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MOLECULAR MECHANISMS OF IRON TRANSPORT ACROSS PLASMA MEMBRANE IN MAMMALIAN CELLS

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LIST OF ABBREVIATIONS

ACO1	aconitase 1
ALD	alcoholic liver disease
Dcytb	duodenal cytochrome b-like
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
HAMP	hepcidin antimicrobial peptide
HCP1	heme carrier protein 1
HFE	hemochromatosis gene (“high-Fe”)
HHC	hereditary hemochromatosis
HIF	hypoxia-inducible factor
HRE	HIF-responsive element
HRG-1	heme responsive gene-1
IDA	iron deficiency anemia
IRE	iron-responsive element
IREB	iron-responsive element-binding protein 2
IRP	iron regulatory protein
IRP1	iron regulatory protein 1
IRP2	iron regulatory protein 2
mRNA	messenger ribonucleic acid
NTBI	non-transferrin-bound iron
PCR	polymerase chain reaction
STEAP	six transmembrane epithelial antigen of prostate
STEAP3	six transmembrane epithelial antigen of prostate 3
TfR1	transferrin receptor 1
UTR	untranslated region
VDAC2	voltage-dependent anion-selective channel 2
ZIP14	Zrt/Irt-like protein 14

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 transport 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.

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 iron transport 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.

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+}). Iron is involved in oxygen transfer and thanks to its ability to exist in two stable oxidative states it can also function as a donor or acceptor of electrons. 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 [1-3].

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. Although the total body iron content is therefore 3 to 4 g, on average, a maximum of 2 mg of iron is absorbed and subsequently lost from the organism each day [4, 5]. Since there is no active mechanism of iron release 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. For these reasons, not only iron absorption, but also iron transport and storage within the body are very strictly regulated processes [4, 6-8].

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 [9-12]. Both heme and non-heme iron are absorbed into the organism via enterocytes in the duodenum and proximal jejunum [13-16].

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 [17] but later disputed, since the molecule was actually established as a proton-coupled folate transporter. However, the role of HCP1 as an apical heme transporter has been reestablished with dual functions as a heme/folate importer with high affinity for folate [18]. Imported heme is metabolized in intestinal cells by heme oxygenase-1 and -2, which produces carbon monoxide, biliverdin, and free ferrous iron [14, 19, 20]. Free iron becomes part of the labile iron pool, is stored in a ferritin, or transported from the enterocyte.

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 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 [21-24]. Ferroportin is the only known protein mediating iron export from cells. As well as DMT1, ferroportin is a transmembrane molecule with affinity for ferrous iron [25-27].

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) [28-30]. Ferrous iron is then transported across the apical membrane of the enterocytes by the DMT1 importer or other possible metal transporters [31, 32]. 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 transmembrane ferroxidase hephaestin or plasma ceruloplasmin [33, 34]. In circulation, ferric iron is bound by plasma transferrin and delivered within the organism.

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 uptake via transferrin receptor mediated endocytosis [35, 36]. However, the physiological relevance of non-transferrin low-molecular-mass iron uptake has also been clearly demonstrated especially in iron overload states [37-41]. Moreover, heme iron can be transported within the organism too [42].

1.3.1 Transport of transferrin-bound iron

When present in serum, two ferric ions are bound to the iron-free transferrin molecule (apo-transferrin) with relatively high affinity [43, 44]. 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 [35, 45]. Transferrin receptor 2 is present in the serum too, but its affinity toward transferrin is significantly lower [46]. Complexes of holo-transferrin/TfR1 are accumulated on the membrane and internalized in endosomes. After internalization, ferric iron is released from complexes in endosomes due to decreased endosomal pH. It is reduced into ferrous iron and transported to the cytoplasm via DMT1 molecules [47-50].

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. 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 [7, 8, 21, 51- 53].

Similarly, heme transport within organism also corresponds with heme absorption via enterocytes. However, 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, 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 [54].

Since reticuloendothelial macrophages are responsible for iron recycling from red blood cells, a special mechanism of its uptake is involved here. 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. Once in macrophages, iron can be stored in ferritin deposits or returned into the circulation by ferroportin [5].

1.4 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.

1.4.1 Homeostasis of body iron level

Whole-body iron homeostasis is primarily mediated by the active form of the regulatory peptide hepcidine [55, 56]. 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 [57]. Besides ferroportin, other iron transport molecules such as DMT1 and TfR1 have been suggested as hepcidin targets [58-60]. The HAMP gene (hepcidin antimicrobial peptide), which encodes hepcidin is predominantly expressed in hepatocytes [61, 62]. Its expression responds to body iron levels via tissue iron stores and transferrin saturation; however it also responds to erythropoiesis, hypoxia, inflammation, and others [56, 63-65].

1.4.2 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 [66, 67]. 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 [68]. IRP binding to IRE, located in the 5'UTR, causes translational inhibition and thus decreased levels of the target protein [69]. On the other hand, IRP/IRE interaction in 3'UTR mediates mRNA stabilization and thus increases protein levels [70].

Similarly another local regulatory mechanism involving HIF/HRE interaction can participate in cellular iron homeostasis [71]. 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 during hypoxia [72, 73]. As the HRE sequences are usually located in enhancer or promotor regions, HIF/HRE regulation affects gene expression at transcriptional level.

1.5 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.

1.5.1 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 [74]. 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.

Primary iron deficiency can result from DNA mutations in several genes coding for various proteins involved in iron transport and metabolism and are represented by e.g. thalassemia, iron refractory iron deficiency anemia or sideroblastic anemia. 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.

Thalassemia is autosomal recessive disorder caused by abnormal formation of hemoglobin and therefore of red blood cells. Patients suffering of thalassemia therefore synthetize lower amounts of hemoglobin resulting in lower numbers of red blood cells in circulation and anemia [75]. Iron refractory iron deficiency anemia (autosomal recessive) is caused by mutations in gene encoding for the matriptase-2 protein. Malfunction of matriptase-2 results in increased hepcidin production, subsequent degradation of ferroportin and insufficient absorption of iron into the body [76, 77]. The origin of sideroblastic anemia can be a congenital genetic disorder as well as have non-genetic causes. Anyway, sideroblasts are produced instead of healthy red blood cells during disease. Therefore, iron is available in the body but it is not incorporated into hemoglobin [78].

Disorders with secondary iron deficiency are mainly accounted for by iron deficiency anemia and anemia of chronic disease. Both diseases are also the most prevalent form of anemias [79]. Especially, since iron deficiency due to inadequate nutrition is the most common cause of microcytic anemia, iron deficiency anemia is one of the major health problems worldwide [80, 81].

1.5.2 Iron overload associated disorders

Iron accumulation in organism, production of reactive oxygen species and subsequent oxidative stress leads to a limitation of cell functions that can affect many different tissues in the body [82]. Iron-induced oxidative stress and subsequent cell death particularly damages parenchymatic organs that contain cells with numerous mitochondria and have high respiratory activity such as hepatocytes, cardiomyocytes and pancreatic β-cells. Therefore organ damage resulting in fibrosis, cirrhosis, hepatocellular carcinoma, heart failure, glucose intolerance or diabetes mellitus can be seen in patients with 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 [83]. 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 (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 hemochromatosis

gene (HFE). HFE gene protein products are involved in the regulation of iron transport by hepcidin but the exact mechanism is not yet completely known [84-86].

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. [8]. However, the most common cause of secondary iron accumulation in the liver is alcohol abuse [87-89]. 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 [90-93].

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 [13-16].

Heme is thought to be imported into enterocytes via the heme carrier protein 1 [18]. Within the cell, heme is metabolized by heme oxygenase molecules while carbon monoxide, biliverdin, and free ferrous iron are produced [14, 19, 20]. Non-heme iron, usually present in nutrients in its ferric form, is reduced to ferrous iron by Dcytb (duodenal cytochrome b) and transported into the cell by DMT1 (divalent metal transporter 1) [21-24, 28-30]. Once inside the enterocyte the iron can be utilized, stored in ferritins, or exported into the bloodstream by ferroportin [25-27]. In the circulation, ferrous iron is re-oxidized by hepcidin or ceruloplasmin and bound to transferrin as the main transport molecule for iron within the organism [33, 34]. Transferrin-bound iron is absorbed into individual cells via transferrin/transferrin receptor complex-mediated endocytosis [35, 36]. Additionally, non-transferrin-bound iron (NTBI) transport across plasma membrane also takes place within the organism, but its significance increases especially during pathological states [8, 37, 51]. 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 [55, 56]. The negative regulatory mechanism involves hepcidin binding to ferroportin, the only known iron exporter, and subsequent ferroportin internalization and degradation [57]. The local regulation of iron homeostasis involves post-transcriptional regulatory mechanisms mainly involving iron regulatory protein (IRP)/iron-responsive element (IRE) interactions [66, 67]. Lately, hypoxia-

inducible factor (HIF)/HIF-responsive element (HRE) regulation has also been considered [71].

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. 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.

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. 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 in mRNA levels, 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 [68].

Despite some changes in the expression of tested molecules on mRNA level in HEP-G2 and K562 cells, 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. We detected significant increase in iron uptake under iron deprivation in K562 cells. Together with the involvement of protein synthesis, we expected participation of some others NTBI transport molecules in this system [94].

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 [95]. 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 [31, 32, 96, 97]. Molecules such as lipocalin 2 or VDAC2 should be also discussed [94, 98, 99]. 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 [100, 101].

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. 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. 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. 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. 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.

Increased mRNA levels of DMT1 and ferroportin were also found in HHC patients after treatment. 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. 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. We also tested the expression of HFE and TfR1 genes. 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 [102]. However, this theory has recently been doubted [103, 104]. 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. 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. Hepcidin suppression can be caused directly by ethanol or indirectly via regulatory molecules [90, 93]. As mentioned above, with these changes 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.

4 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 DMT1 (divalent metal transporter 1), Dcytb (duodenal cytochrome b), 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, HFE (hemochromatosis gene), and transferrin receptor 1 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. 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. However, due to a significant increase in iron uptake under iron deprivation in K562 cells, we suspect involvement of some other transport molecules.

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. 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. 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.

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