess specific mechanisms of iron export [19].

Maintaining of iron homeostasis within the cell concerns regulation of proteins involved in iron transport across plasma membrane, as well as proteins involved in iron utilization and storage. Post-transcriptional regulation plays a key role here. The cytosolic iron regulatory proteins (IRPs) 1 and 2 have the ability to sense and control the intracellular iron concentration by binding to iron responsive elements (IREs) of genes encoding key iron-related proteins [20–22]. In iron-depleted states, IRPs binding to multiple IREs in the 3′-untranslated region (UTR) of transferrin receptor 1 (TfR1) mRNA stabilizes mRNA transcript against degradation, while IRPs binding to single IRE in the 5′-UTR of mRNAs encoding ferritin H and L chains or ferroportin inhibits translation process [1]. It leads to increased cellular iron uptake and decreased intracellular iron storage in ferritin, and finally it results in increased levels of intracellular iron [16]. A single IRE was also identified in the 3′-UTR of one isoform of DMT1. On the other hand, increased iron levels favor the conversion of IRP1 from its active RNA binding form into a Fe-S cluster containing cytoplasmic aconitase that lacks IRE binding activity, as well as the proteasomal degradation of IRP2. IRPs are active in various types of cells including intestinal cells (Caco-2 cells), erythroid cells (K562 cells), and liver cells (HEP-G2 cells) [23, 24].

Key role in regulation of iron homeostasis within whole organism is played by peptide hepcidin which is mainly expressed in the liver. Hepcidin regulates serum iron level via the modulation of iron absorption through enterocytes and iron release from hepatocytes and macrophages. This regulation is based on hepcidin binding to ferroportin which induces ferroportin internalization and subsequent degradation. Increased serum iron level leads to increased expression of hepcidin on transcriptional level and vice versa. Several molecular mechanisms mediated by iron-saturated transferrin are involved here [2, 16].

It was shown that mammalian cells can take up iron from low-molecular-mass sources via an alternative transferrin-independent mechanism(s) [25–29]. Most of low-molecular-mass iron in plasma seems to be in the form of ferric citrate [30]. As transferrin becomes saturated in iron overload states, excess iron is found as non-transferrin iron and it probably plays an important role in hepatocyte iron loading in hereditary hemochromatosis and other iron overload conditions. However, the mechanism of non-transferrin ferric iron uptake by mammalian cells, with the exception of enterocytes, still remains obscure.

The aim of this study was to compare the expression of proteins potentially involved in non-transferrin iron transport across membranes (DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin) in various types of human cells (Caco-2, K562, and HEP-G2) under extracellular iron deficiency and excess of extracellular non-transferrin iron. Cell lines employed represent the main tissue types involved in iron exchange. Caco-2 cell line (human colorectal carcinoma) represents a model of intestinal epithelial cells which are responsible for iron absorption into the circulation. K562 cell line (human erythroleukemia) represents erythroid cells which are the major iron-utilizing cells in the body. HEP-G2 cells (human hepatocellular carcinoma), a model of hepatocytes, represent the major iron storage cells. We demonstrated that changes in iron availability, i.e., iron deficiency and iron excess, affect the expression of DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin on mRNA as well as on protein level in a cell type-specific manner. We also demonstrated that changes of the expression on mRNA level do not often correlate with relevant changes on protein level.

Materials and methods

Materials

Goat polyclonal antibodies NRAMP 2 (N-20) and Hephaestin (N-20) against human DMT1 and hephaestin from Santa Cruz Biotechnology (Santa Cruz, CA, USA), goat polyclonal anti-Cytochrome *b* reductase 1 antibody (EB06633) against human Dcytb from Everest Biotech (Oxfordshire, UK), rabbit polyclonal antibody MTP11-A against human ferroportin from Alpha Diagnostic International (San Antonio, TX, USA), rabbit polyclonal antibody A0031 against human ceruloplasmin from DakoCytomation (Glostrup, Denmark), and mouse monoclonal antibody Anti-Actin (clone AC-40) against human actin (Sigma-Aldrich, St. Louis, MO, USA) were used.

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Cells and culture conditions

The human cell line Caco-2 (colorectal carcinoma) was obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA), the human cell line K562 (erythroleukemia) from M. Wessling-Resnick (Harvard School of Public Health, Boston, MA, USA), and the human cell line HEP-G2 (hepatocellular carcinoma) from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cells were maintained in the FBS medium [28], based on the RPMI 1640 medium and supplemented with 10% fetal bovine serum (containing also bovine transferrin and ceruloplasmin), at 37°C in a humidified atmosphere of 5% CO_2 in air.

In the experiments, defined media were employed. Iron-free medium was a defined serum-free medium, based on RPMI 1640 and containing supplements substituting serum, without addition of any iron source as described previously [28]. Ferric citrate medium contained in addition 500 μM ferric citrate as a source of iron [31]. Transferrin medium contained in addition 5 μg/ml of iron-saturated human transferrin as a source of iron [27]. On the basis of our previous studies [23, 29], we suppose that 24-h preincubation in iron-free medium leads to certain iron deprivation of the cells and 24-h preincubation in ferric citrate medium represents an excess of iron for the cells.

The defined transferrin medium was used as a control medium in the case of K562 cells because these cells can easily be adapted to long-term growth in a defined medium. The transferrin medium provides the cells with a sufficient amount of iron similar to the medium supplemented with 10% fetal bovine serum [29]. The FBS medium supplemented with 10% fetal bovine serum was used as a control medium for Caco-2 and HEP-G2 cells. These cells cannot be adapted to long-term growth in a defined medium.

Real-time PCR

Total RNA was prepared from Caco-2, K562, and HEP-G2 cells using the RNAeasy MiniKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA was reverse transcribed in a 10 μl reaction using the reverse transcription kit TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) with random primers according to the manufacturer's instructions. Transcribed cDNA was subjected to real-time quantitative PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using a commercially available kits Sybr green PCR Master Mix (Applied Biosystems) for Dcytb, hephaestin, ceruloplasmin, and GAPDH, and Taq Man Universal PCR Master Mix (Applied Biosystems) for DMT1 and ferroportin. Commercially available TaqMan® Gene Expression Assay for hepcidin was obtained from Applied Biosystems and used according to the manufacturer's instructions. Amplification of DMT1(IRE), Dcytb, ferroportin, hephaestin, ceruloplasmin, and GAPDH cDNA was performed with primers and probes shown in Table 1. For the amplification of DMT1(IRE), Dcytb and ferroportin cDNA, previously described primers and probes were used [32, 33]. Primers and probes were purchased from Generi Biotech (Hradec Kralove, Czech Republic) and Applied Biosystems, respectively. All data were normalized to the amount of

Table 1 Sequences of used primers and probes

Gene	Primer/probe	Sequence
DMT1(IRE) [26]	Forward primer	5′-GTG GTC AGC GTG GCT TAT CTG-3′
	Reverse primer	5′-GATGCT TAC CGT ATG CCC ACA GT-3′
	Probe	5′-FAM TGT TCT ACT TGG GTT GGC AAT GTT TGA TTG C TAMRA-3′
Dcytb [27]	Forward primer	5′-GTC ACC GGC TTC GTC TTC A-3′
	Reverse primer	5′-CAG GTC CAC GGC AGT CTG TA-3′
Ferroportin [26]	Forward primer	5′-TGA CCA GGG CGG GAG A-3′
	Reverse primer	5′-GAG GTC AGG TAG TCG GCC AA-3′
	Probe	5′-FAM CAC AAC CGC CAG AGA GGA TGC TGT G TAMRA-3′
Hephaestin[a]	Forward primer	5′-GGG AAT GGC ACA ACC AGT CT-3′
	Reverse primer	5′-CCA GGA GCC CAT CCT TGT T-3′
Ceruloplasmin[a]	Forward primer	5′-AAC CCT GGA GAA TGG ATG CTC-3′
	Reverse primer	5′-TTG CAA ACC GGC TTT CAG A-3′
GAPDH[b]	Forward primer	5′-GTC GGA GTC AAC GGA TTT GG-3′
	Reverse primer	5′-AAA AGC AGC CCT GGT GAC C-3′

[a] Our own sequences

[b] Sequences provided by Dr. M. Kahle (Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague)

GAPDH cDNA in the sample. The expression of housekeeping gene GAPDH was not affected significantly by changes in iron availability. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression employing the ABI Prism 7000 SDS Software Version 1.1 (Applied Biosystems).

Western blot analysis

Western blot analysis of the levels of DMT1, Dcytb, ferroportin, hephaestin, ceruloplasmin, and actin was carried out with some modifications as described in detail previously [34]. Proteins of whole cell lysates separated by SDS-PAGE were blotted onto 0.2 μm nitrocellulose membrane for 2 h at 0.25 A using MiniProtean II blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% BSA in TBS (100 mM Tris–HCl, 150 mM NaCl, pH 7.5) for 40 min (DMT1, Dcytb, ferroportin, and hephaestin) or with 5% non-fat milk in TBS for 20 min (ceruloplasmin and actin). Tween-20 (0.1 %)/TBS was used for washing. The washed membrane was incubated with relevant primary antibody. Antibodies against human DMT1, Dcytb, ferroportin, hephaestin, ceruloplasmin, and actin (loading control) (see Materials) were used as primary antibodies. After the incubation (overnight, 4°C), the washed membrane was incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology. The horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using the Supersignal reagent from Pierce (Rockford, IL) and LAS 1000 CCD device (Fuji).

Statistical analysis

Data concerning mRNA levels are presented as mean ± SEM of four or three experimental values obtained in independent experiments. Statistical significance of differences, when comparing obtained data, was determined using paired Student's t test for two groups. $P < 0.05$ was considered statistically significant.

Results

Effect of iron deficiency and excess on mRNA level

We tested the effect of iron deficiency, i.e., the incubation in iron-free medium, and the effect of high level of non-transferrin iron, i.e., the incubation in ferric citrate medium containing 500 μM ferric citrate, on mRNA level of DMT1 (IRE isoform), Dcytb, ferroportin, hephaestin, and ceruloplasmin in Caco-2, K562, and HEP-G2 cells employing quantitative real-time PCR.

After 24-h preincubation of Caco-2 cells in iron-free medium when comparing with the preincubation in control medium, we detected certain increase of the mRNA level of DMT1, Dcytb, ferroportin, and hephaestin. However, this increase was not shown to be statistically significant. The preincubation of Caco-2 cells in ferric citrate medium, when compared with control, led to certain statistically non-significant decrease of the mRNA level of DMT1 and to a statistically significant increase (about 40–60%) of the mRNA level of Dcytb, ferroportin, and hephaestin. When comparing the effect of iron deficiency (the preincubation in iron-free medium) versus the effect of high level of non-transferrin iron (the preincubation in ferric citrate medium), we detected a statistically significant increase in DMT1 mRNA level. The mRNA level increased from the relative quantity about 0.6 under iron excess to the relative quantity about 1.4 under iron deficiency (Fig. 1).

In the case of K562 cells, we did not detect any ceruloplasmin mRNA. The preincubation of the cells for 24 h in iron-free medium, when compared with control, led to a statistically significant decrease of the mRNA level of DMT1 (about 20%) and ferroportin (about 40%). The preincubation in ferric citrate medium resulted in certain statistically non-significant increase of the mRNA level of DMT1 and Dcytb and to a statistically significant increase (about 70%) of ferroportin mRNA level in comparison with control. When comparing the effect of iron deficiency versus the effect of high level of non-transferrin iron, we detected a statistically significant decrease in the mRNA level of both DMT1 and ferroportin. DMT1 mRNA level decreased from the relative quantity about 1.4 under iron excess to the relative quantity about 0.8 under iron deficiency. For ferroportin it was even a decrease from about 1.7 under iron excess to about 0.6 under iron deficiency (Fig. 2).

We did not detect any hephaestin mRNA in HEP-G2 cells. These cells did not significantly change the mRNA level of DMT1, Dcytb, ferroportin, and ceruloplasmin after 24-h preincubation in iron-free medium. After the preincubation in ferric citrate medium when comparing with control, we detected some statistically non-significant increase of ferroportin mRNA level and a statistically significant decrease (about 20%) of ceruloplasmin mRNA level. Comparison of the effect of iron deficiency versus the effect of high level of non-transferrin iron showed a statistically significant decrease in ferroportin mRNA level. The mRNA level decreased from the relative quantity about 1.4 under iron excess to the relative quantity about 0.8 under iron deficiency (Fig. 3).

Effect of iron deficiency and excess on protein level

Detected statistically significant differences in the mRNA level of the studied genes due to changed availability of

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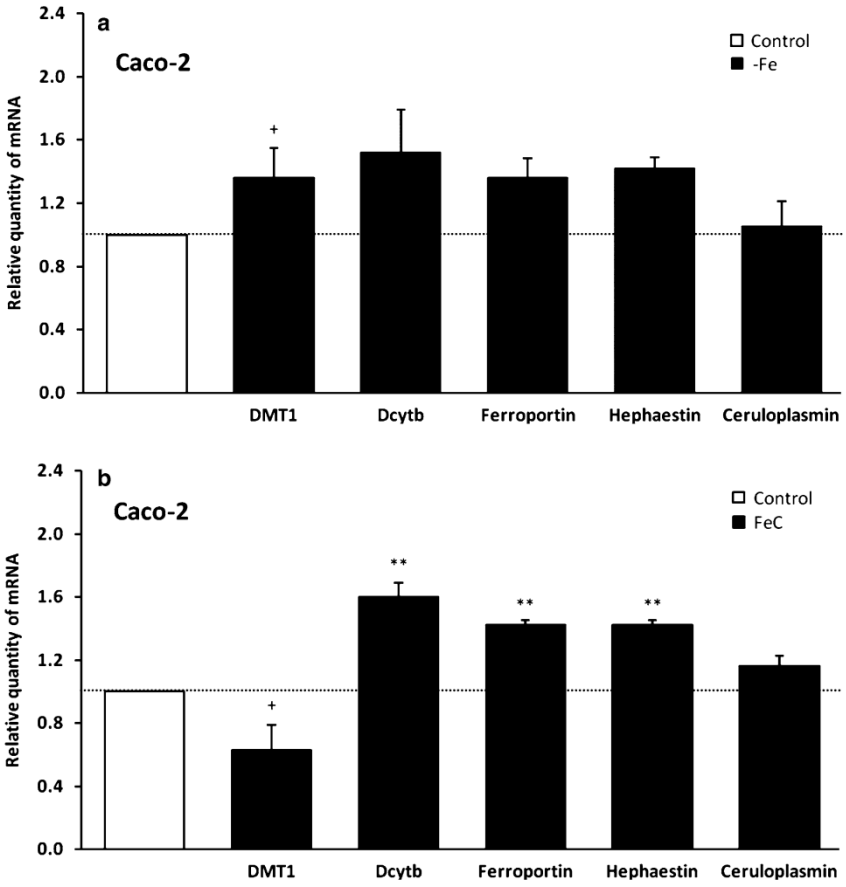

Fig. 1 (**a**) Effect of iron deficiency (iron-free medium) and (**b**) effect of high level of non-transferrin iron (ferric citrate medium) on mRNA levels of DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin in Caco-2 cells. The effect of 24-h preincubation of the cells in defined iron-free medium (−Fe) as well as in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control FBS medium (Control), containing 10% fetal bovine serum as a source of iron, on mRNA level was determined by quantitative real-time PCR. Relative quantity of mRNA in the cells preincubated in iron-free medium or in ferric citrate medium is compared with relative quantity of mRNA in the control cells as a calibrator. The mean ± SEM of four or three independent experiments is shown. **Statistically significant difference ($P < 0.01$) when compared with the control. $^{+}$Statistically significant difference ($P < 0.05$) when (**a**) the effect of iron deficiency and (**b**) the effect of high level of non-transferrin iron is compared

iron (see previous chapter) were also tested on protein level, employing western blot analysis, in order to confirm upregulation or downregulation resulting in real change on protein level.

Statistically significant difference in the mRNA level of DMT1 in Caco-2 cells (see Fig. 1), when comparing the effect of iron deficiency (the preincubation in iron-free medium) versus the effect of high level of non-transferrin iron (the preincubation in ferric citrate medium), was confirmed on protein level. DMT1 level after 24-h preincubation in iron-free medium was significantly higher when compared with the preincubation in ferric citrate medium (Fig. 4). Statistically significant increase of the mRNA level of Dcytb, ferroportin, and hephaestin in Caco-2 cells after the preincubation in ferric citrate medium (see Fig. 1), when compared with control, correlated with increased protein level only in the case of ferroportin and hephaestin. Ferroportin level was significantly increased and hephaestin level was slightly increased. However, we did not find any effect of the preincubation in ferric citrate medium on protein level of Dcytb (Fig. 5).

Statistically significant decrease of the mRNA level of both DMT1 and ferroportin in K562 cells (see Fig. 2), when comparing the effect of iron deficiency versus the effect of high level of non-transferrin iron, was not followed by corresponding change in the protein level. Protein levels of DMT1 and ferroportin were found unchanged here (Fig. 4).

Statistically significant decrease of ferroportin mRNA level in HEP-G2 cells was shown when comparing the effect of iron deficiency versus the effect of high level of non-transferrin iron (see Fig. 3). This decrease was not confirmed on protein level (Fig. 4). On the other hand, detected statistically significant decrease of ceruloplasmin mRNA level (see Fig. 3), when comparing the effect of high level of non-transferrin iron with control, was confirmed on protein level. Ceruloplasmin level was somewhat decreased after the preincubation in ferric citrate medium (Fig. 5).

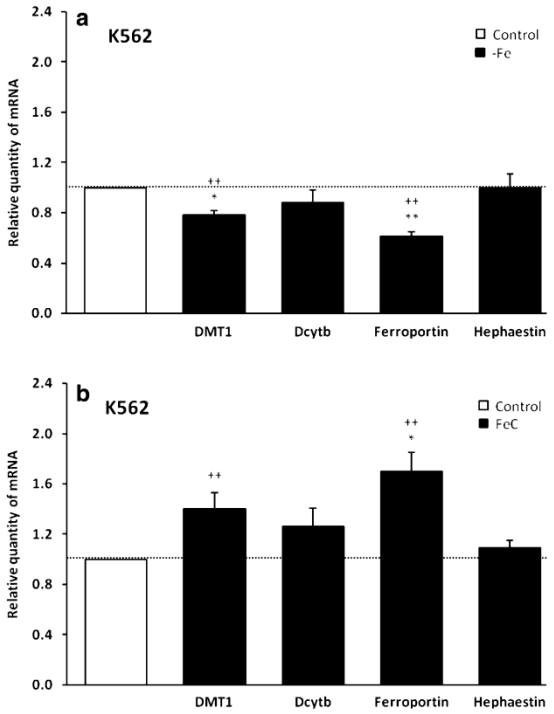

Fig. 2 (**a**) Effect of iron deficiency (iron-free medium) and (**b**) effect of high level of non-transferrin iron (ferric citrate medium) on mRNA levels of DMT1, Dcytb, ferroportin, and hephaestin in K562 cells. The effect of 24-h preincubation of the cells in defined iron-free medium (−Fe) as well as in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control transferrin medium (Control), containing 5 μg/ml of transferrin as a source of iron, on mRNA level was determined by quantitative real-time PCR. Relative quantity of mRNA in the cells preincubated in iron-free medium or in ferric citrate medium is compared with relative quantity of mRNA in the control cells as a calibrator. The mean ± SEM of four or three independent experiments is shown. *Statistically significant difference ($P < 0.05$), **statistically significant difference ($P < 0.01$) when compared with the control. $^{++}$Statistically significant difference ($P < 0.01$) when (**a**) the effect of iron deficiency and (**b**) the effect of high level of non-transferrin iron is compared

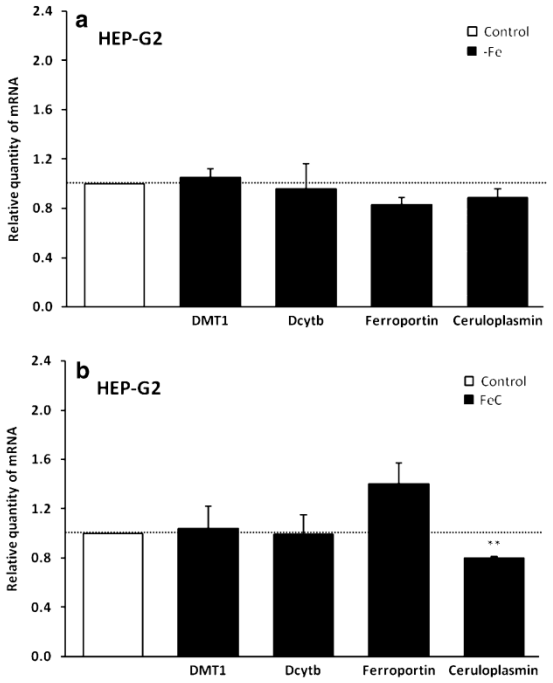

Fig. 3 (**a**) Effect of iron deficiency (iron-free medium) and (**b**) effect of high level of non-transferrin iron (ferric citrate medium) on mRNA levels of DMT1, Dcytb, ferroportin, and ceruloplasmin in HEP-G2 cells. The effect of 24-h preincubation of the cells in defined iron-free medium (−Fe) as well as in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control FBS medium (Control), containing 10% fetal bovine serum as a source of iron, on mRNA level was determined by quantitative real-time PCR. Relative quantity of mRNA in the cells preincubated in iron-free medium or in ferric citrate medium is compared with relative quantity of mRNA in the control cells as a calibrator. The mean ± SEM of four or three independent experiments is shown. **Statistically significant difference ($P < 0.01$) when compared with the control. $^{+}$Statistically significant difference ($P < 0.05$) when (**a**) the effect of iron deficiency and (**b**) the effect of high level of non-transferrin iron is compared

Effect of iron deficiency and excess on hepcidin mRNA level

In the case of liver HEP-G2 cells, we also tested the effect of iron deficiency (the preincubation in iron-free medium) and the effect of high level of non-transferrin iron (the preincubation in ferric citrate medium) on hepcidin mRNA level.

After 24-h preincubation of HEP-G2 cells in iron-free medium we detected dramatic statistically significant decrease (approximately 100-fold) of hepcidin mRNA level when compared with control medium. We detected even more pronounced decrease (approximately 300-fold) of hepcidin mRNA level after the preincubation in ferric citrate medium (Fig. 6).

Discussion

We compared the expression of proteins potentially involved in non-transferrin iron transport across cell membranes (DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin) in various types of human cells (Caco-2, K562, and HEP-G2) under extracellular iron deficiency and excess of extracellular non-transferrin iron employing quantitative real-time PCR (mRNA level) and western blot analysis (protein level). The absorption of dietary

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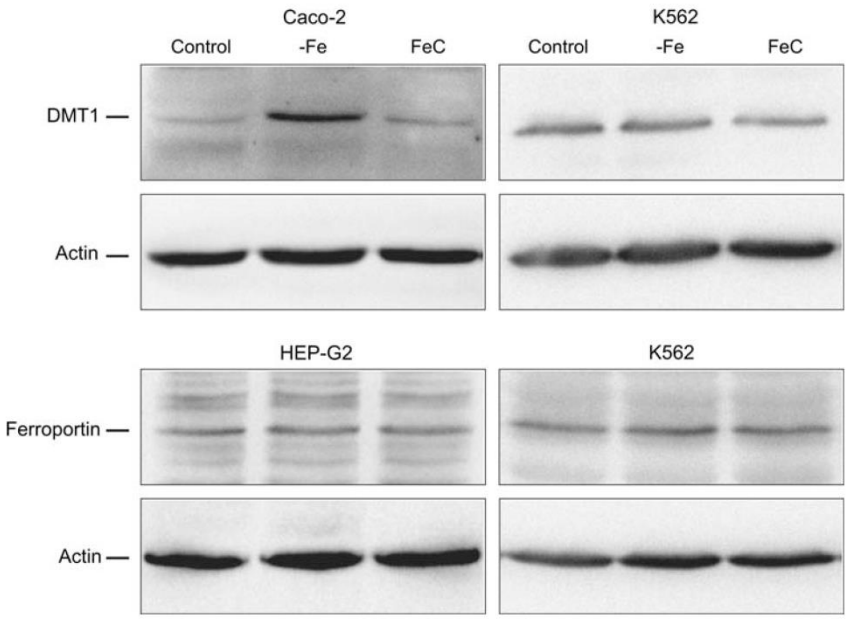

Fig. 4 Effect of iron deficiency (iron-free medium) and effect of high level of non-transferrin iron (ferric citrate medium) on the level of DMT1 in Caco-2 cells, the level of DMT1 and ferroportin in K562 cells, and the level of ferroportin in HEP-G2 cells. The effect of 24-h preincubation of the cells in defined iron-free medium (−Fe) as well as in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control FBS medium (Control) (Caco-2, HEP-G2) or in control transferrin medium (Control) (K562), containing 10% fetal bovine serum or 5 μg/ml of transferrin as a source of iron, on protein level was determined by western blot analysis employing relevant antibodies (see Materials and methods). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of at least three independent experiments

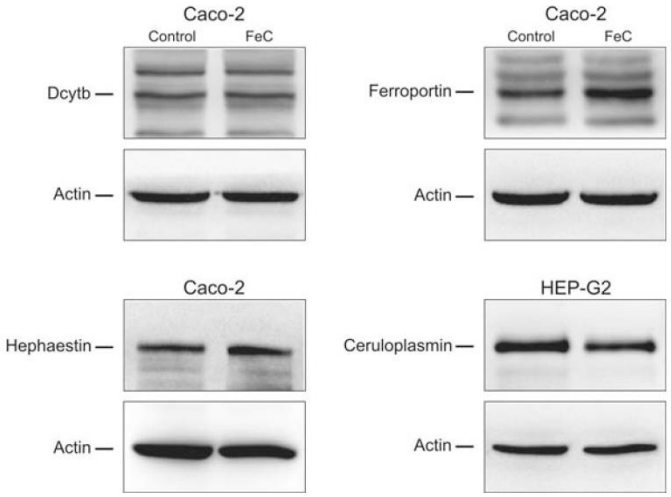

Fig. 5 Effect of high level of non-transferrin iron (ferric citrate medium) on the level of Dcytb, ferroportin, and hephaestin in Caco-2 cells and the level of ceruloplasmin in HEP-G2 cells. The effect of 24-h preincubation of the cells in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control FBS medium (Control), containing 10% fetal bovine serum as a source of iron, on protein level was determined by western blot analysis employing relevant antibodies (see Materials and methods). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of at least three independent experiments

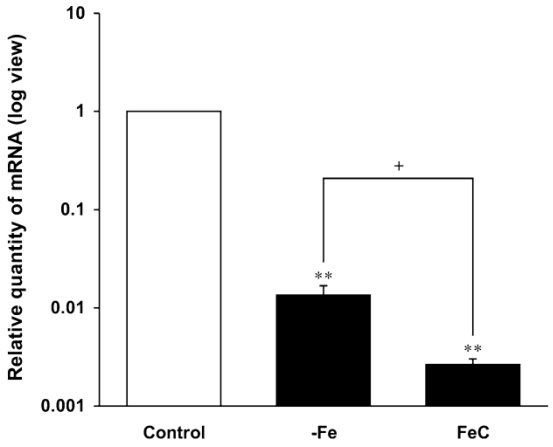

Fig. 6 Effect of iron deficiency (iron-free medium) and effect of high level of non-transferrin iron (ferric citrate medium) on mRNA level of hepcidin in HEP-G2 cells. The effect of 24-h preincubation of the cells in defined iron-free medium (−Fe) as well as in defined ferric citrate medium (FeC), containing 500 μM ferric citrate as a source of iron, and in control FBS medium (Control), containing 10% fetal bovine serum as a source of iron, on mRNA level was determined by quantitative real-time PCR. Relative quantity of mRNA in the cells preincubated in iron-free medium or in ferric citrate medium is compared with relative quantity of mRNA in the control cells as a calibrator. The mean ± SEM of four independent experiments is shown. **Statistically significant difference ($P < 0.01$) when compared with the control. $^{+}$Statistically significant difference ($P < 0.05$) when the effect of iron deficiency (−Fe) and the effect of high level of non-transferrin iron (FeC) is compared

non-heme iron is carried out by duodenal enterocytes which represent the major regulatory site controlling iron homeostasis in mammals. The absorbed iron is released into the circulation. A significant portion of the iron in the circulation is taken up by developing erythrocytes and incorporated into hemoglobin. Certain amount of the absorbed iron is stored in other types of cells, predominantly in hepatocytes. Thus, Caco-2 cells represent type of cells responsible for iron absorption. K562 cells represent the major iron-utilizing cells and HEP-G2 cells represent the major iron storage cells.

There are plentiful publications concerning the effect of iron availability on the expression of proteins involved in iron transport across plasma membrane in various types of cells. Studies presenting these data were carried out employing both models involving cell lines in vitro as well as in vivo models. Most of mentioned data just represent the expression on mRNA level and only significantly smaller portion of the results represents the expression on protein level or both mRNA and protein level. However, the data are often differing or contradictory, even in the case of cells of the same tissue origin, probably due to various experimental models and conditions used. Therefore, we decided to compare response of all molecules potentially involved in iron transport on mRNA and protein level in cell lines representing main tissue types involved in iron exchange under the same experimental conditions. Furthermore, we demonstrated in this study that changes in the expression on mRNA level do not often correlate with changes on protein level (see Results). Therefore, we would like to stress that it can be highly speculative to make physiological interpretations just on the basis of mRNA data.

Concerning Caco-2 cells, one would expect increased expression of the iron transport molecules under iron deprivation and decreased expression under iron excess as a general pattern. Our findings are in agreement with this pattern only in the case of DMT1 when regarding the expression on both mRNA and protein level (see Figs. 1, 4). These findings are consistent with results of previous studies [4, 35, 36]. It has been proposed that DMT1 expression in enterocytes is regulated via the control of mRNA stability by the iron-dependent binding of IRP1 to the IRE of DMT1 mRNA, analogous to the iron-responsive upregulation of transferrin receptor under iron deprivation [1]. We showed that iron excess unexpectedly led to a significant increase of the mRNA level but not the protein level of Dcytb. A statistically significant change on Dcytb mRNA level under iron deficiency was not found (see Figs. 1, 5). It could correspond to the previous finding that Cybrd1 (murine Dcytb homolog) is not perhaps an essential component of the intestinal iron absorption mechanism [8]. However, significant increase of duodenal expression of Dcytb mRNA and protein was detected under factors stimulating iron absorption such as iron deficiency, chronic anemia, and hypoxia [5]. Changes of ferroportin and hephaestin expression in Caco-2 cells appear to be in opposite direction under iron excess than expected. We demonstrated that the levels of ferroportin and hephaestin mRNAs as well as proteins were significantly increased in response to iron excess (see Figs. 1, 5). In contrast to this, it was shown previously that iron excess decreases ferroportin levels in the small intestine and Caco-2 cells [9, 11, 37]. Within the organism, ferroportin is negatively post-translationally regulated by hepcidin and this regulation is mediated by iron-saturated transferrin as a sensor of iron status. High iron level corresponds with high hepcidin level [16, 38, 39]. In our experimental system we use defined media without any serum, i.e., without any hepcidin and transferrin. It means that in spite of the fact that there is an excess of non-transferrin iron, Caco-2 cells can interpret the situation, concerning hepcidin regulation of ferroportin expression, as an iron deficiency. Membrane-bound ferroxidase hephaestin is mainly involved in iron transfer from intestinal enterocytes into the circulation. Iron release from non-intestinal cells was shown to require ceruloplasmin

ferroxidase activity [12, 17]. There are some differing data concerning hephaestin expression in duodenum under iron overload [33, 40, 41], thus it is hard to interpret our data concerning slight upregulation of hephaestin in response to iron excess now. The fact that we detected ceruloplasmin mRNA in Caco-2 cells (see Fig. 1) was rather surprising because the absence of ceruloplasmin mRNA in the duodenum was previously demonstrated [42–45].

Erythroid K562 cells represent functionally different type of cells than Caco-2 where a high rate of iron uptake is required for optimal heme synthesis. Unfortunately, there are not too many studies in the literature concerning the effect of iron availability on the expression of discussed proteins in erythroid cells. We showed that iron excess led to a significant increase of the mRNA level of DMT1 and ferroportin in comparison with iron deficiency that resulted in a decrease of the DMT1 and ferroportin mRNA levels. However, these findings do not correspond to unchanged protein levels (see Figs. 2, 4). We observed entirely opposite pattern of the expression of DMT1 mRNA in K562 cells when compared with Caco-2 cells. In K562 cells, low mRNA level of DMT1 correlates with low iron level in medium and vice versa. It was shown that the DMT1 IRE form is expressed predominantly by epithelial cell lines, whereas the non-IRE form is expressed by blood cell lines [46]. However, the expression of DMT1 IRE form by erythroid cells was also shown [47]. In contrast to our findings, it was reported that ferroportin expression is not modulated by iron availability in K562 cells [19]. We did not detect any significant effect of iron availability on Dcytb expression here (see Fig. 2). It could indicate that Dcytb does not play a substantial role in iron transport of non-intestinal cells.

In HEP-G2 cells representing iron storage cells, increased uptake and decreased export of iron under iron excess as well as decreased uptake and increased export of iron under iron deficiency could be expected. However, we did not obtain any supportive data for this presumption, maybe with the exception of ceruloplasmin. We showed that the level of ceruloplasmin mRNA and also the level of ceruloplasmin protein were decreased in response to iron excess (see Figs. 3, 5). It was reported that iron overload results in decreased expression of ceruloplasmin protein in the liver [48] and that the treatment of cultured HEP-G2 cells with iron chelators markedly increases ceruloplasmin mRNA level and protein synthesis [49]. DMT1 mRNA level and also Dcytb mRNA level were not affected by changed iron availability (see Fig. 3). It was demonstrated previously that the expression of the IRE-containing mRNA of DMT1 is upregulated in the small intestine under iron deficiency. On the other hand, its expression in hepatoma cells was shown to be mostly independent of iron status [50, 51]. The pattern of ferroportin expression was rather against the presumption. Iron excess led to a significant increase of the mRNA level, but not protein level, of ferroportin in comparison to iron deficiency (see Figs. 3, 4). However, ferroportin expression could be, at least partly, deregulated in liver cells due to the absence of working hepcidin regulatory mechanism. Hepcidin expression is regulated by several mechanisms on transcriptional level [2, 16]. Therefore, we also assessed the expression of hepcidin in HEP-G2 cells on mRNA level under our experimental conditions. Surprisingly, we found a dramatic decrease of hepcidin mRNA level not only under iron deficiency but also under iron excess (see Fig. 6). It is in agreement with experimental data of several other studies [52–54]. The regulation of hepcidin expression is mediated by iron-saturated transferrin as mentioned above. In our experiment we used defined media without any transferrin and thus the liver cells can interpret the conditions as a severe iron deficiency with the dramatic decrease of hepcidin mRNA expression even there is an excess of non-transferrin iron in reality. The fact that we did not detect any hephaestin mRNA in HEP-G2 cells (see Fig. 3) was not too surprising because low levels of hephaestin mRNA were found in liver [12].

Taken together, as to our knowledge this is the first study where the expression (on mRNA as well as on protein level) of the most relevant proteins of non-transferrin iron transport across cell membranes (DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin) is compared under identical experimental conditions of iron deficiency and excess of non-transferrin iron in cell lines which represent the main functional cell types involved in iron handling. Our data show that changes in iron availability, i.e., iron deficiency and iron excess, affect the expression of DMT1, Dcytb, ferroportin, hephaestin, and ceruloplasmin in a cell type-specific manner. However, demonstrated responses of tested cells to changed availability of non-transferrin iron do not often correspond to expected behavior of cells with particular physiological role. The reason for that discrepancy, when studies with cell lines in vitro are performed, could be the absence of systemic iron homeostasis regulation in organism. It is also important to notice that not every alteration of the expression found on mRNA level correlates with a relevant alteration of the expression on the protein level.

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3.5. PAPER 3

DUODENAL EXPRESSION OF IRON TRANSPORT MOLECULES IN PATIENTS WITH HEREDITARY HEMOCHROMATOSIS OR IRON DEFICIENCY

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Duodenal expression of iron transport molecules in patients with hereditary hemochromatosis or iron deficiency

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Abstract

Disturbances of iron metabolism are observed in chronic liver diseases. In the present study, we examined gene expression of duodenal iron transport molecules and hepcidin in patients with hereditary hemochromatosis (HHC) (treated and untreated), involving various genotypes (genotypes which represent risk for HHC were examined), and in patients with iron deficiency anaemia (IDA). Gene expressions of DMT1, ferroportin, Dcytb, hephaestin, HFE and TFR1 were measured in duodenal biopsies using real-time PCR and Western blot. Serum hepcidin levels were measured using ELISA. DMT1, ferroportin and TFR1 mRNA levels were significantly increased in post-phlebotomized hemochromatics relative to controls. mRNAs of all tested molecules were significantly increased in patients with IDA compared to controls. The protein expression of ferroportin was increased in both groups of patients but not significantly. Spearman rank correlations showed that DMT1 *versus* ferroportin, Dcytb *versus* hephaestin and DMT1 *versus* TFR1 mRNAs were positively correlated regardless of the underlying cause, similarly to protein levels of ferroportin *versus* Dcytb and ferroportin *versus* hephaestin. Serum ferritin was negatively correlated with DMT1 mRNA in investigated groups of patients, except for HHC group. A decrease of serum hepcidin was observed in IDA patients, but this was not statistically significant. Our data showed that although untreated HHC patients do not have increased mRNA levels of iron transport molecules when compared to normal subjects, the expression is relatively increased in relation to body iron stores. On the other hand, post-phlebotomized HHC patients had increased DMT1 and ferroportin mRNA levels possibly due to stimulated erythropoiesis after phlebotomy.

Keywords: DMT1 • ferroportin • Dcytb • hephaestin • TFR1 • HFE • hepcidin • hemochromatosis • iron deficiency • gene expression

Introduction

HFE-linked hereditary hemochromatosis (HHC) is one of the most common autosomal recessive diseases among Caucasians. It occurs with a frequency of 1 in 300–400 individuals and a heterozygote carrier rate of 8–10% in populations with a northern European origin [1]. The HFE protein is one of the regulatory molecules associated with iron metabolism. Inadequate function of this protein leads to excessive absorption of iron in the duodenum resulting in serious damage to organs, *e.g.* liver cirrhosis, diabetes mellitus and/or cardiomyopathy. Homozygosity for the C282Y mutation (c.845G>A) in the HFE gene is present in more than 80% of hemochromatosis patients with a northern European origin [1–3]. H63D (c.187 C>G), another mutation in HFE gene, is not associated with the same level of iron loading as C282Y and is found in hemochromatosis patients especially when combined with C282Y (compound heterozygotes).

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However, a small number of H63D homozygote patients have been reported [1, 2, 4]. S65C (c.193 A>T), a third mutation of the HFE gene, is also occasionally found in hemochromatosis patients, although the role of this mutation is not clear.

Iron homeostasis is regulated primarily by iron absorption. The process of dietary iron absorption in the duodenum starts with reduction of ferric iron Fe^{3+} to the ferrous Fe^{2+} form, on the apical surface of enterocytes, by duodenal cytochrome b reductase (Dcytb). Ferrous iron is then transported across the membrane into the cell by an apical iron transporter – divalent metal ion transporter (DMT1). Once in the cell, iron is stored in ferritin or transferred across the basolateral membrane *via* ferroportin (FPN1). Ferrous iron is then oxidized to the ferric form by hephaestin (Hp), a multi-copper oxidase, before entering the circulation by binding to the transferrin [5].

HFE affects the interaction of transferrin-bound iron with transferrin receptor (TFR1) thereby modulating iron uptake. HFE interacts with TFR1 in such a way that binding of HFE to TFR1 lowers its affinity for iron-transferrin, resulting in a reduction of cellular iron uptake [6, 7]. Except of TFR1, HFE also forms complexes with TFR2 [8], which is mainly expressed in hepatocytes. The HFE/TFR2 complex is thought to serve as a body iron sensor, which regulates hepcidin expression [9].

Regulation of iron homeostasis in organisms is mainly mediated by a small 25 amino acid peptide hepcidin [10, 11], which is, mostly, synthesized in the liver. Hepcidin appears to act by binding to and internalization of ferroportin and its subsequent degradation [12, 13] which results in reduced iron efflux from enterocytes. It has also been shown that, in intestinal cells, hepcidin mediates a transcriptional response resulting in decreased duodenal DMT1 expression and decreased apical iron uptake [14, 15].

There have been relatively few human's studies with regard to the expression of duodenal iron transport molecules associated with iron overload (hemochromatosis) or iron deficiency, and the studies that have been done, produced conflicting results [16–22]. Moreover, these studies usually focused on mRNA levels only, with hepcidin not being measured.

The aim of the present study was to examine the expression profile of duodenal iron transport molecules on both mRNA and protein levels and to investigate how expression is influenced by hepcidin. Furthermore, we analysed the relationship among expressions of these iron transport molecules and indices of iron metabolism. This study was conducted in patients with HHC (treated and untreated) with different genotypes (only genotypes which represent a risk for hemochromatosis were examined), in patients with iron deficiency anaemia, and control subjects.

Materials and methods

Patients

The study included 60 individuals (31 male, 29 female), mean age of 54.3 years, ranging from 25 to 82 years. Patients were recruited at our outpatient department between 2005 and 2009. Hemochromatosis (HHC) patients (N = 14) were diagnosed on the basis of risk genotype (C282Y homozygosity, C282Y/H63D compound heterozygosity and H63D homozygosity) and persistently raised iron indices, defined as serum ferritin > 250 $\mu g\ l^{-1}$ for male patients and serum ferritin > 200 $\mu g\ l^{-1}$ for female patients or transferrin saturation > 45%. The untreated HHC group (N = 5) had duodenal biopsies taken prior to phlebotomy treatment. Porphyria cutanea tarda was diagnosed in one HHC patient. Patients with iron deficiency anaemia (IDA) (N = 16) were defined as those with a serum ferritin < 20 $\mu g\ l^{-1}$, low haemoglobin (men < 13 g dl^{-1} and women < 11.5 g dl^{-1}) and transferrin saturation (<16%). The control group (N = 30) had upper GI endoscopy, as part of the evaluation of their dyspeptic symptoms, and presented with iron parameters that were within normal range (serum iron 11–26 $\mu mol\ l^{-1}$, serum ferritin male 30–250 $\mu g\ l^{-1}$, female 30–200 $\mu g\ l^{-1}$, transferrin saturation 20–45%). DNA for HFE genotyping was available in 10 controls, 16 IDA and 13 HHC patients. C282Y, H63D and S65C mutations of the HFE gene were analysed using the PCR-RFLP method, as described previously [23]. Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the Third Faculty of Medicine and conducted in accordance with the Helsinki Convention.

Sample collection

Duodenal biopsy samples were obtained from 60 individuals during gastrointestinal endoscopy. For mRNA analysis, samples were stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) at −20°C and for protein analysis, samples were stored at −80°C.

Quantitative (real-time) polymerase chain reaction

Total RNA was extracted from RNAlater stored duodenal biopsy samples using a RNAeasy MiniKit with DNase treatment (Qiagen, Hilden, Germany) following the manufacturer's instructions. After estimation of RNA integrity, using gel electrophoresis, and determination of concentration of each sample, two samples were found to be insufficient for further analysis. Next, total RNA was reverse transcribed in an 80 μl reaction using a reverse transcription kit TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with random primers; the procedure followed the manufacturer's instructions. Transcribed cDNA was subjected to real-time quantitative PCR in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using commercially available kit Taq Man Universal PCR Master Mix (Applied Biosystems) for DMT1 (IRE variants), FPN1, TFR1, HFE and GAPDH. Sybr green PCR Master Mix (Applied Biosystems) was used for detection of Dcytb and Hp. Primers and probes were designed intron spanning to avoid co-amplification of genomic DNA. Amplification of DMT1, Dcytb and Hp cDNA was completed following the previously described techniques [21, 24]. For the amplification of FPN1, TFR1, HFE and GAPDH, Applied Biosystems pre-designed gene expression assays were used. The part numbers for the assays were as follows: FPN1 – Hs00205888_m1, TFR1 – Hs00174609_m1, HFE – Hs00373474_m1, GAPDH – Hs99999905_m1. Amplification conditions were identical for all analysed molecules. Initial denaturation at 50°C for 2 min. and then 95°C for 10 min. was followed by 40 amplification cycles at 95°C for 15 sec. and 60°C for 1 min. Each sample was examined in triplicate. All data were normalized to the amount of GAPDH cDNA in the sample. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression.

Western blot analysis

Due to a limited amount of biological material, Western blot analyses were performed only on duodenal iron transporters and coupled oxidoreductases (DMT1, FPN1, Dcytb, Hp). After protein extraction from duodenal biopsy samples using RIPA buffer (Sigma-Aldrich) and determination of concentration (Thermo Scientific Pierce BCA Protein Assay Kit; Thermo Fisher Scientific Inc., Rockford, IL, USA), six samples were found to be insufficient for further analysis by Western blot. Due to the small amount of tissue obtained from three of the biopsies, only Hp and DMT1 could be evaluated in these samples.

Western blot analysis of DMT1 (IRE variants), Dcytb, FPN1, Hp and actin (loading control) levels was carried out with some modifications as described, in detail, previously [25]. Proteins separated by SDS-PAGE were blotted onto a 0.2 μm nitrocellulose membrane for 2 hrs at 0.25 A using a MiniProtean II blotting apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% BSA in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 25 min. (DMT1), 40 min. (ferroportin), 60 min. (Dcytb), 45 min. (Hp) or in 5% non-fat milk in TBS for 20 min. (actin). A solution of 0.1% Tween-20/TBS was used for washing. Washed membranes were incubated with the relevant primary antibody. Goat polyclonal antibodies NRAMP 2 (N-20) and Hephaestin (N-20) against human DMT1 and Hp from Santa Cruz Biotechnology (Santa Cruz, CA, USA), goat polyclonal anti-Cytochrome b reductase 1 antibody (EB06633) against human Dcytb (Everest Biotech, Upper Heyford, UK), rabbit polyclonal antibody MTP11-A against human ferroportin from Alpha Diagnostic International (San Antonio, TX, USA) and mouse monoclonal antibody (A3853) against human actin (Sigma-Aldrich) were used. After incubation (overnight, 4°C), the washed membrane was incubated for 1 hr with corresponding horseradish peroxidase-conjugated secondary antibody. Antibodies were diluted in 5% BSA (non-fat milk)/0.1% Tween-20/TBS. The horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using Supersignal reagent from Pierce (Rockford, IL, USA) and a LAS 1000 CCD (Fuji). Band intensities were quantified by densitometry and the data were analysed with the use of ImageJ software (version 1.42q; NIH, USA, available on http://rsb.info.nih.gov/ij/).

ELISA

Serum for hepcidin measurement was available in 30 patients. Bioactive hepcidin was measured in serum samples of 8 controls, 13 IDA and 9 HHC patients using a newly developed commercial kit enzyme-linked immunosorbent assay (ELISA) specific for the mature peptide (EIA-4705) (DRG International Inc., NJ, USA) according the manufacturer's instructions. In both patients and controls, blood samples were drawn between 7.30 and 8.30 a.m. after overnight fasting.

Statistical analysis

Data are expressed as mean ± S.E.M. Comparison of the data between groups was performed using the ANOVA and LSD test. When the variables were not normally distributed, the Mann-Whitney test or the Kruskal-Wallis test followed by multiple comparison tests, were used as appropriate. Correlation was assessed using the Spearman rank method. Statistical analysis was done using the Statistica program (version 9; StatSoft, Tulsa, OK, USA) and the GraphPad Prism program (version 5.00; GraphPad Software, Inc., San Diego, CA, USA). The significance level was set at 0.05.

Results

Clinical and laboratory characteristics of patients

Clinical characteristics and laboratory parameters of each patient group (HHC treated and untreated, IDA, controls) are shown in Table 1. As for the HFE gene analysis, in the HHC group, eight patients were C282Y homozygotes, four patients were compound heterozygotes C282Y/H63D and one patient was an H63D homozygote. In the IDA group, two patients were H63D heterozygotes and one was an H63D homozygote. Analysed control subjects were wild type for all three tested mutations.

RNA expression

The gene expression at the mRNA level was measured in DMT1, FPN1, Dcytb, Hp, TFR1 and HFE. DMT1 qPCR was performed on the DMT1(IRE) transcripts, because these splice variants are the isoforms that respond to variations in cell iron content [26]. Because the IRE and non-IRE sequences are localized in last exons, we could not further distinguish between 1A and 1B variants, which vary in first exons. The 1A isoform of DMT1 mRNA is most actively expressed in duodenal and kidney cells and also participates in iron regulation, whereas the 1B isoform has broad distribution, which correlates with the role of DMT1 in the release of iron from endosome. Anyway, both of these mRNA variants are expressed in the proximal duodenum, where systemic iron absorption is regulated [26].

There was a significant difference in the expression of all analysed molecules across groups at the mRNA level (Kruskal-Wallis test). Further examination of the data using multiple comparison tests found a significant increase in the expression of all analysed molecules in IDA patients when compared to controls: DMT1 (5.36-fold, $P = 0.0003$), FPN1 (2.58-fold, $P < 0.0001$), Dcytb (1.39-fold, $P = 0.0019$), Hp (1.34-fold, $P = 0.0175$), TFR1 (2.41-fold, $P = 0.0002$), HFE (1.28-fold, $P = 0.0052$). When the HHC group and controls were analysed, no significant difference in the expression of any molecules was detected, although DMT1, FPN1 and TFR1 mRNA were increased in HHC group (3.30-fold, $P = 0.5514$, 1.48-fold, $P = 0.4815$, 1.62-fold, $P = 0.5740$, respectively) (Fig. 1A).

To evaluate the situation in the HHC group, we divided the HHC group into untreated (N = 5) and treated (N = 9) patients. A significant increase of DMT1 and TFR1 mRNA expression was observed in the treated HHC patients compared to untreated patients (5.14 *versus* 0.36, $P = 0.0451$, 2.23 *versus* 0.65, $P = 0.0451$, respectively). Although FPN1 gene expression had the same pattern as DMT1, the increase did not reach statistical

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Table 1 Clinical characteristics and iron phenotype in patient groups

Variable	Control N = 30	IDA N = 16	HHC Total N = 14	HHC Treated N = 9	HHC Untreated N = 5
Age [yrs]	57.40	51.69	50.71	54.22	44.40
	±2.85	±4.32	±3.69	±4.97	±4.43
Gender [M/F]	18/12	1/15***	12/2	4/1	8/1
Serum iron	17.55	3.18	28.77	23.73	28.77
[μmol l^{-1}]	±0.67	±0.34***	±3.82*	±4.59	±3.82***
Serum ferritin	151.00	16.43	1075.00	923.10	1349.00
[μg l^{-1}]	±13.83	±4.52***	±433.90*	±638.20	±467.00***
Transferrin	31.22	6.24	57.46	44.81	95.40
Saturation [%]	±0.96	±1.28***	±10.32	±10.69	±3.14**
ALT	0.54	0.36	0.91	0.93	0.87
[μkat l^{-1}]	±0.05	±0.05*	±0.15**	±0.20*	±0.25
AST	0.47	0.36	0.69	0.73	0.62
[μkat l^{-1}]	±0.03	±0.04*	±0.10*	±0.15	±0.08*
Hb	14.00	8.38	14.63	14.41	15.02
[g dl^{-1}]	±0.31	±0.31***	±0.40	±0.47	±0.77
Ht	41.48	27.06	42.78	42.01	44.16
[%]	±0.93	±0.93***	±1.15	±1.33	±2.21

Data are presented as arithmetic mean ± S.E.M. ALT: alanine aminotransferase (EC 2.6.1.2); AST: aspartate aminotransferase (EC 2.6.1.1); Hb: haemoglobin; Ht: hematocrit. Normal ranges: serum iron (11–26 μmol l^{-1}), serum ferritin (male 30–250 μg l^{-1}, female 30–200 μg l^{-1}), transferrin saturation 20–45%, ALT (0.1–0.75 μkat l^{-1}); AST (0.1–0.75 μkat l^{-1}), Hb (male 13.0–18.0 g dl^{-1}, female 11.5–16.0 g dl^{-1}), Ht (male 38–54%, female 35–47%). Statistical significant differences as compared with the control group are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

significance. However, when treated patients were compared to controls, higher levels of DMT1, TFR1 and also FPN1 mRNA were observed (5.14-fold, $P = 0.0115$, 2.23-fold, $P = 0.0115$, 1.90-fold, $P = 0.0484$, respectively) (Fig. 2A). To investigate the effect of different HFE genotypes, representing the risk for hemochromatosis, the HHC group was divided according to genotype, *i.e.* C282Y/C282Y, C282Y/H63D and H63D/H63D. However, no significant changes in the expression levels were found among C282Y/C282Y, C282Y/H63D and H63D/H63D individuals.

Protein expression

To investigate whether the changes in mRNA expression translated into changes in protein content, we examined DMT1, FPN1, Dcytb and Hp protein levels using Western blotting. No statistically significant differences in expression of any molecule were found across the studied groups of patients. Even though the FPN1 protein level was increased in both groups of patients, this increase did not reach statistical significance when compared to controls: IDA (1.37-fold, $P = 1.000$), HHC (1.45-fold, $P = 1.000$) (Fig. 1B).

When the situation in the HHC group was analysed, no differences between treated and untreated patients were found (Fig. 2B). Further analysis of hemochromatosis patients, studying the role of different genotypes, detected no changes in gene expression in C282Y or H63D homozygotes, or C282Y/H63D compound heterozygotes.

Relationship among gene expressions of tested molecules

The association among Dcytb, Hp, DMT1, FPN1, TFR1 and HFE gene expressions was investigated using the Spearman rank correlation. When all individuals were analysed together, there was a positive relationship among mRNA expressions of all tested molecules except between HFE *versus* DMT1, and HFE *versus* Hp. The strongest correlations were found between DMT1 *versus* FPN1

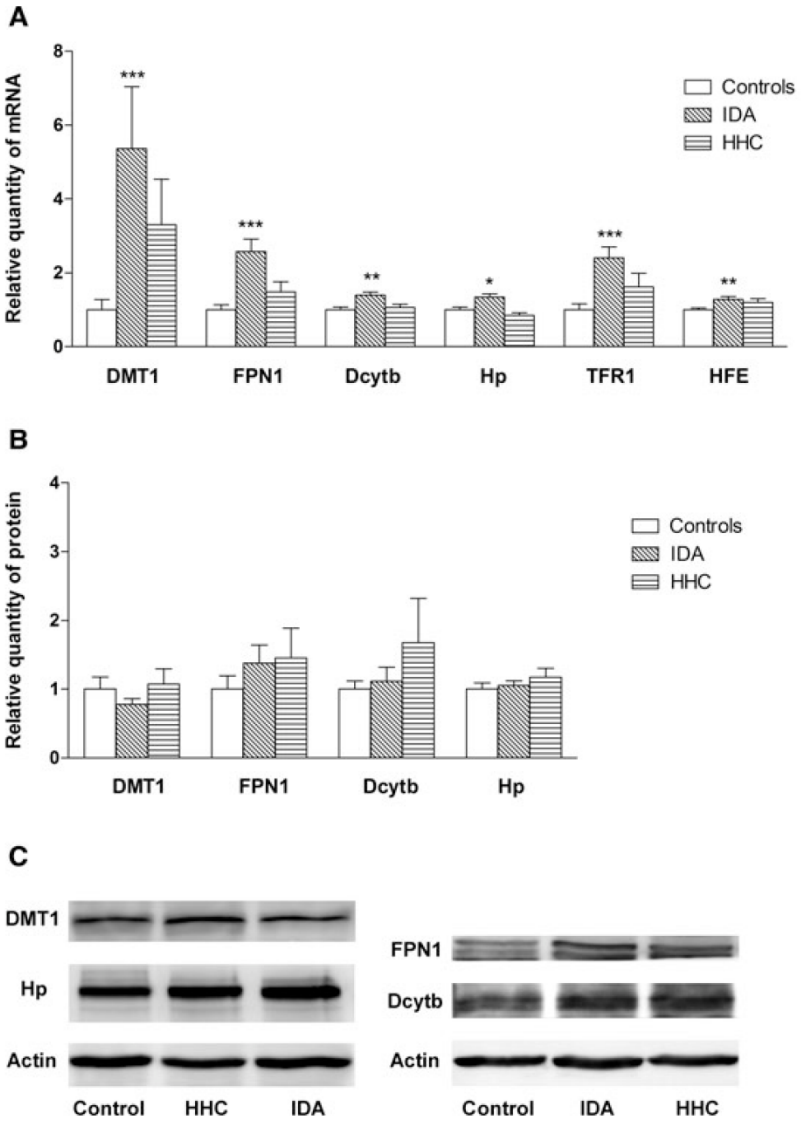

Fig. 1 Gene expression of the analysed molecules in controls, iron deficiency anaemia and hemochromatosis patients. (**A**) mRNA expression of DMT1, FPN1, Dcytb, Hp, TFR1, HFE. (**B**) Protein expression of DMT1, FPN1, Dcytb, Hp. (**C**) Western blot analysis of DMT1, FPN1, Dcytb, Hp and actin (loading control). Results are depicted as means ± S.E.M. Statistical significant differences as compared with the control group are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(r = 0.849, $P < 0.0001$), between Dcytb *versus* Hp (r = 0.733, $P < 0.0001$) and between DMT1 *versus* TFR1 (r = 0.746, $P < 0.0001$) (Table 2). When gene expression on the protein level was studied, a positive relationship between the following genes was found: Dcytb *versus* Hp (r = 0.434, $P = 0.0015$), Dcytb *versus* FPN1 (r = 0.681, $P < 0.0001$), DMT1 *versus* Hp (r = 0.378, $P = 0.0053$) and FPN1 *versus* Hp (r = 0.457, $P = 0.0007$) (Table 3).

mRNA level and protein level correlated with each other in DMT1 (r = 0.334, $P = 0.0167$) and Hp (r = 0.302, $P = 0.0299$) when all groups were analysed together.

When correlations in HHC, IDA and controls were investigated separately, positive relationships were found, as summarized in Tables 2 and 3. The strongest correlations at the mRNA level were found between DMT1 *versus* FPN1, Dcytb *versus* Hp and DMT1 *versus* TFR1 in all groups. With respect to protein expression, the strongest correlations were detected between Dcytb *versus* FPN1 (Tables 2 and 3). To evaluate the effect of the phlebotomy treatment the data were analysed separately for HHC subgroups (treated and untreated patients). However, the correlations found in HHC group were lost in HHC subgroups very

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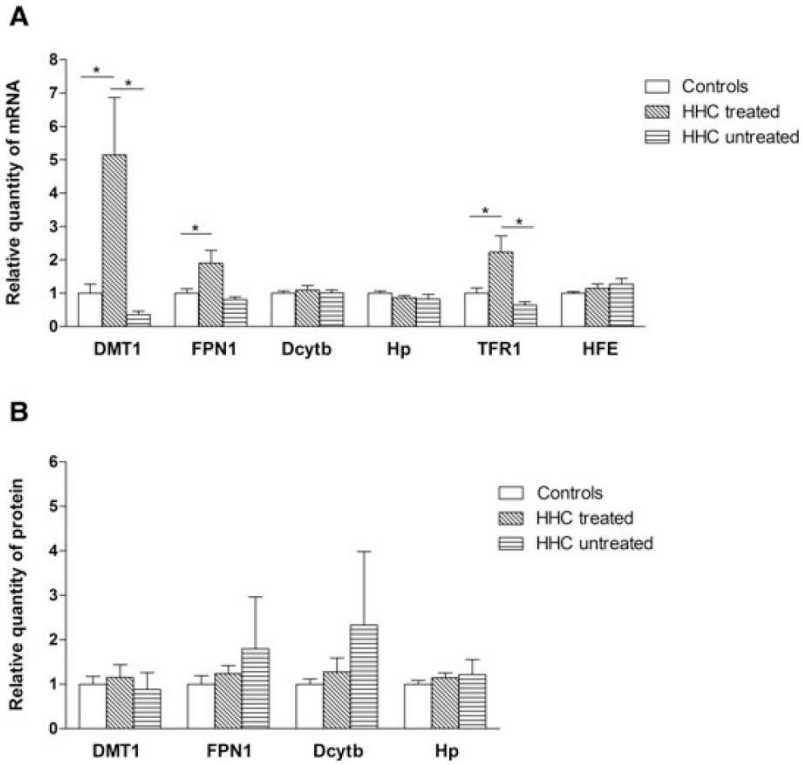

Fig. 2 Gene expression of the analysed molecules in controls, treated and untreated HHC patients. (**A**) mRNA expression of DMT1, FPN1, Dcytb, Hp, TFR1, HFE. (**B**) Protein expression of DMT1, FPN1, Dcytb, Hp. Results are depicted as means ± S.E.M. Statistical significant differences are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

probably because of the small sample size in these subgroups (treated HHC patients N = 9, untreated HHC patients N = 5) (Tables 2 and 3).

Relationship among gene expressions of tested molecules and iron parameters

We also investigated the relationship between gene expression of analysed molecules and serum iron parameters. When all patients, regardless of the underlying disease, were examined, negative correlations were found between serum ferritin and mRNA of Hp ($r = -0.646$, $P < 0.0001$), FPN1 ($r = -0.636$, $P < 0.0001$), DMT1 ($r = -0.609$, $P < 0.0001$), TFR1 ($r = -0.530$, $P = 0.0005$) and Dcytb ($r = -0.466$, $P = 0.0028$). Serum iron correlated at the mRNA level with FPN1 ($r = -0.405$, $P = 0.0105$) and Hp ($r = -0.402$, $P = 0.0112$). The same applies to correlations between transferrin saturation and the mRNA of FPN1 ($r = -0.534$, $P = 0.0008$), Hp ($r = -0.429$, $P = 0.0091$), TFR1 ($r = -0.420$, $P = 0.0106$), Dcytb ($r = -0.380$, $P = 0.0221$), and DMT1 ($r = -0.369$, $P = 0.0267$). There was no correlation between HFE mRNA and any of the tested iron parameters. When protein expression was examined in the cohort of all patients, no correlation was found between any protein and any iron parameter.

With respect to HHC, no inverse relationship between any of the tested molecules at the mRNA or protein level and serum ferritin or serum iron or transferrin saturation was detectable except for a positive correlation between HFE mRNA and transferrin saturation ($r = 0.690$, $P = 0.0186$). When the IDA group was studied separately serum ferritin correlated with Dcytb mRNA ($r = -0.533$, $P = 0.0405$), DMT1 mRNA ($r = -0.587$, $P = 0.0213$) and DMT1 protein ($r = -0.586$, $P = 0.0276$). Transferrin saturation correlated with Dcytb protein ($r = -0.785$, $P = 0.0005$) and FPN1 protein ($r = -0.745$, $P = 0.0014$) in the IDA group. Among controls, we found an inverse relationship between serum ferritin and DMT1 mRNA expression ($r = -0.772$, $P = 0.0053$) and FPN1 and Dcytb protein expression ($r = -0.766$, $P = 0.0159$, $r = -0.700$, $P = 0.0358$, respectively). Serum iron correlated with DMT1 protein expression ($r = -0.641$, $P = 0.0182$), and transferrin saturation with DMT1 protein expression ($r = -0.910$, $P = 0.0017$) in controls.

Transferrin saturation and serum ferritin, serum iron and serum ferritin, and serum iron and transferrin saturation were positively correlated with each other in all subjects ($r = 0.827$, $r = 0.799$, $r = 0.938$, respectively, $P < 0.0001$ for all comparisons listed).

Table 2 Associations among gene expression of analysed molecules on mRNA level

mRNA		HFE	TFR1	FPN1	DMT1	Hp
Dcytb	HHC total	0.071	0.495	0.648*	0.374	0.631*
	HHC treated	−0.333	0.476	0.619	0.286	0.619
	HHC untreated	0.400	0.600	0.700	0.000	0.800
	IDA	0.341	0.209	0.394	0.324	0.670**
	Controls	0.319	0.365	0.364**	0.186	0.790***
	All	0.375**	0.574***	0.650***	0.458***	0.733***
Hp	HHC total	0.178	0.587*	0.505	0.335	
	HHC treated	−0.452	0.762*	0.524	0.381	
	HHC untreated	0.600	0.400	0.700	0.200	
	IDA	−0.147	0.068	0.132	0.153	
	Controls	0.414*	0.330	0.593***	0.149	
	All	0.256	0.462***	0.564***	0.343**	
DMT1	HHC total	−0.220	0.637*	0.846***		
	HHC treated	−0.595	0.381	0.810*		
	HHC untreated	0.600	0.400	0.300		
	IDA	−0.035	0.620*	0.794***		
	Controls	0.250	0.634***	0.683***		
	All	0.218	0.746***	0.849***		
FPN1	HHC total	−0.341	0.551			
	HHC treated	−0.595	0.333			
	HHC untreated	0.100	0.100			
	IDA	0.003	0.841***			
	Controls	0.367	0.523**			
	All	0.270*	0.716***			
TFR1	HHC total	−0.044				
	HHC treated	−0.191				
	HHC untreated	0.800				
	IDA	−0.147				
	Controls	0.604***				
	All	0.408**				

Spearman rank correlation, statistical significant differences are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Hepcidin analysis

To get more insight into the regulation of iron absorption, the relationship between serum hepcidin and the expression of the analysed molecules was studied as well. Sera for hepcidin measurements were available in 30 individuals. A decrease in hepcidin levels was observed in IDA patients compared to controls, but this was not statistically significant (30.65 ng ml^{-1} *versus* 36.93 ng ml^{-1}, $P = 0.2935$). The same applies to comparison of HHC patients to controls (34.26 ng ml^{-1} *versus* 36.93 ng ml^{-1}, $P = 0.7360$). When the situation in the HHC group was analysed, no differences between treated and untreated patients or patients with different genotypes were found. The relationship between serum hepcidin level and the gene expression, at both the mRNA or protein level, was analysed but with the exception of HFE mRNA ($r = -0.524$, $P = 0.0036$) no correlation was found. In addition,

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Table 3 Associations among gene expression of analysed molecules on protein level

Proteins		FPN1	DMT1	Hp
Dcytb	HHC total	0.648*	0.119	0.533
	HHC treated	0.262	−0.262	0.024
	HHC untreated	0.700	0.800	0.900
	IDA	0.814***	−0.521*	0.379
	Controls	0.601**	0.213	0.360
	All	0.681***	0.003	0.434**
Hp	HHC total	0.302	0.593*	
	HHC treated	−0.619	0.583	
	HHC untreated	0.900	1.000	
	IDA	0.243	−0.043	
	Controls	0.580**	0.490*	
	All	0.457***	0.378**	
DMT1	HHC total	0.014		
	HHC treated	−0.691		
	HHC untreated	0.800		
	IDA	−0.154		
	Controls	0.176		
	All	0.018		

Spearman rank correlation, statistical significant differences are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

hepcidin levels did not correlate with any of measured iron parameter regardless of study group. Nevertheless, the small sample size does not permit to rule out definitely a possibility of serum hepcidin decrease in hemochromatics and IDA patients or correlations of serum hepcidin with gene expressions or iron indices. The gender distribution (see Table 1) may be another confounding factor.

Discussion

In this study, the gene expression of DMT1, FPN1, Dcytb and Hp at both the mRNA and protein levels were investigated in duodenal biopsy samples. In addition, the expression of HFE and TFR1 was examined at the mRNA level. To get a complete view of iron transport in duodenum, the role of serum hepcidin was studied as well. The study was conducted on a cohort of treated and untreated HHC patients with different genotypes, which represent a risk for hemochromatosis (C282Y/C282Y, C282Y/H63D and H63D/H63D), patients with iron deficiency anaemia, and controls. The current study is, to our knowledge, the first to investigate duodenal gene expression of iron transport molecules at both the mRNA and protein level, together with serum hepcidin levels among the above mentioned groups of patients.

Previously published reports on duodenal expression of iron transporters in HHC subjects have found mixed results [16–22]. Some authors reported major increases in the gene expression of DMT1 and FPN1 compared to controls, whereas other authors found no increase in the expression of these molecules. However, earlier studies did not distinguish between treated and untreated HHC patients. In our HHC patients, there were no significant differences in gene expression at the mRNA and protein levels when compared to controls. A significant increase of DMT1, FPN1 and TFR1 mRNA was, however, detected in the subgroup of treated HHC patients. Similar observations were documented in some previous studies [17, 18, 20] but not others [19, 21, 22]. Although the expression of iron transport genes was not different from controls in untreated HHC subjects, it seems to be inappropriately high relative to the degree of body iron overload. Some authors have suggested that hemochromatics initially load iron in similar amounts as seen in iron deficiency and when the body iron stores increase to some limit, the stores regulator (now recognized as hepcidin) is stimulated to down-regulate iron loading [17]. In post-phlebotomized subjects, the increased level of DMT1 and FPN1 could be explained by the fact that duodenal iron uptake is increased in response to increased erythropoiesis after phlebotomy. Duodenal enterocytes behave as if iron deficient, which can manifest as increased transferrin receptor mRNA expression in these patients. As others have found [16, 18, 20], Hp and Dcytb were not up-regulated in HHC patients, regardless of therapy, and do not seem to play a significant role in the increase of iron absorption observed in HHC patients.

HHC patients with different genotypes (C282Y/C282Y, C282Y/H63D and H63D/H63D) express different severities of clinical signs of the disease [27]. Our goal was to examine the effect of different genotypes on expression levels of iron transport genes. Variable expression could explain different levels of iron absorption resulting in different degrees of iron accumulation. However, when the effect of these genotypes in HHC patients was studied, no significant differences in gene expression at the mRNA or protein or hepcidin levels were observed. Yet, the small size of the HHC genotype groups does not permit definite conclusions in this respect.

In agreement with previous studies [17, 20–22, 28] our patients with iron deficient anaemia showed significantly increased mRNA gene expression of all tested molecules (DMT1, FPN1, Dcytb, Hp, TFR1). This probably represents a compensatory mechanism to increase iron absorption in response to the body need for iron. The minor increase in the HFE expression, seen in these patients, supports the idea that the major site of HFE action is in the liver. The crypt cell hypothesis proposed that HFE acts as a regulator of iron homeostasis by sensing the amount of body iron on the basolateral membrane of enterocytes in conjunction with TFR1 [29]. However, with the discovery of hepcidin, the liver is now thought to be a centre for iron regulation and the HFE/TFR2 complex is supposed to transmit the signal to induce hepcidin expression [8, 9].

In our study, only the unchanged expression of mRNA (in untreated HHC patients) was paralleled by the same pattern at the protein level, whereas significant increases of DMT1 and

FPN1 at the mRNA level, seen in IDA and treated HHC patients, was not followed by significant increase at the protein level, although some elevation of FPN1 was detected. In contrast to our findings, some studies have shown, using Western blot and immunohistochemistry, that differences in DMT1 [17] and FPN1 [21] mRNA expression are reflected by corresponding changes in the expression of the respective proteins. However, in a later study by this group on Dcytb and Hp [22], the mRNA did not correspond to the respective proteins. A similar incongruence between mRNA gene expression of duodenal iron transport genes and the respective proteins was seen in our previous study, which used cell lines [30]. Of note, all the above-mentioned molecules have IRE (iron responsive element) sequences. DMT1 and TFR1 (which was increased as well) bear an IRE within its 3′UTR and are stabilized upon IRP (iron regulatory protein) binding, whereas in ferroportin, this sequence is localized in its 5′UTR and IRP binding inhibits translation. It was shown using animal models that specific intestinal depletion of IRP1 and IRP2 decreases DMT1 and increases FPN1, resulting in the death of the duodenal cells [31]. This demonstrates the important role of the IRP/IRE system in post-transcriptional regulatory mechanisms of DMT1 and FPN1. Therefore, although hepcidin is considered a major regulatory molecule of DMT1 and FPN1 gene expression, the IRE/IRP mechanism probably acts as a fine tuner, which modulates the expression of these proteins. In addition, decreased protein stability under specific conditions in particular patients (*e.g.* degree of iron overload, presence of possible genetic modifiers) must be considered. Recently, Ndfips adaptor proteins, in association with ubiquitin ligase WWP2, were shown to enhance ubiquitination and subsequent degradation of DMT1, thus reducing the amount of DMT1 protein [32]. Nevertheless, to date, the majority of gene expression human studies of duodenal iron transporters have focused on the mRNA level only [16, 18–20, 28]. This inconsistency between mRNA and protein expression emphasizes the importance of complex analyses of both mRNA and protein, through which post-transcriptional regulatory mechanisms can be found.

Similar to previous studies, we observed a strong positive correlation between DMT1 and FPN1, Dcytb and Hp, DMT1 and TFR1 at the mRNA level in all studied groups suggesting coordinated regulation of these genes. It seems that this integral regulation is also retained in HHC individuals and is not disturbed by HFE gene mutations.

Results obtained from studies examining the relationship between duodenal iron transporters and iron metabolism parameters are inconsistent. In the present study there were significant inverse correlations between mRNA expression of some duodenal iron transporters and iron indices. The relationship between DMT1 mRNA and serum ferritin in controls and IDA patients suggests that body iron stores play a role in the regulation of duodenal expression of this transporter. Nevertheless, this correlation was not found in HHC patients. This lack of correlation may be due to a disturbance in iron homeostatic signalling in HHC patients, probably *via* alteration of HFE function, which normally forms the HFE/TFR2 iron sensing complex involved in the hepcidin regulatory pathway [9]. Our observations are in agreement with some studies [19, 21, 22], whereas other studies have demonstrated a significant inverse relationship between serum ferritin and DMT1 expression in patients with HHC, iron deficiency and controls [20]. In addition, some studies did not find any correlation at all [16–18].

To get more insight into the regulation of iron metabolism, we measured bioactive hepcidin in patients' sera. Hepcidin is now recognized as the key regulator of iron metabolism. It was found to be up-regulated by iron overload and down-regulated by iron deficiency [11, 33–35]. It has been documented in some reports that hepcidin is decreased in HHC [36–38] and IDA patients and is inversely correlated with duodenal FPN1 and DMT1 expression in animal models [39]. On the other hand, in a study by Gehrke *et al.* [40], liver hepcidin expression was not significantly decreased in hemochromatosis patients. In our current study, while serum hepcidin in IDA patients was decreased in comparison to controls, this difference did not reach statistical significance. Moreover, no decrease in hepcidin was detected in HHC patients when compared to controls, even when divided into treated and untreated groups. Another unexpected observation was that the hepcidin level did not negatively correlate with FPN1 or DMT1 gene expression (at the mRNA or protein level) and iron indices. Frazer *et al.* [33] made similar observations. In their study there was no correlation between serum hepcidin concentrations and serum ferritin, soluble transferrin receptors, or haemoglobin concentrations, all of which are commonly used markers of iron status [33]. The failure to demonstrate an association between hepcidin levels and increased FPN1 and DMT1 expression in our HHC and IDA patients may be due to the small sample of patients and their diversity (*i.e.* varying degree of iron overload and genetic background). Moreover, data regarding hepcidin in relationship to duodenal transport molecules usually come from mRNA levels in the liver; whereas we determined hepcidin protein levels from serum. It is known that hepcidin can also be regulated at the post-translational level, during hepcidin maturation, which occurs *via* furin convertase [41]. In addition, α2-macroglobulin was recently identified as a specific hepcidin-binding molecule. A complex of α2-macroglobulin and hepcidin could influence the determination of hepcidin during diagnostic testing [42].

In conclusion, the results of the current study indicate that duodenal mRNA expression of DMT1, FPN1 and TFR1 is increased in post-phlebotomized HHC patients. This elevation was not observed in untreated HHC patients, although their mRNA expression of iron transport molecules was relatively increased in relation to body iron stores. The different genotypes, representing risk for HHC, do not seem to influence the expression of duodenal iron transport molecules. Serum hepcidin was not decreased in our HHC patients. Our observations show that mRNA and protein expression of duodenal iron transport molecules do not always correlate with each other; therefore it is necessary to assess protein levels in such studies as well. Observed positive correlations among Dcytb, Hp, DMT1, FPN1 and TFR1 mRNA indicate the presence of coordinated regulation of these genes. Further studies are needed to elucidate the complex system of pathogenesis of iron homeostasis in iron metabolism diseases.

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Author contributions

M. Dostalikova-Cimburova, K. Kratka, J. Kovar and J. Horak designed the research study; M. Dostalikova-Cimburova, K. Balusikova, J. Chmelikova and J. Neubauerova performed the research (performed molecular analyses); K. Kratka, V. Hejda and J. Hnanicek performed the research (examined patients, collected the clinical data and biological material); M. Dostalikova-Cimburova and J. Vranova analysed the data; M. Dostalikova-Cimburova wrote the paper; K. Kratka, K. Balusikova, J. Chmelikova, V. Hejda, J. Hnanicek, J. Neubauerova, J. Vranova, J. Kovar and J. Horak critically revised the manuscript.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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3.6. PAPER 4

ROLE OF DUODENAL IRON TRANSPORTERS AND HEPCIDIN IN PATIENTS WITH ALCOHOLIC LIVER DISEASE

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Role of duodenal iron transporters and hepcidin in patients with alcoholic liver disease

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Abstract

Patients with alcoholic liver disease (ALD) often display disturbed iron indices. Hepcidin, a key regulator of iron metabolism, has been shown to be down-regulated by alcohol in cell lines and animal models. This down-regulation led to increased duodenal iron transport and absorption in animals. In this study, we investigated gene expression of duodenal iron transport molecules and hepcidin in three groups of patients with ALD (with anaemia, with iron overload and without iron overload) and controls. Expression of DMT1, FPN1, DCYTB, HEPH, HFE and TFR1 was measured in duodenal biopsies by using real-time PCR and Western blot. Serum hepcidin levels were measured by using ELISA. Serum hepcidin was decreased in patients with ALD. At the mRNA level, expressions of *DMT1*, *FPN1* and *TFR1* genes were significantly increased in ALD. This pattern was even more pronounced in the subgroups of patients without iron overload and with anaemia. Protein expression of FPN1 paralleled the increase at the mRNA level in the group of patients with ALD. Serum ferritin was negatively correlated with *DMT1* mRNA. The down-regulation of hepcidin expression leading to up-regulation of iron transporters expression in the duodenum seems to explain iron metabolism disturbances in ALD. Alcohol consumption very probably causes suppression of hepcidin expression in patients with ALD.

Keywords: DMT1 • FPN1 • DCYTB • HEPH • TFR1 • HFE • hepcidin • alcoholic liver disease • iron • gene expression

Introduction

Iron metabolism disturbances are common findings in patients with chronic alcohol consumption. It ranges from anaemia to iron overload [1–3]. The pathogenesis of anaemia in alcoholic liver disease (ALD) is complex; it includes hypersplenism with splenic pooling and increased destruction of erythrocytes, blood loss because of gastrointestinal bleeding as a complication of alcoholic cirrhosis, which can lead to anaemia with iron deficiency [4–8]. Inadequate diet with nutrient deficits, in alcoholic patients, can result in anaemia with megaloblastic and sideroblastic features [9]. Additionally, anaemia of chronic disease can occur in patients with chronic ALD [10, 11]. Conversely, some alcoholics develop iron overload [1, 3, 12]. It has been documented that chronic alcohol consumption in moderate to excess amounts leads to elevated serum ferritin concentration and transferrin saturation, and can result in increased hepatic iron stores [13]. Additionally, increased intestinal iron absorption has been observed in patients with chronic alcoholic disease [14]. Both iron and ethanol individually cause oxidative stress and lipid peroxidation and the cumulative effects of ethanol and iron on liver cell damage, in patients with ALD, exacerbate liver injury [15–17].

As there is no physiological way of eliminating excess iron, iron homoeostasis is regulated primarily by iron absorption. The process of intestinal non-haeme iron uptake starts with the reduction in ferric iron to the ferrous form by, the brush border enzyme, ferrireductase DCYTB (CYBRD1, duodenal cytochrome b reductase 1) [18]. Iron is then transported across the apical membrane of duodenal enterocytes

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by means of the divalent metal ion transporter 1 (DMT1, SLC11A2, NRAMP2, DCT1) [19, 20]. From the enterocyte, iron is exported across the basolateral membrane by ferroportin (FPN1, SLC40A1, IREG1, MTP1) [21–23]; a ferroxidase hephaestin (HEPH) oxidizes iron to its soluble and non-reactive ferric state, which is then delivered to transferrin [24]. HFE, responsible for hereditary haemochromatosis, is another regulatory protein associated with iron metabolism [25]. HFE affects the interaction of transferrin-bound iron with the transferrin receptor (TFR1, TFRC). Binding of HFE to TFR1 lowers its affinity for iron-transferrin, resulting in a reduction of cellular iron uptake [26, 27]. HFE forms complexes also with transferrin receptor 2 (TFR2) [28]. The HFE/TFR2 complex is thought to serve as an iron sensor that regulates hepcidin expression [29]. Hepcidin, a key hormone in the regulation of iron metabolism [30], is produced mainly in the liver. Hepcidin appears to act *via* binding to and internalization of ferroportin and its subsequent degradation [31, 32]; although, evidence that hepcidin inhibits apical uptake *via* DMT1 is also available [33]. Hepcidin was found to be up-regulated by iron overload and down-regulated by iron deficiency anaemia and hypoxia [30, 34–36]. It has been documented that hepcidin is decreased in patients with haemochromatosis [37–39]. In addition to its response to iron homoeostasis, hepcidin is induced by inflammation [30]. Recently, with the use of animal models, ethanol was shown to down-regulate the expression of hepcidin in the liver which resulted in elevated expression of the iron transporters DMT1 and ferroportin in the duodenum [40, 41]. Deregulation of hepcidin synthesis may be one of the causes of iron disturbances during chronic alcohol consumption.

So far, the effect of ethanol on iron uptake *via* duodenal iron transporters and its relation to hepcidin have only been analysed using cell lines and animal models [40–43]. Therefore, the aim of this study was to evaluate the expression of duodenal iron transporters both on mRNA and protein levels and their relation to hepcidin in alcoholic patients either with anaemia, iron overload or normal iron stores.

Materials and methods

Patients

A total of 54 individuals (35 male, 19 female), mean age of 57.4 years, ranging from 25 to 82 years were enrolled in the study. The diagnosis of ALD ($N = 24$) was based on patients' history of consumption of more than 30 g alcohol per day, presence of elevated serum AST (aspartate aminotransferase, EC 2.6.1.1) or ALT (alanine aminotransferase, EC 2.6.1.2) and GGT (gamma-glutamyltransferase, EC 2.3.2.2) activity and sonographically observed fatty changes in the liver (liver steatosis). According to laboratory parameters, ALD patients were categorized into three subgroups: ALD with anaemia ($N = 8$), ALD with iron overload ($N = 6$) and ALD without overload ($N = 10$). First, patients were divided according to the presence of anaemia (haemoglobin levels <11 g/dl). These patients had minor decreases in serum iron parameters, however, they did not meet criteria for iron deficiency anaemia (serum ferritin <20 μg/l, haemoglobin <11.0 g/dl and transferrin saturation <16%). Patients without anaemia were then divided according to the presence of iron overload, defined as elevated ferritin levels (cut-off: 200 μg/l for women and 250 μg/l for men) or increased transferrin saturation (cut-off = 45%). The control group ($N = 30$) had an upper GI (gastrointestinal) endoscopy to evaluate their dyspeptic symptoms and their iron parameters were in normal ranges (serum iron 11–26 μmol/l, serum ferritin male 30–250 μg/l, female 30–200 μg/l, transferrin saturation 20–45%). Controls were participants of another study by our group concerning the gene expression in haemochromatosis [44]. To analyse the effect of *HFE* gene mutations, the genotyping for C282Y, H63D and S65C mutations of *HFE* gene was performed using the PCR-RFLP method, as described previously [45]. DNA for *HFE* genotyping was available from 23 ALD patients and from 10 controls. Patients were recruited at our outpatient department between 2005 and 2009. Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the Third Faculty of Medicine, Charles University and conducted in accordance with the Helsinki Convention.

Sample collection

Duodenal biopsy samples were obtained from 54 individuals during GI endoscopy. For RNA analysis, samples were stored at −20°C in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) prior to RNA isolation and for protein analysis at −80°C prior to protein isolation.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from RNAlater-stored duodenal biopsies by using an RNAeasy MiniKit (Qiagen, Hilden, Germany), and included DNAse digestion according to manufacturer's instructions. After estimation of RNA integrity by using gel electrophoresis and determination of each sample concentration, one sample was found to be inadequate for further analysis. The following analyses were carried out as previously described in detail [44]. Briefly, cDNA synthesis was performed using a reverse transcription kit TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) with random primers according to the manufacturer's instructions. Real-time quantitative PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and commercially available kits: Taq Man Universal PCR Master Mix and Sybr green PCR Master Mix (Applied Biosystems). For the amplification of *FPN1*, *TFR1*, *HFE* and *GAPDH cDNA*, Applied Biosystems pre-designed gene expression assays were used: *FPN1* – Hs00205888_m1, *TFR1* – Hs00174609_m1, *HFE* – Hs00373474_m1 and *GAPDH* – Hs99999905_m1. For amplification of *DMT1*, *DCYTB* and *HEPH* cDNA, previously described analyses were used [46]. *DMT1* analysis was performed on the *DMT1(IRE)* variants because these are the isoforms that are regulated by iron status in cell [47]. All data were normalized to the amount of *GAPDH* cDNA in the sample. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression.

Western blot analysis

Because of the limited amount of biological material, Western blot analyses were performed only on duodenal iron transporters and coupled oxido-reductases (DMT1, ferroportin, DCYTB and hephaestin). After protein extraction from duodenal biopsy by using the RIPA buffer (Sigma-Aldrich) and determination of concentration, seven samples were found to be inadequate for further analysis with Western blot. A

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small amount of tissue, obtained by biopsy, was detected in two samples; therefore, the Western blot analysis could only be performed for hephaestin and DMT1 detection in these samples.

Western blot analyses of DMT1 (IRE variants), DCYTB, ferroportin, hephaestin and actin (loading control) levels were performed using goat polyclonal antibodies NRAMP 2 and Hephaestin against human DMT1 and hephaestin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-Cytochrome b reductase 1 antibody against human DCYTB (Everest Biotech, Upper Heyford, UK), rabbit polyclonal antibody MTP11-A against human ferroportin (Alpha Diagnostic International, San Antonio, TX, USA) and mouse monoclonal antibody against human actin (Sigma-Aldrich). Analysis was carried out as previously described [44]. Briefly, proteins separated using SDS-PAGE were blotted onto a 0.2 μm nitrocellulose membrane for 2 hrs at 0.25 A, by using a Mini-Protean II blotting apparatus (Bio-Rad, Hercules, CA, USA). The membrane for DMT1, ferroportin, DCYTB and hephaestin was blocked with 5% BSA in TBS (100 mM Tris-HCl, 150 mM NaCl, pH = 7.5), whereas, for actin, 5% non-fat milk in TBS was used. The washed membrane was incubated with the relevant primary antibody. After incubation (overnight, 4°C), the washed membrane was incubated for 2 hrs with the corresponding horseradish peroxidase-conjugated secondary antibody, which was then detected by using enhanced chemiluminescence with Supersignal reagent (Pierce, Rockford, IL, USA) and a LAS 1000 CCD device (Fuji; Fujitsu Limited, Tokyo, Japan). Band intensities were quantified by densitometry and the data were analysed by using ImageJ software (version 1.42q; NIH, USA, available on http://rsb.info.nih.gov/ij/).

ELISA

To evaluate the role of hepcidin, a subgroup of 24 individuals, whose serum was available for hepcidin analysis, was created. It represented samples from eight controls and 16 ALD patients, which were further divided into the following subgroups: ALD with anaemia ($N = 8$), without overload ALD ($N = 6$), and ALD with iron overload ($N = 2$). Mature bioactive hepcidin was measured in serum samples by using a commercial ELISA (EIA-4705) kit (DRG International Inc., Springfield, NJ, USA) according to the manufacturer's instructions. In both patients and controls, blood sample was drawn between 7.30 and 8.30 a.m. after overnight fasting.

Statistical analysis

Data are expressed as mean ± SEM. Because of non-normality of the measured variables, the non-parametric Mann–Whitney test or the Kruskal–Wallis ANOVA with multiple comparison tests were used as needed. Correlations were assessed using the Spearman rank method. *P* value less than 0.05 was considered significant. Statistical analyses were performed using the Statistica program (Version 9; StatSoft, Tulsa, OK, USA) and the GraphPad Prism program (Version 5.00; GraphPad Software, Inc., San Diego, CA, USA).

Results

Clinical and laboratory characteristics of patients

Laboratory parameters of controls and ALD group are shown in Table 1. DNA genotyping showed that among ALD patients, one was found to be a C282Y/H63D compound heterozygote, one was a H63D homozygote, three were H63D heterozygotes and one was a S65C heterozygote. Tested controls were wild-type for all three tested mutations. In further analyses, gene expression values of individuals with *HFE* gene mutations were not outliers or extremes and were within the non-outlier range of values measured in their respective subgroups.

RNA expression

Gene expression at the mRNA level was measured for *DMT1*, *FPN1*, *DCYTB*, *HEPH*, *HFE* and *TFR1*. A significant increase in the ALD group was found when *DMT1*, *FPN1* and *TFR1* were examined (2.51-fold, $P = 0.0147$, 1.54-fold, $P = 0.0342$, and 2.02-fold, $P = 0.0011$, respectively). Gene expression of the other tested molecules (*DCYTB*, *HEPH* and *HFE*) was not significantly different in ALD patients compared to controls (Fig. 1A).

To study the ALD group in more detail, we divided the group into three subgroups: (*i*) patients with the signs of iron overload, (*ii*) patients without iron overload and (*iii*) patients with anaemia according to parameters defined in Materials and Methods. A significant increase in *DMT1* and *FPN1* mRNA was found in the ALD subgroup without iron overload compared to ALD with iron

Table 1 Clinical characteristics and iron metabolism phenotype in patients and controls

Variable	ALD ($N = 24$)	Controls ($N = 30$)	*P*-value
Age (years)	57.38 ± 1.57	57.40 ± 2.85	0.4431
Gender	17M/7F	18M/12F	0.5673
Serum iron (μmol/l)	18.03 ± 1.94	17.55 ± 0.67	0.6240
Serum ferritin (μg/l)	296.22 ± 96.11	151.0 ± 13.83	0.9640
Transferrin saturation (%)	32.22 ± 4.88	31.22 ± 0.96	0.4455
Hb (g/dl)	12.66 ± 0.44	14.00 ± 0.31	0.0138
Ht (%)	37.16 ± 1.16	41.48 ± 0.93	0.0058
ALT (μkat/l)	0.62 ± 0.05	0.54 ± 0.05	0.0478
AST (μkat/l)	0.81 ± 0.09	0.47 ± 0.03	0.0003

Data are presented as arithmetic mean ± SEM. Hb: haemoglobin; Ht: haematocrit; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Normal ranges: serum iron (11–26 μmol/l), serum ferritin (male 30–250 μg/l, female 30–200 μg/l), transferrin saturation 20–45%, Hb (male 13.0–18.0 g/dl, female 11.5–16.0 g/dl), Ht (male 38–54%, female 35–47%), ALT (0.1–0.75 μkat/l) and AST (0.1–0.75 μkat/l).

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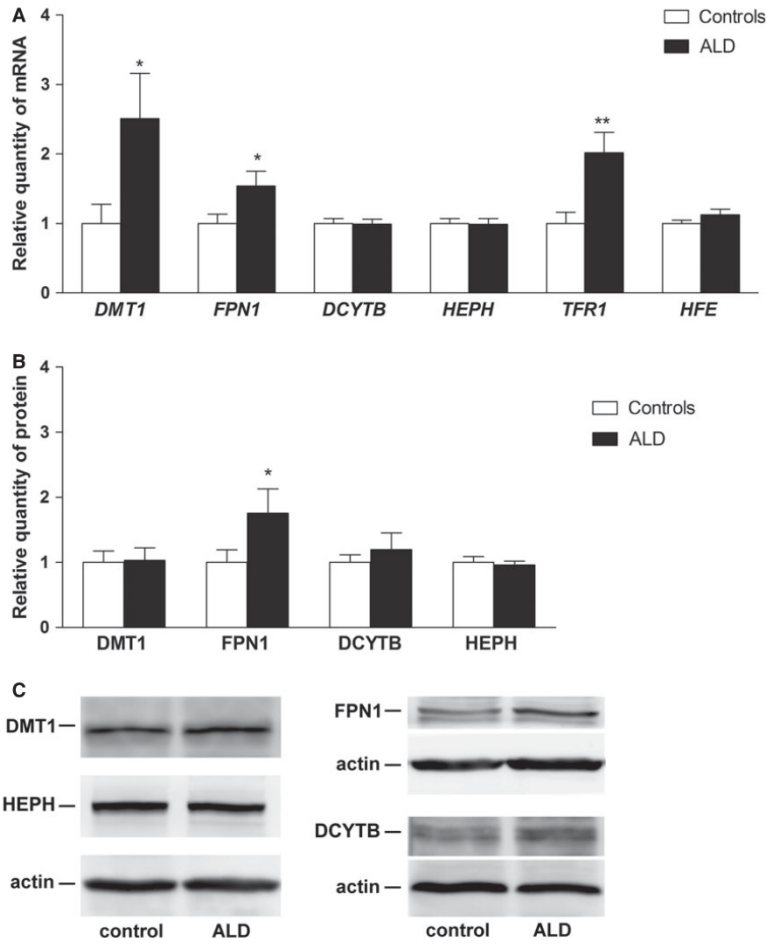

Fig. 1 Gene expression of the analysed molecules in controls and patients with alcoholic liver disease (ALD). (**A**) mRNA expression of *DMT1*, *FPN1*, *DCYTB*, *HEPH*, *TFR1* and *HFE*. (**B**) Protein expression of DMT1, FPN1, DCYTB and HEPH. (**C**) Western blot analysis of DMT1, FPN1, DCYTB, HEPH and actin (loading control). Results are depicted as means ± SEM. Statistically significant differences as compared with the control group are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

overload (3.02 *versus* 0.34, $P = 0.0312$ and 1.72 *versus* 0.70, $P = 0.0420$, respectively). Moreover, a significant elevation in *DMT1* and *TFR1* mRNA expression was observed in ALD patients without iron overload compared to controls (3.02-fold, $P = 0.0479$, and 1.91-fold, $P = 0.0066$, respectively). Although *FPN1* gene expression was also increased compared to controls, the increase did not reach statistical significance (1.72-fold, $P = 0.0599$). When the ALD subgroup with anaemia was examined, a significant increase in *DMT1*, *FPN1* and *TFR1* mRNA levels was observed compared with controls (3.50-fold, $P = 0.0018$, 1.93-fold, $P = 0.0115$ and 2.84-fold, $P = 0.0075$, respectively). The same applies to comparison of the ALD anaemia subgroup to the ALD iron overload patients when *DMT1* and *FPN1* mRNA were analysed (3.50 *versus* 0.34, $P = 0.0007$ and 1.93 *versus* 0.70, $P = 0.0080$, respectively). Additionally, the *HFE* mRNA level was elevated in ALD patients with iron overload compared with controls (1.40-fold, $P = 0.0376$) (Fig. 2A–F).

Protein expression

To investigate whether the changes in mRNA expression correlated with changes in protein expression, we examined DMT1, ferroportin, DCYTB and hephaestin levels by using Western blotting.

There was a significant increase in ferroportin protein expression in ALD patients compared with controls (1.76-fold, $P = 0.0495$). Unchanged *DCYTB* and *HEPH* mRNA expression was paralleled by unchanged respective protein expression (1.20-fold, $P = 0.8292$ and 0.96-fold, $P = 0.8898$, respectively). DMT1 protein expression was not different in the ALD group compared with controls (1.03-fold, $P = 0.3879$) (Fig. 1B). Further analysis of the ALD group, divided based on iron overload or anaemia, revealed no significant differences in protein expression. The ferroportin protein expression had the same pattern as seen at the mRNA level; however, the significance found in the ALD group was lost

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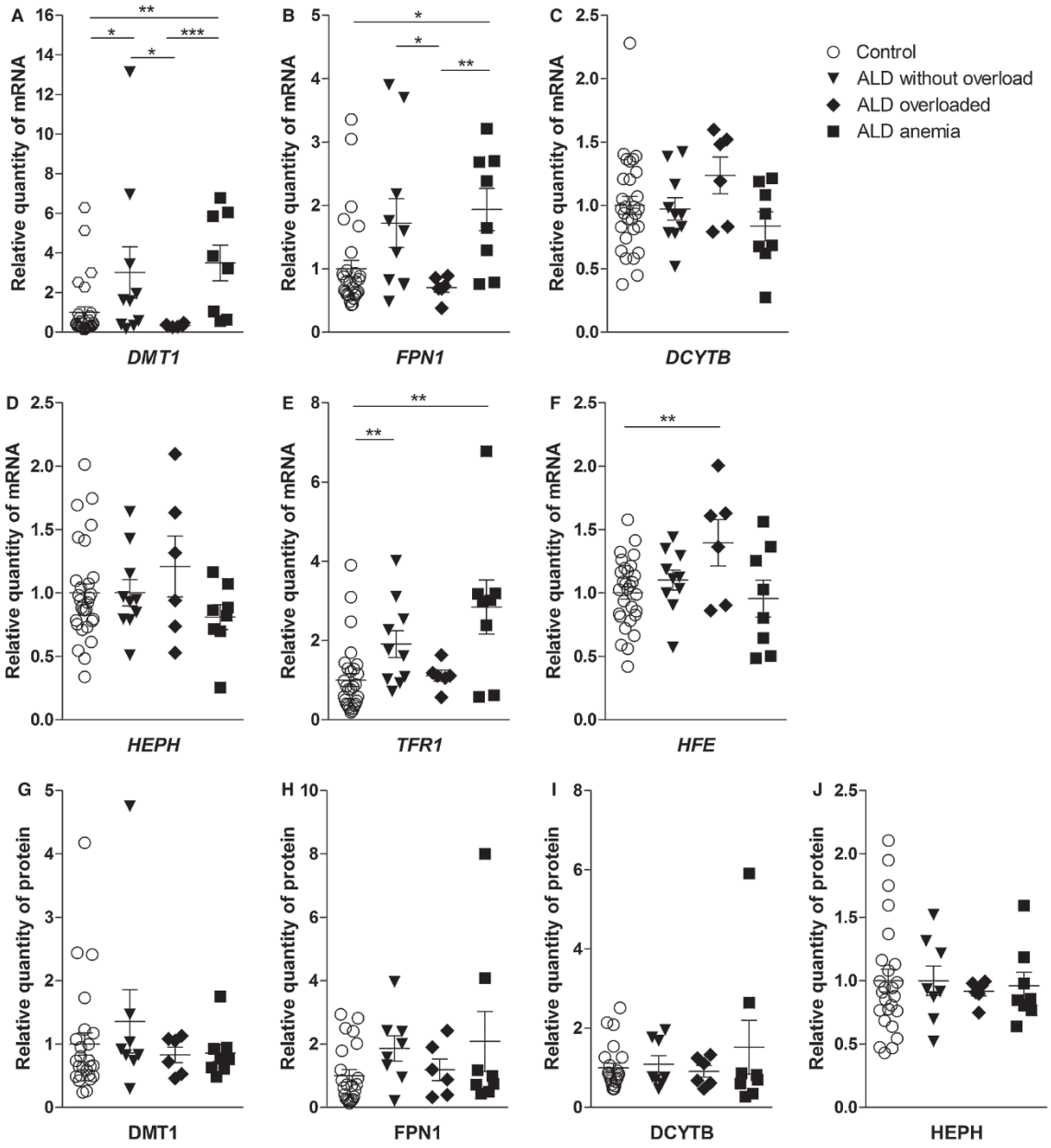

Fig. 2 Gene expression of the analysed molecules in controls, patients with alcoholic liver disease without iron overload (ALD without overload), with iron overload (ALD overloaded), and anaemia (ALD anaemia). (**A**) mRNA expression of *DMT1*, (**B**) *FPN1*, (**C**) *DCYTB*, (**D**) *HEPH*, (**E**) *TFR1*, (**F**) *HFE*. (**G**) Protein expression of DMT1, (**H**) FPN1, (**I**) DCYTB, (**J**) HEPH. Results are depicted as means ± SEM. Statistically significant differences are indicated by $*P < 0.05$, $** P < 0.01$ and $*** P < 0.001$.

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and the increase in ferroportin protein in ALD patients without iron overload and anaemia did not reach statistical significance compared with controls (1.90-fold, $P = 0.0746$ and 2.08-fold, $P = 0.1685$, respectively; Fig. 2G–J).

Relationships among gene expression of tested molecules

Relationships among DMT1, FPN1, DCYTB, HEPH, TFR1 and HFE expression were analysed by using the Spearman rank correlation. When all individuals regardless of underlying disease were evaluated, there was a positive association among mRNA expression of the tested molecules. The strongest correlation was found between *DMT1* and *FPN1* ($r = 0.778$, $P < 0.0001$), between *DCYTB* and *HEPH* ($r = 0.687$, $P < 0.0001$) and between *DMT1* and *TFR1* ($r = 0.660$, $P < 0.0001$; Table 2).

When the gene expression, at the protein level, in the cohort of all individuals was investigated, a positive relationship between the following proteins was found: DCYTB and ferroportin ($r = 0.632$, $P < 0.0001$), DMT1 and hephaestin ($r = 0.580$, $P < 0.0001$), ferroportin and hephaestin ($r = 0.482$, $P = 0.0008$), DCYTB and hephaestin ($r = 0.391$, $P = 0.0080$) and DMT1 and DCYTB ($r = 0.314$, $P = 0.0358$; Table 3).

When all individuals were analysed together, a correlation between gene expression at the mRNA level and the protein level was found only for hephaestin ($r = 0.338$, $P = 0.0215$).

When correlations in ALD and controls were investigated separately, positive relationships were found, and are summarized in Tables 2 and 3.

Table 2 Associations among analysed molecules—mRNA level

	mRNA				
	HFE	***TFR1***	***HEPH***	***DCYTB***	***FPN1***
DMT1					
ALD	ns	0.613**	ns	ns	0.863***
Controls	ns	0.634***	ns	ns	0.683***
All	ns	0.660***	ns	ns	0.778***
FPN1					
ALD	ns	0.593**	ns	ns	
Controls	ns	0.523**	0.593***	0.364**	
All	ns	0.591***	0.447***	0.322*	
DCYTB					
ALD	0.600**	ns	0.570**		
Controls	ns	ns	0.790***		
All	0.468***	ns	0.687***		
HEPH					
ALD	ns	ns			
Controls	0.414*	ns			
All	0.399**	0.315*			
TFR1					
ALD	ns				
Controls	0.604***				
All	0.452***				

Spearman rank correlation, only statistically significant data are shown, statistically significant differences are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, ns - not significant.

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Table 3 Associations among analysed molecules—protein level

	Proteins			
	HEPH	**DCYTB**	**FPN1**	**Hepcidin**
DMT1				
ALD	0.792***	0.471*	ns	ns
Controls	0.490*	ns	ns	ns
All	0.580***	0.314*	ns	ns
Hepcidin				
ALD	ns	ns	ns	
Controls	ns	ns	ns	
All	ns	ns	ns	
FPN1				
ALD	0.428*	0.719***		
Controls	0.580**	0.601**		
All	0.482***	0.632***		
DCYTB				
ALD	0.485*			
Controls	ns			
All	0.391**			

Spearman rank correlation, only statistically significant data are shown, statistically significant differences are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, ns - not significant.

Relationships among gene expression of tested molecules and iron parameters

The association between gene expression of the analysed molecules and serum iron parameters was also tested. When all participants were examined together, a correlation was found between serum ferritin and the mRNA of *DMT1* ($r = -0.484$, $P = 0.0038$) and *FPN1* ($r = -0.447$, $P = 0.0080$). Serum iron correlated at the mRNA level with *FPN1* ($r = -0.344$, $P = 0.0373$). The same applies to the correlation between transferrin saturation and mRNA: *DMT1* ($r = -0.451$, $P = 0.0108$), *FPN1* ($r = -0.432$, $P = 0.0153$) and *TFR1* ($r = -0.384$, $P = 0.0328$). There were no correlations between *DCYTB*, *HEPH* and *HFE* and any of tested iron parameters. When protein expression was examined in the cohort of all participants, no correlation was found between any protein or iron parameter.

With respect to ALD patients analysed separately, there were relationships between serum ferritin and *DMT1* mRNA ($r = -0.465$, $P = 0.0252$) and *FPN1* mRNA ($r = -0.448$, $P = 0.0318$). Transferrin saturation correlated with *DMT1* mRNA ($r = -0.465$, $P = 0.0289$) and serum iron with *TFR1* mRNA ($r = -0.414$, $P = 0.0495$) in ALD patients. Among controls, we found inverse relationship of serum ferritin and *DMT1* mRNA expression ($r = -0.772$, $P = 0.0053$) and ferroportin and DCYTB protein expression ($r = -0.700$, $P = 0.0358$, $r = -0.766$, $P = 0.0159$, respectively). Serum iron correlated with DMT1 protein expression ($r = -0.641$, $P = 0.0182$), and transferrin saturation correlated with DMT1 protein expression ($r = -0.910$, $P = 0.0017$).

Transferrin saturation *versus* serum ferritin and transferrin saturation *versus* serum iron were correlated with each other in all individuals ($r = 0.747$, $r = 0.863$, $P < 0.0001$ for both comparisons listed). The same also applies to correlations between serum iron *versus* serum ferritin ($r = 0.500$, $P = 0.0031$).

Hepcidin analysis

To evaluate the regulation of iron metabolism in more detail, the relationship of serum hepcidin and expression of analysed molecules was studied as well. The sera for hepcidin measurements were available from 24 individuals (ALD $N = 16$, controls $N = 8$). A significant decrease in serum hepcidin was observed in ALD patients compared with controls (23.16 ng/ml *versus* 36.93 ng/ml, $P = 0.0010$) (Fig. 3A). When the ALD group was divided based on iron overload or anaemia, significant changes in serum hepcidin levels were found in all ALD subgroups compared with controls: ALD patients without iron overload (22.67 ng/ml *versus* 36.93 ng/ml, $P = 0.0081$) and ALD patients with anaemia (24.30 ng/ml *versus* 36.93 ng/ml, $P = 0.0027$; Fig. 3B). Because of small number of samples in the ALD subgroup with iron overload ($N = 2$), this subgroup was not evaluated, although decreased hepcidin levels were also detected in this group (20.10 ng/ml). Interestingly, when these subgroups were compared to each other, no differences were detected (Fig. 3B). The relationship between serum hepcidin level and gene expression both at the RNA and protein levels was analysed, but no correlation was found. In addition, hepcidin levels did not correlate with any of the measured iron parameters regardless of data analysis techniques or grouping methods, *i.e.* ALD and controls separately or all participants taken together.

Discussion

In this study, iron metabolism in alcoholic patients with normal iron indices, iron overload and anaemia was examined. Gene expression of molecules participating in iron absorption in duodenum (DMT1, ferroportin, DCYTB and hephaestin) was analysed both at mRNA and protein levels. Additionally, *TFR1* and *HFE* mRNA expression was investigated. Serum hepcidin, a key regulator of iron metabolism, was evaluated as well. This study is, to our knowledge, the first to investigate duodenal gene expression of iron transport molecules both at the mRNA and protein level, together with the serum hepcidin level in ALD patients.

The effect of alcohol consumption on hepcidin expression has been previously demonstrated [40, 41]. It has been shown, by using animal models, that alcohol down-regulates hepcidin expression,

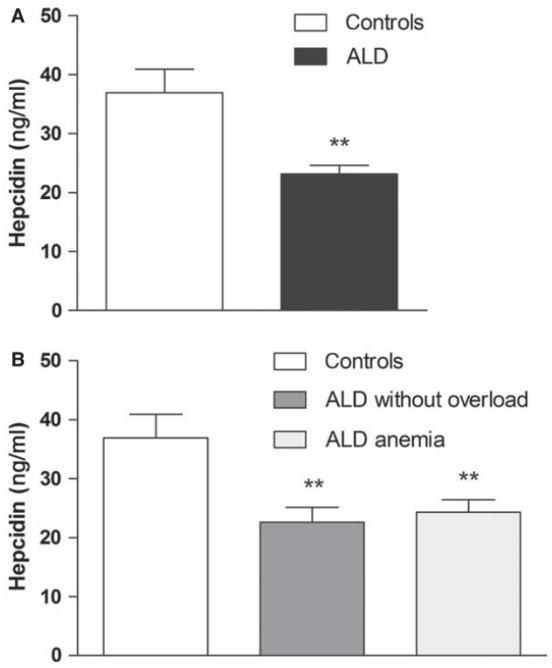

Fig. 3 Serum hepcidin levels (**A**) in controls and patients with alcoholic liver disease (ALD) (**B**) in controls, patients with alcoholic liver disease without iron overload (ALD without overload) and anaemia (ALD anaemia). Results are depicted as means ± SEM. Statistically significant differences as compared with the control group are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

which affects ferroportin and DMT1 and leads to increased iron absorption in the duodenum. It has been documented that ethanol down-regulates hepcidin promoter activity and DNA binding activity of transcription factor C/EBPα [40, 41]. In the present study, hepcidin levels were decreased in ALD patients compared with controls, supporting the abovementioned mechanism of alcohol on hepcidin expression in humans. We found a significant elevation of FPN1 at both the mRNA and protein level and an increase in *DMT1* and *TFR1* mRNA in ALD patients. However, the elevation of *DMT1* mRNA was not paralleled by the elevation of DMT1 protein. It could be speculated that some post-transcriptional mechanisms are involved *e.g.* IRE/IRP system [48], Ndfips/WWP2 system responsible for ubiquitination and degradation of DMT1 [49], or PAP7 which inhibition was shown to cause a reduction in the expression of DMT1 (IRE) protein but not mRNA [50]. The regulation by miRNA can also be considered but the only miRNA documented to affect DMT1 acts on the non-IRE variant [51]. Also, the heterogeneity of patients' genetic background or the small sample size can be the reason of this discrepancy. Further analysis of the ALD subgroups showed that at the mRNA level, there was a significant increase in *DMT1* and *FPN1* in ALD patients without iron overload compared with ALD with iron overload and a significant increase in *DMT1* and *TFR1* mRNA when compared with controls. Additionally, hepcidin levels in these subgroups were significantly lower compared with controls. Thus, it can be hypothesized that in ALD patients, chronic alcohol consumption decreases hepcidin levels, which results in up-regulation of *DMT1* and *FPN1* mRNA in patients with yet normal iron indices. It could be expected that when iron stores become increased, the iron burden is sensed to be inappropriately high by the 'iron status' hepcidin regulatory pathway (probably HJV/BMP and/or HFE/TFR2 pathway) and decreased hepcidin levels tend to normalize to compensate iron absorption. This would lead to suppression of increased *DMT1* and *FPN1* mRNA levels in ALD patients with iron overload as seen in our study. However, we did not detect this normalization of hepcidin levels in the ALD iron overload subgroup, probably because of limited number of serum samples; thus we can only speculate at this point. It can be argued that these two factors—alcohol, which tends to decrease hepcidin expression and physiological regulation, which tends to compensate for high iron stores by increasing hepcidin expression, act on various regulatory pathways and this interaction finally establishes some sort of balance; an analogous situation has been documented in treated and untreated haemochromatosis patients [52–54]. This is in agreement with another study where the effect of alcohol together with iron was investigated [42]. In the study, animal models were used and the results showed that iron elevated and alcohol decreased liver hepcidin expression. Alcohol was shown to suppress up-regulation of *hepcidin* mRNA in iron-overloaded rats to levels similar to those in control animals. The duodenal ferroportin expression was elevated in alcohol-treated mice. When both factors were investigated together duodenal ferroportin expression was increased and reached levels between alcohol-treated and iron-treated mice. In addition, the iron-induced increases in the DNA binding activity of C/EBPα were diminished by alcohol to levels found in controls. Similar observations using *HFE* knockout mice were documented in another study [43], which also suggested that alcohol decreases hepcidin expression independently of the HFE pathway, possibly by alcohol-induced hypoxia.

We also investigated ALD patients with anaemia. The pathogenesis of anaemia in ALD is complex: it may include splenic pooling and haemolytic anaemia caused by hypersplenism; chronic bleeding into the gastrointestinal tract resulting in iron deficiency; secondary malnutrition, leading to anaemia with folic acid deficiency; anaemia as a consequence of the direct toxic effect of alcohol on erythrocyte precursors in bone marrow [10]. These symptoms lead to hypoxia, anaemia and iron deficiency, which all inhibit hepcidin synthesis *via* several pathways and corresponding mediators: hypoxia inducible factor α, erythropoietin (EPO), growth differentiation factor 15 and twisted gastrulation protein homologue 1. In iron deficiency, the regulation pathway for iron status includes activity of the BMP/HJV and HFE/TFR2 complex [55, 56]. On the other hand, anaemia of chronic disease can occur in patients with chronic ALD [10], when hepcidin expression is induced by inflammatory stimuli (*e.g.* IL-6) [55, 56]. Finally, with chronic alcohol consumption, the effect of ethanol on hepcidin promoter activity and the DNA binding activity of transcription factor C/EBPα, must also be considered [41]. All these factors may have played a role in our ALD patients with anaemia and the final level of hepcidin represented the combined effect of all these various pathways. We also found decreased hepcidin level in our ALD patients

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with anaemia, which was consistent with the abovementioned facts. The effect of increased hepcidin synthesis because of anaemia of chronic disease seems to be minor, especially when the erythroid demand for iron is thought to be a more powerful regulator of hepcidin expression than inflammation [57]. Consequently, these patients displayed increased mRNA expression of *DMT1* and *FPN1* in the duodenum, demonstrating the efforts of the organism to increase iron absorption in response to iron needs associated with enhanced erythropoiesis. However, these elevations were not detected at the protein level of DMT1 and FPN1. Although the expression of FPN1 protein was increased, it did not reach statistical significance. Surprisingly, serum hepcidin levels were similar in ALD patients with anaemia (when the effect of anaemia plus ethanol affects hepcidin synthesis) and without anaemia (when only ethanol is implicated). We were not able to distinguish between the effect of erythropoietic stimulation, iron deficiency and ethanol when analysing the combined effect of all factors playing a role in the pathogenesis of anaemia in our ALD patients. It seems that the effect of various mediators on hepcidin expression pathway is not simply additive, and crosstalk among the different pathways, can be hypothesized, to influence different signal transduction intensities. On the other hand, EPO has been proposed to act directly to repress hepcidin through EPOR-mediated regulation of C/EBPα [58], which is also affected by ethanol. It could be possible that this pathway can suppress hepcidin synthesis, but only to some limit; and once the C/EBPα is influenced by a mediator, others are unable to exert their full influence. However, the small sample size of our associated ALD subgroup did not permit definite conclusions in this respect.

TFR1 mRNA was increased in ALD patients with anaemia and without iron overload. The increase of *TFR1* in ALD patients without overload cannot be explained by iron deficiency and anaemia as in the ALD anaemia subgroup [59], thus the effect of ethanol needs to be considered. It has been shown that ethanol exposure can increase the expression of TFR1 in hepatocytes [60, 61]. The increase in TFR1 expression is partially because of the increased activity of iron regulatory proteins (IRPs) linked to the oxidative stress of ethanol metabolism [60]. It can be speculated that a similar mechanism is implicated in duodenal cells; however, this mechanism has yet to be elucidated. In addition, if ethanol can affect *TFR1* mRNA expression in duodenal cells it may also play a role in the expression of other iron transport molecules.

We detected a strong positive correlation between *DMT1* and *FPN1*; *DCYTB* and *HEPH*; and *DMT1* and *TFR1* at the mRNA level, which suggests a coordinated regulation of these genes. We also investigated the association between serum iron parameters and duodenal iron transporters. Significant inverse correlations between *DMT1* mRNA and serum ferritin, in controls and ALD patients, were detected confirming that body iron stores play a role in the regulation of duodenal expression of this transporter. With regard to controls, our observations are in agreement with several studies [54, 62]; however, there were other studies that did not find any correlation at all [52, 53].

In conclusion, our results demonstrate that serum hepcidin levels are decreased by alcohol consumption resulting in increased expression of iron transporter ferroportin at the mRNA and protein level and *DMT1* at the mRNA level in duodenum of ALD patients. Detailed analyses revealed that these changes were observed in ALD patients without iron overload and ALD patients with anaemia. The increase in duodenal *TFR1* mRNA expression is a consequence of anaemia and probably also an effect of ethanol on duodenal cells. Positive correlations among *DCYTB*, *HEPH*, *DMT1*, *FPN1* and *TFR1* mRNA indicate coordinated regulation of these genes. Further research is required to elucidate the complex pathogenesis of ALD and the effect of ethanol and oxidative stress derived from its metabolism in duodenal cells deserves further investigation.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Author contribution

Dostalikova-Cimburova M., Kratka K., Kovar J., and Horak J. designed the research study; Dostalikova-Cimburova M., Balusikova K., Chmelikova J., and Neubauerova J. performed the research (performed molecular analyses); Kratka K., Hejda V., Hnanicek J. performed the research (examined patients, collected the clinical data and biological material); Dostalikova-Cimburova M. and Vranova J. analysed the data; Dostalikova-Cimburova M. wrote the paper; Kratka K., Balusikova K., Chmelikova J., Hejda V., Hnanicek J., Neubauerova J., Vranova J., Kovar J., and Horak J. critically revised the manuscript.

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4. UNPUBLISHED DATA AND FURTHER RESEARCH

Besides the data included in our papers, we have some additional unpublished data concerning the iron transport and metabolism. Our planned further studies represent a natural continuation of previous studies presented in this thesis.

4.1. STUDIES *IN VITRO*

4.1.1. IRON OVERLOAD EFFECT ON CELL DAMAGE AND DEATH

High iron levels in cells and iron accumulation in organism may arise from various causes (Cox and Peters, 1980). Surplus iron is stored particularly in cells of parenchymatic organs such as the pancreas, heart, and liver and can lead to the reduction of cellular functions, cell damage or even cell death and serious organ injury (Britton *et al.*, 1994; Hower *et al.*, 2009). Various cell types have a similar basic mechanism of iron-caused cell damage due to iron's ability to produce ROS followed by oxidative stress and probably reduced antioxidant enzymes levels in cells (Cooksey *et al.*, 2004; McClain *et al.*, 2006; Bresgen *et al.*, 2010). However, the current knowledge suggests that iron-induced endoplasmic reticulum (ER) stress could play a role in apoptosis induction in hepatocytes and cardiomyocytes (Lou *et al.*, 2009).

Although the mechanism of cell death induction resulting from iron accumulation is not fully elucidated yet, only a limited number of studies are currently dealing with this

topic. In our recent project, employing experiments with mammalian cell lines, we are testing the activation of pathways of cell death induction due to iron overload in cell models of the above mentioned tissue types, i.e. NES2Y cells (pancreatic β-cells), H9c2 cells (cardiomyocytes), and HEP-G2 cells (hepatic cell line). Iron overload is simulated by increased ferric citrate levels in the culture media of tested cells.

Our pilot data confirmed that high levels of iron in culture medium results in increased iron intake into cells and leads to the formation of reactive oxygen species and oxidative stress. Additionally, persistent oxidative stress can subsequently result in ER stress and autophagy activation. Unless the high iron levels are sufficiently compensated, cellular damage becomes more severe and the cell undergoes cell death. Some preliminary data concerning NES2Y cells are shown in Fig. 4.1.

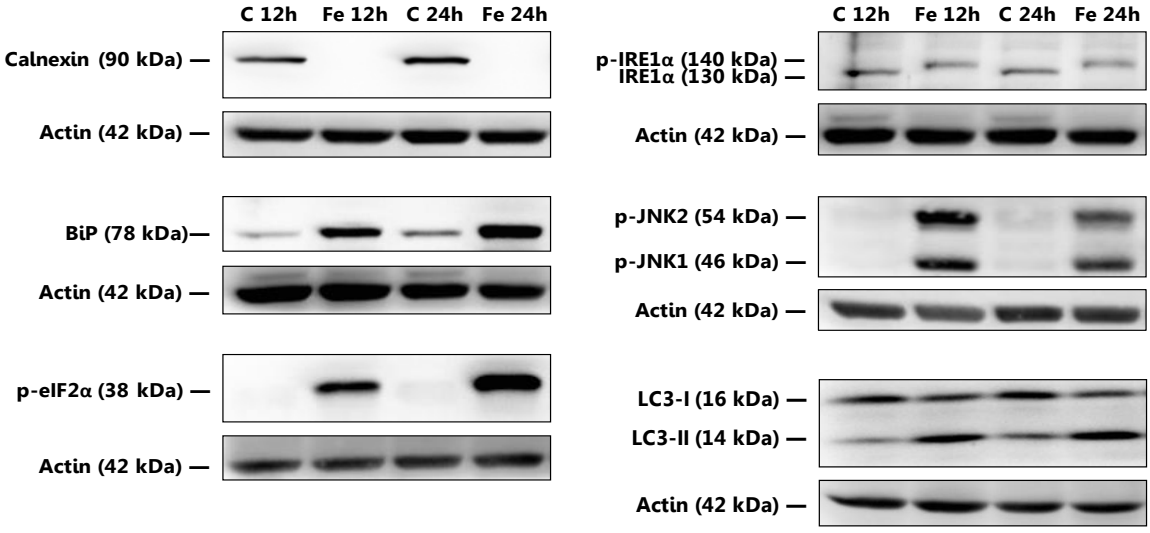

Fig. 4.1. Protein levels of ER stress markers (Calnexin, BIP, p-eIF2α, p-IRE1α, p-JNK) and autophagy marker (LC3) in NES2Y cells. Protein levels in control cells (C) and cells after 12 h and 24 h incubation with 20 μM ferric citrate (Fe) were determined using western blot analysis. Actin levels were used as a loading control. One representative western blot analysis is shown.

4.1.2. ETHANOL EFFECT ON IRON HOMEOSTASIS

In relation to a previous study, we wanted to focus on the mechanism of secondary iron overload and the cell damage due to the ethanol abuse. It seems that ethanol exposure affects iron homeostasis predominantly via changed expression of iron-related proteins regulated by the redox status of the cell (Suzuki *et al.*, 2002; Harrison-Findik, 2007). Prolonged effect of ethanol-induced oxidative stress together with increased iron levels, however, can lead to other consequences such as ER stress (Chen *et al.*, 2008). ER stress is induced via disruption of ER homeostasis and accumulation of misfolded proteins in the ER lumen. Increased amount of defective proteins with subsequent increased expression of molecular chaperons and the activation of signaling pathways can cause cell death induction. On the other hand, cells can adapt to the presence of mild ER stress via permanently induced expression of chaperones. Recent studies suggest that cellular iron homeostasis can be affected by ER stress through the regulation of hepcidin (Vecchi *et al.*, 2009) (summarized in Fig. 4.2.).

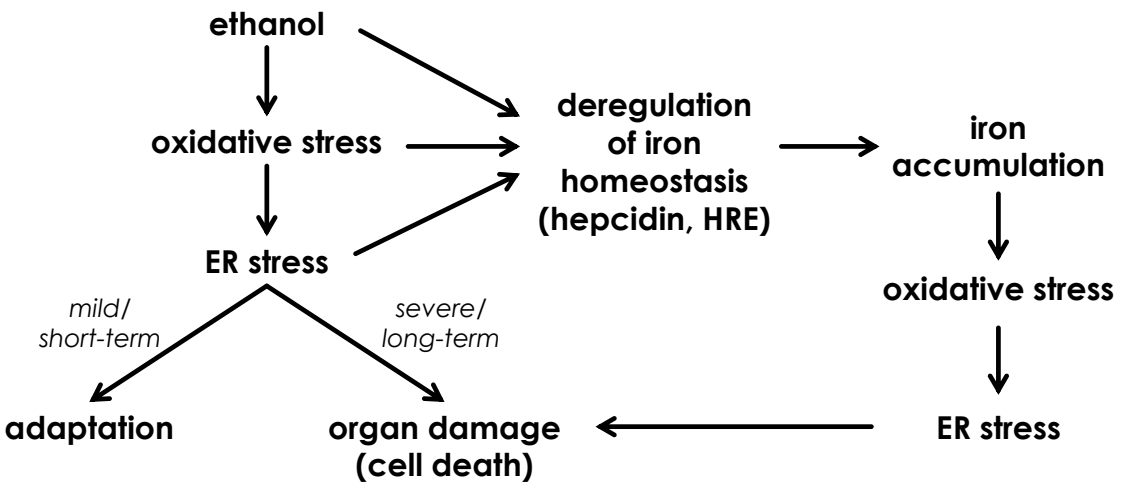

Fig. 4.2. Scheme of ethanol effect on iron homeostasis and cell injury.

Since ethanol absorption into the blood takes place mainly in the stomach, small intestines, and colon, and since the main metabolism site of ethanol is the liver, we focused on enterocytes and hepatocytes in our experiments. For this purposes, HEP-G2 and Caco-2 cell lines cultivated under log-term ethanol exposure were established in our laboratory (Balusikova and Kovar, 2013). Some preliminary data are shown in Fig. 4.3.

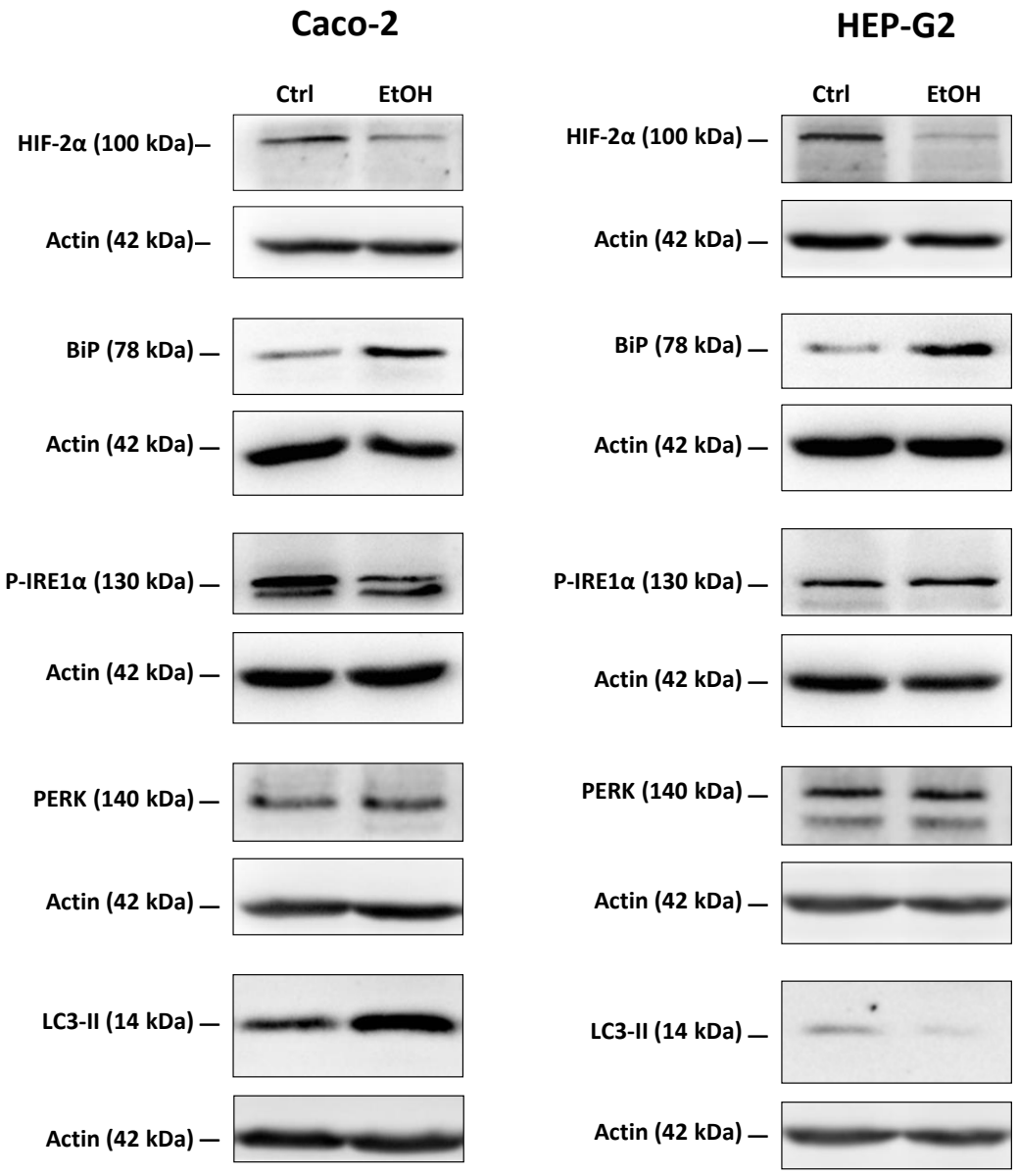

Fig. 4.3. Protein levels of HIF-2α, ER stress markers (BIP, p-IRE1α, PERK) and autophagy marker (LC3B) in Caco-2 and HEP-G2 cells. Protein levels of control cells (Ctrl) and cells after long-term incubation with ethanol (EtOH) (Balusikova and Kovar, 2013) were determined using western blot analysis. Actin levels were used as a loading control. One representative western blot analysis is shown.

4.2. STUDIES *IN VIVO*

4.2.1. HEME-IRON UPTAKE BY DUODENAL ENTEROCYTES IN IDA, HHC AND ALD PATIENTS

As with our former studies, we decided to test the expression of heme transport and metabolism related molecules in duodenal biopsies of the previously discussed group of patients (see paper 3 and 4). Therefore, the expression of heme carrier protein 1 (HCP1) and heme oxygenase molecules (HO-1 and HO-2) was analyzed in patients with iron deficiency anemia (IDA), hereditary hemochromatosis (HHC), alcoholic liver disease (ALD), and controls. Only mRNA levels of target molecules were tested until now and no significant changes in HCP1 expression was found in any of the groups of patients compared to controls (Fig. 4.4.a). The expression of HO-1 was decreased in HHC patients (Fig. 4.4.b) whereas HO-2 mRNA levels were increased in patients with ALD (Fig. 4.4.c). Analysis of protein levels are in progress.

4.2.2. CELLULAR REGULATION OF IRON UPTAKE BY DUODENAL ENTEROCYTES IN IDA, HHC AND ALD PATIENTS

In relation to a planned analysis as described in earlier chapter and previously discussed results, we planned to analyze expression of molecules involved in cellular regulation of iron homeostasis in enterocytes of patients with iron-related disorders (see paper 3 and 4). In order to complete our insight into the complex mechanisms of iron absorption into the body by the intestine, we are testing in ongoing analysis the expression of molecules involved in iron uptake regulation such as transferrin receptor 2 (TfR2), iron regulatory protein 1 and 2 (IRP1 and IRP2), hypoxia-inducible factor 1α, 2α, and 1β (HIF-1α, HIF-2α, HIF-1β), von Hippel-Lindau tumor supressor (VHL), prolyl hydroxylase domain (PHD), and others.

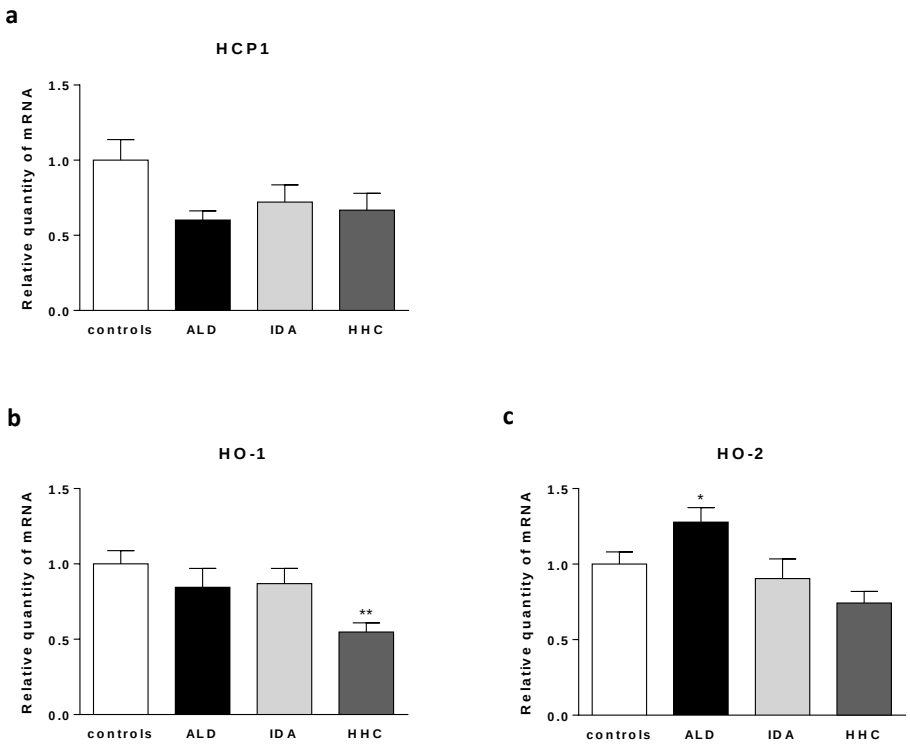

Fig. 4.4. Relative mRNA level of heme carrier protein (HCP1) (**a**), hemoxygenase-1 (HO-1) (**b**) and hemoxygenase-2 (HO-2) (**c**) in patients with alcoholic liver disease (ALD), iron deficiency anemia (IDA) and hereditary hemochromatosis (HHC) compared to controls. The relative quantity of mRNA was assessed using real-time PCR with control group of patients as calibrator. The mean ± standard error of the mean (SEM) of each group of patients is shown. Statistical significance when compared to controls using Mann-Whitney nonparametric test: * $P < 0.05$, ** $P < 0.01$.

4.2.3. EXPRESSION OF IRON TRANSPORT-RELATED MOLECULES IN DIFFERENT LEVELS OF DUODENUM

Until recently, a detailed analyzes of the localization of iron uptake into the organism in intestine were performed on animal samples only (Conrad *et al.*, 1966; Raffin *et al.*, 1974; Muir and Hopfer, 1985; Chowrimootoo *et al.*, 1992). Therefore, we wanted to determine whether the expression of molecules involved in the absorption of iron into the body differs at individual levels of duodenum in humans. That is, whether there is a predilection site of iron absorption or whether there exists a gradient of that absorption in duodenum.

Analysis was performed with samples of duodenal mucosa obtained using duodenal biopsies from healthy controls (patients with normal iron metabolism parameters). Samples were collected from 10 patients at the following levels: closely post-bulbar, 1-2 cm below the papilla of Vater and at the distal duodenum. We analyzed the gene expression of molecules involved in iron transport to and from the enterocyte (DMT1, Dcytb, ferroportin, hephaestin, HCP1, HO-1 and -2) at the mRNA level using real-time PCR and at the protein level by western blot analysis.

At the mRNA level, significantly different expression of DMT1, ferroportin and HCP1 at individual positions of duodenum was found (Fig. 4.5.a-c). The DMT1 expression at post-bulbar position gradually decreased up to 73% at papilla of Vater and then fell to 62% at the distal duodenum. The expression of ferroportin decreased to 78% from post-bulbar position to the distal duodenum. On the other hand, the expression of HCP1 was increased to 125% at Vater papilla and to 181% at the distal duodenum when compared with post-bulbar position. For the other tested molecules, statistically significant differences were not detected. At the protein level, decreasing expression of transporters DMT1 and ferroportin at advancing positions along the duodenum were also observed (Fig. 4.5.d-g). In both cases, the changes were statistically significant but in comparison with the level of mRNA less pronounced. DMT1 as well as ferroportin protein levels decreased up to 74% and to 77% at the distal duodenum, respectively, compared to post-bulbar position. Protein levels of HCP1, HO-1 and HO-2 molecules were not tested yet. It seems there is a gradient of iron uptake into the body both at the level of iron absorption into the enterocytes as well as at the level of iron release from the enterocytes. Surprisingly, our results suggest that this gradient is the opposite comparing heme and non-heme iron absorption.

4.2.4. IRON TRANSPORT AND ITS REGULATION IN VEGAN POPULATION

People consuming food without any meat limitations have higher body iron stores compared with people which are not consuming animal food (vegans). Since body iron stores positively correlates with the intake of especially red meat, together with the fact

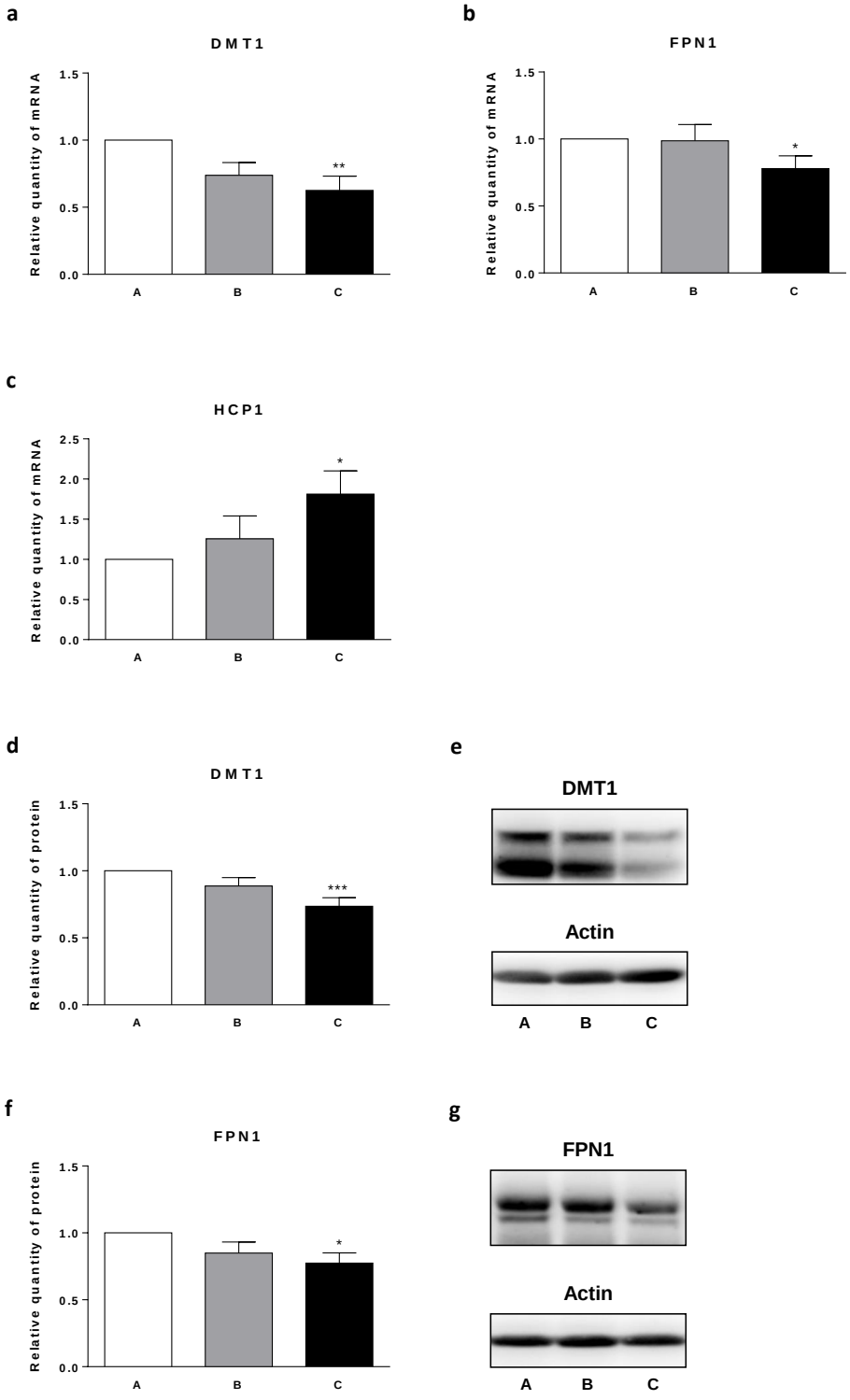

Fig. 4.5. Relative amount of divalent metal transporter 1 (DMT1), ferroportin (FPN1) and heme carrier protein (HCP1) on mRNA (**a**-**c**) and/or protein (**d** and **f**) levels at individual positions of duodenum of healthy controls: A – post-bulbal, B – papilla of Vater, C – distal duodenum. The relative quantity of mRNA and protein was assessed using real-time PCR and western blot analysis, respectively. Position A values were used as the calibrator. Actin levels were used as a loading control. One representative western blot analysis for DMT1 (**e**) and ferroportin (**g**) is shown. The mean ± standard error of the mean (SEM) of each group is shown. Statistical significance when compared to A using the Mann-Whitney nonparametric test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that higher iron stores positively correlate with insulin resistance, it can be assumed that the lower iron uptake in vegans is one of the factors increasing insulin sensitivity (Craig, 2010). A key hormone involved in iron homeostasis is hepcidin, which functions as a negative regulator of iron absorption by enterocytes and is also responsible for release of iron from the monocyte-macrophage system into circulation (Nicolas *et al.*, 2002a; Ganz, 2004; Nemeth *et al.*, 2004; Dunn *et al.*, 2007). However, to this time, no studies have tested the hepcidin levels in a population of vegans.

Our study included 57 vegans and 52 corresponding controls. Exclusion criteria were under 18 years of age and the presence of any chronic disease with an impact on the intermediary metabolism. Each subject underwent a collection of peripheral blood under basal conditions and the following parameters of iron metabolism and glucose homeostasis were determined in serum: hepcidin, pro-hepcidin, ferritin, iron binding capacity, transferrin, serum iron level, blood glucose, insulin, and C-peptide.

Concerning our results, a significant difference was found between the groups in the levels of hepcidin (vegans 5.72 ng/ml vs. control 9.63 ng/ml, $p < 0.0001$) (Fig. 4.6.a) and pro-hepcidin (vegans 288.0 ng/ml vs. control 431.5 ng/ml, $p < 0.0001$) (Fig. 4.6.b).

These results showed that vegans, compared with the controls, have lower serum hepcidin and pro-hepcidin levels. This corresponds to an effort by the organism to increase the intake of iron through enterocytes, but also increases the release of iron into circulation at the level of individual cells. These facts, together with the known negative association between vegan diet and insulin resistance may indicate that lower dietary intake of iron assists in preventing development of insulin resistance.

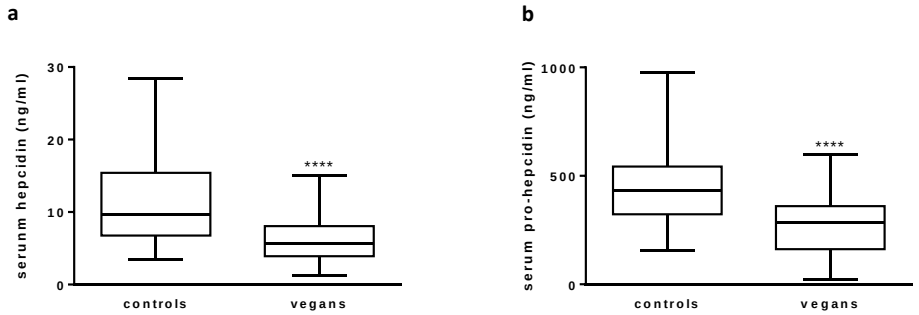

Fig. 4.6. Serum levels of hepcidin (**a**) and pro-hepcidin (**b**) in tested groups of controls and vegan population. The hepcidin levels were assessed using commercial ELISA kit. The median, interquartile range, minimum and maximum of each group is shown. Statistical significance when compared to controls using the Mann-Whitney nonparametric test: **** $P < 0.0001$.

5. CONCLUSIONS

In our studies we were focused on mechanisms of non-heme and non-transferrin-bound iron (NTBI) transport across plasma membranes of human cells and its regulations. Nevertheless, heme transport and metabolism are as well in our interest in our ongoing studies.

Although the main importance of transferrin-independent iron transport across plasma membrane takes place in iron uptake into the body, its participation in cellular iron trafficking across plasma membranes within the organism should also be considered. Therefore, we aimed on known molecules involved in NTBI transport in both enterocytes and non-intestinal cells. The expression of duodenal cytochrome b (Dcytb), divalent metal transporter 1 (DMT1), ferroportin, hephaestin, and ceruloplasmin molecules were tested under conditions of iron deficiency or excess *in vitro* as well as *in vivo*. Moreover, some of the regulatory molecules such as hepcidin, hemochromatosis gene (HFE), and transferrin receptor 1 (TfR1) were also tested.

For *in vitro* analyzes (paper 1, paper 2), we employed three main types of cells involved in iron handling. The Caco-2 cell line represented intestinal cells responsible for iron absorption into the body, the K562 cell line represented erythroid cells as major iron-utilizing cells, and HEP-G2 cells as a model of hepatocytes represented the major iron storage cells.

We detected stimulation of NTBI iron uptake in K562 cells under conditions of iron deficiency. Although we demonstrated that increased iron uptake was dependent on protein synthesis, none of the tested molecules was probably involved in this cellular response (paper 1). We also demonstrated that different iron availability affects the expression of tested molecules in cell-type specific manners. Because of *in vitro* conditions in these experiments, the mechanisms of cellular regulation of iron homeostasis are only involved here. In Caco-2 cells, the protein levels of DMT1 and

ferroportin were increased under conditions of iron deficiency and excess, respectively. It could correspond with increased iron absorption or efflux according to the given conditions. On the other hand, just slight or no changes at all were detected on proteins levels in HEP-G2 and K562 cells (paper 2). However, due to a significant increase in iron uptake under iron deprivation in K562 cells, we suspect involvement of some other transport molecules (paper 1).

In the case of *in vivo* studies (paper 3, paper 4), expression of NTBI transport molecules was assessed in duodenal biopsies of a defined group of patients with iron-related disorders. Iron deprivation was represented by patients with iron deficiency anemia (IDA), primary iron overload was represented by individuals with hereditary hemochromatosis (HHC), and secondary iron overload was represented by patients with alcoholic liver disease (ALD). Subjects with no evidence of disturbed iron homeostasis were used as healthy controls.

In IDA and HHC patients, a statistically insignificant decrease in serum hepcidin levels was detected. Increased mRNA levels of DMT1, ferroportin and TfR1 in IDA and treated HHC patients suggest IRP/IRE regulation of this genes expression as a result of iron deprivation. Unfortunately, these changes were not confirmed with regard to protein level (paper 3). For individuals with ALD, our results demonstrated that serum hepcidin levels were decreased by alcohol consumption. Since very complex regulation of iron homeostasis in ALD patients resulting in different iron levels in organism, this group had to be divided into ALD patients with iron deprivation, iron overload and normal iron status. After patient classification into ALD subgroups, changes in DMT1, ferroportin, and TfR1 on mRNA level were observed in ALD patients with anemia and normal iron status, as a consequence of iron deprivation and probably as a direct effect of ethanol (paper 4). However, some positive correlations among Dcytb, hephaestin, DMT1, ferroportin and TFR1 mRNA in all groups of patients indicate coordinated regulation of expression of these genes (paper 3, paper 4).

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7. PAPERS UNRELATED WITH TOPIC

1. **Balusikova K.**, Kovar J.
   Alcohol dehydrogenase and cytochrome P450 2E1 can be induced by long-term exposure to ethanol in cultured liver HEP-G2 cells.
   In Vitro Cell Dev Biol Anim. 49(8):619-625, 2013. doi: 10.1007/s11626-013-9636-y.
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2. Němcová-Fürstová V., **Balušíková K.**, Šrámek J., James R.F., Kovář J.
   Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells.
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3. Jelínek M., **Balušíková K.**, Kopperová D., Němcová-Fürstová V., Šrámek J., Fidlerová J., Zanardi I., Ojima I., Kovář J.
   Caspase-2 is involved in cell death induction by taxanes in breast cancer cells.
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4. Pavlíková N., Bartoňová I., **Balušíková K.**, Kopperová D., Halada P., Kovář J.
   Differentially expressed proteins in human MCF-7 breast cancer cells sensitive and resistant to paclitaxel.
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5. Jelínek M., **Balušíková K.**, Schmiedlová M., Němcová-Fürstová V., Šrámek J., Stančíková J., Zanardi I., Ojima I., Kovář J.

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   IF 2014: 2.766

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