Effect of acute phase cytokines on iron uptake in hepatocytes and differential localization of Lipocalin-2 and Transferrin receptors in rat hepatic and extra hepatic organs

Dissertation

for the award of the degree

"Doctor of Philosophy" Ph.D.

Division of Mathematics and Natural Sciences

of the Georg-August-Universität Göttingen

within the doctoral program Biology

of the Georg-August University School of Science (GAUSS)

submitted by

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Göttingen, 2014

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ABBREVIATIONS

Ab	Antibody
APP	Acute phase proteins
APR	Acute phase response
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Copy desoxyribonucleic acid
CRP	C-reactive protein
Ct	Threshold cycle
dd H_2O	Double distilled water
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
Dcytb	Duodenal cytochrome B reductase
DMT1	Divalent metal transporter 1
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
FCS	Fetal calf serum
FTH	Ferritin heavy chain
FTL	Ferritin light chain
Fpn1	Ferroportin 1
g	Gravity
GFP	Green fluorescent protein
gp	Glycoprotein
GRE	Glucocorticoid response element
HIF	Hypoxia induced factor
HRP	Horseradish peroxidase
Hepc	Hepcidin

Heph	Hephaestin
HFE	Hemochromatosis gene
Hjv	Hemojuvelin
IgG	Immunoglobulin G
IL	Interleukin
IRP	Iron regulatory protein
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
IRE	Iron responsive elements
IRE-BP1	Iron responsive element binding protein 1
IRE-BP2	Iron responsive element binding protein 2
kDa	Kilodalton
LCN-2	Lipocalin-2
Min	Minutes
MOPS	3-(N-Morpholino)-propanesulfonic acid
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulfonylfluoride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulfonylfluoride
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
RNase	Ribonuclease
ROS	Reactive oxygen species

rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TEMED	N, N, N´, N´-tetramethylethylenediamine
TNF-α	Tumor necrosis factor α
Tris	Tris-(hydroxymethyl)-aminomethane
TAE	Tris acetate EDTA buffer
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TO	Turpentine oil
Tris	Tris-(hydroxymethyl)-aminomethane
UTR	Untranslated region
UV	Ultraviolet

1   INTRODUCTION

1.1   Iron an essential element of Life

With few exceptions, almost every cell needs iron as an essential cofactor for biochemical processes, i.e. oxygen transport, energy metabolism, mitochondrial functions, myelin production and DNA synthesis (Camaschella, 2013;Hentze et al, 2004;Napier et al, 2005). Due to redox reactivity of iron it can associate with proteins, couple with oxygen and can also transfer electrons or mediate catalytic reactions (Aisen et al, 2001).

An imbalance of body iron can lead to pathological conditions. The most common disorder of iron metabolism associated with iron depletion is iron deficiency anemia, which affects over 30% of the world's population and is especially prevalent in developing countries (DeMaeyer and Adiels-Tegman, 1985). Factors such as inadequate iron intake, blood loss, increased iron requirements, and reduced iron absorption contribute to the progression of iron deficiency (Dreyfuss et al., 2000; Lewis et al., 2005; Stoltzfus et al., 2000). At the other extreme are iron overload disorders such as hemochromatosis which is due to excess iron deposition, primarily in the liver (Pietrangelo, 2010). It shows that iron could be potentially toxic because it catalyses the dissemination of ROS (reactive oxygen species) under aerobic conditions which ultimately generate potentially reactive hydroxyl radicals by Fenton pathway (Koppenol, 1993). The oxidative stress, due to ROS is associated with cellular damage of macromolecules, tissues and certain disease conditions (Galaris et al., 2008; Kell, 2009). However, the acquisition, usage and detoxification of iron is a considerable challenge for cells and organisms, which have evolved various sophisticated mechanisms to comply with their metabolic needs and to

minimize the risk of toxicity concomitantly (Andrews, 2008; De, I et al., 2008; Hentze et al., 2010).

The amount of iron in the body depends on age, gender, nutrition, and general state of health. In normal conditions, two-thirds of total body iron is incorporated into hemoglobin by erythroid cells, while the remaining third is stored in the liver (~1 g iron) or as myoglobin in muscle (300 mg iron) or is found in reticuloendothelial macrophages (600 mg iron) (Andrews, 1999; Olsson and Norrby, 2008). Other tissues contain lower quantities of iron but not negligible.

1.2 Iron metabolism and liver

The liver is a major organ for the synthesis of iron regulatory proteins and its output includes several proteins that play critical roles in iron metabolism. These include iron transport proteins [(Tf, TfR1, TfR2, LCN-2)(Johnson et al., 2007; Kaplan, 2002; Lu et al., 1989; Yang et al., 2002)], iron storage proteins [(Ferritin)(Arosio et al., 2009)] ceruloplasmin, haptoglobin, hemopexin, hepcidin and iron export protein ferroportin 1 (Fig. 1b). A major cell population of the liver, hepatocytes (70% of the cells of liver) is the key cells to regulate iron absorption and reutilization of iron after senescent red cells are phagosytosed and digested by macrophages.

Dietary iron absorption from intestinal lumen (Fig. 1a) and recycling by macrophages (Fig. 1d) is regulated by different physiological factors including iron-load, erythropoiesis and inflammation (De, I et al., 2008). Hepcidin, an antimicrobial peptide secreted by liver, is a key element in the regulation of iron homeostasis (Ganz and Nemeth, 2012; Loreal et al., 2000). Hepcidin is a 25 amino acid peptide secreted by the liver into the circulation (Fig. 1c). In fact, hepcidin synthesis is regulated by iron demand; transcription of hepcidin is increased when iron

stores are high and, conversely, decreased when iron stores are depleted. In different conditions of chronic inflammation, such as arthritis or cancer-associated inflammation, hepcidin production is increased and decreased iron absorption results in an iron-limited erythropoiesis, known as the anemia of chronic inflammation (De, I et al., 2006).

Almost most of the iron in the circulation is transported by plasma Tf and the vast majority of Tf is synthesized in the liver (Ponka et al., 1998). Transferrin (Tf)-iron is transported to the reticuloendothelial system (spleen, liver and bone marrow), to liver parenchymal cells and to all proliferating cells in the body. It carries iron through portal blood to the liver by TfR1 mediated iron uptake mechanism (Hentze et al. 2010) or Tf-independent mechanism (Prus and Fibach 2011). Another transferrin-dependent iron uptake route is via TfR2. Although, the role of TfR1 is well defined for transporting iron across the plasma membrane, the role of TfR2 is not yet clearly understood. TfR2 appears to be involved in systemic iron homeostasis (Fig. 1c) rather than in cellular iron uptake and delivery because mutations in the TfR2 gene or its knockout results in hepatic iron overload (Roetto et al. 2010).

However, there has been convincing evidences that, during disrupted iron homeostasis, iron delivery to cells can take place be alternative mechanism i.e. transferrin-independent pathway. This alternative to transferrin-iron is called non-transferrin bound iron (NTBI). This pool has been documented in a variety of iron overload syndromes when transferrin is saturated (Hentze et al., 2004), including hemochromatosis (69% of patients) and end-stage renal disease (22% of patients) (Breuer et al., 2000b). Furthermore, NTBI can reduce the uptake of Tf-bound iron and vice versa (Graham et al., 1998; Trinder and Morgan, 1997). A recent study has shown that

LCN-2 can deliver iron to kidney cells during development (Yang et al., 2003).
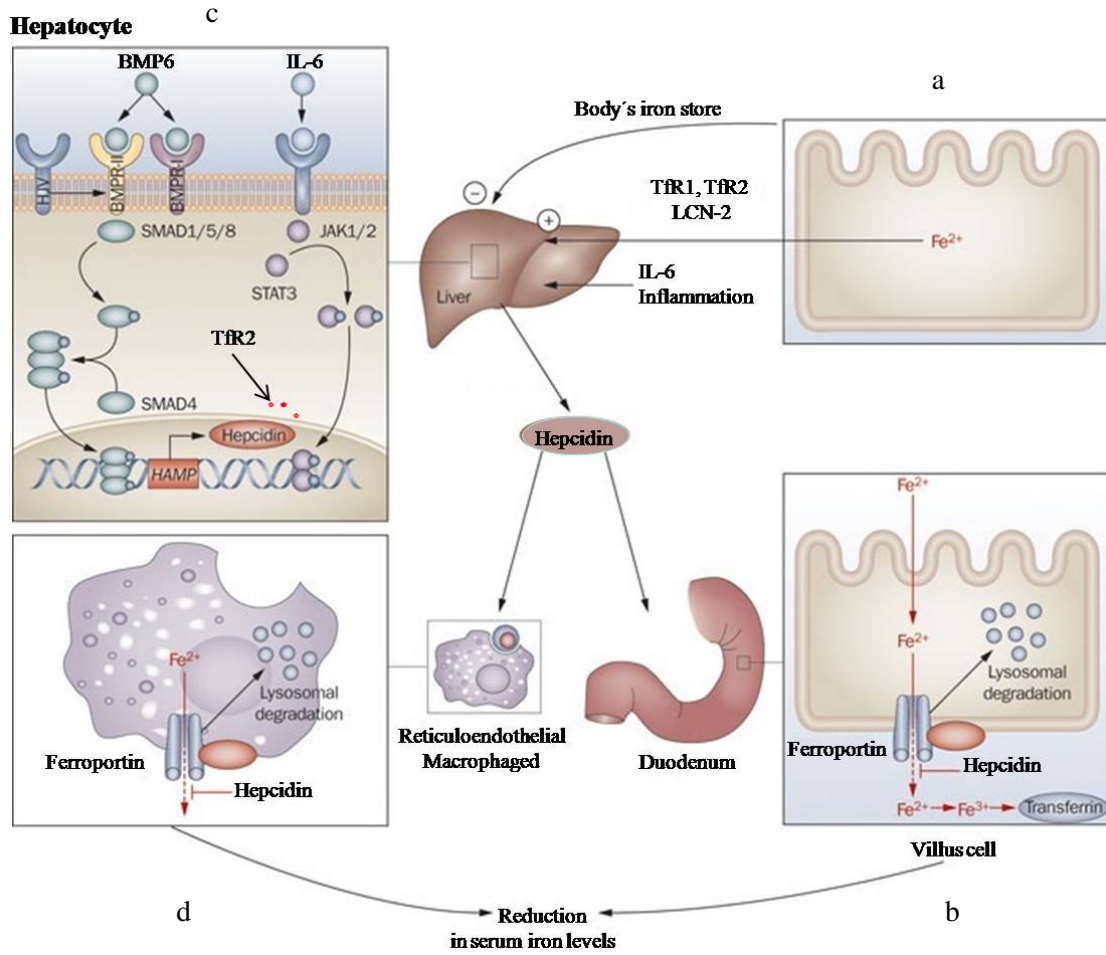
Figure 1: A Schematic overview of iron absorption. From intestinal lumen Fe^{+3} is reduced to Fe^{+2} by reductase enzyme and then transported to enterocytes by DMT1 (a). Ferroportin 1 export this iron into the circulation and transferrin bind the free iron which is transported to Liver via Tf-TfR mediated pathway (b). Hepcidin gene expression is up-regulated during inflammation by pro-inflammatory cytokines mainly IL-6 (involving JAK-dependent activation of STAT3) (c). Hepcidin binds to ferroportin and triggers its lysosomal degradation, leading to a reduction in iron release from enterocytes and macrophages (d). Modified from:Stein et al., 2010.

1.3 Mechanisms of iron uptake

1.3.1 Transferrin bound iron uptake and regulation of TfR

1.3.1.1 Transferrin receptor 1 (TfR1) mediated iron uptake

Many studies on the uptake of Tf-bound iron by perfused liver, by isolated hepatocytes, or by hepatoma cell lines showed existence of both a high-affinity saturable component and a low-affinity unsaturable component (Goldenberg et al., 1991; Morgan, 1991; Trinder et al., 1988). The high-affinity uptake of Tf by hepatic cells is mediated by Tf receptor 1 [(TfR1, figure 2) (Aisen, 2004; Hentze et al., 2010)]. At physiological pH, TfR1 binds diferric Tf with 10-fold higher affinity than mono-ferric Tf and 2000-fold higher affinity than apo-transferrin (Ponka et al., 1998). After Tf binding to TfR1 on the cell surface, endocytosis of this Tf-TfR1 complex takes place through clathrin-coated pits (Fig. 2). These vesicles are then uncoated to become endosomes and are acidified by a proton pumping ATPas (Watkins et al., 1992). After entering the cytosol, iron is released at a pH of around 5.5, a process requiring also reduction (Richardson et al., 2010) and takes advantage of a conformational change in Tf that accompanies its binding to TfR1 (Bali et al., 1991). The ferrireductase Steap3 reduces cytosolic Fe^{3+} to Fe^{2+} (Ohgami et al, 2005), transported by DMT1 or directly in erythroid cell´s mitochondria (Richardson et al., 2010). The affinity of TfR1 for apotransferrin is much higher at the low pH of the endosome than at physiological pH and the two proteins remain bound as the endosome is recycled to the plasma membrane. Apotransferrin is then released into the extracellular milieu where it can again bind iron (Fig. 2). Within the liver, all cell types have Tf receptors as they all need iron to meet their basic metabolic requirements, but quantitatively hepatocytes take up most Tf (Morgan et al., 1986; Sibille et al., 1986). A study demonstrated that hepatocytes expressed three- to four-fold more high-affinity diferric Tf binding sites than nonparenchymal cells which is most likely

TfR1 (Vogel et al., 1987). TfR1 receptor has also been detected on Kupffer cells (Vogel et al., 1987), endothelial cells (Tavassoli et al., 1986), and activated hepatic stellate cells (Bridle et al., 2003). During iron deficiency conditions, cells require more iron and increase both their total cellular complement of TfR1 and the proportion of TfR1 on the plasma membrane (Aisen, 2004; Hirose-Kumagai and Akamatsu, 1989). Some regulation of the TfR1 gene occurs at the transcriptional level, while most regulation is at the level of mRNA stability via the iron regulatory element (IRE)/iron regulatory protein (IRP) system (Eisenstein, 2000). The TfR1 mRNA contains a series of stem-loop structures (iron responsive elements or IREs) in its 3´ un-translated region (UTR) which act as targets for the iron-responsive RNA binding proteins IRP1 and IRP2. When intracellular iron levels decrease, the IRPs bind to the TfR1 3´ UTR and protect the TfR1 mRNA from endonuclease degradation. Consequently more TfR1 is synthesized (Mullner and Kuhn, 1988). The opposite response is observed when cells have an excess of iron and a reduction in TfR1 expression serves to protect the cells from accumulating iron (Rouault, 2006; Wallander et al., 2006). TfR1 levels are much higher in fetal liver than in adult liver (Trinder et al., 1986) and in regenerating liver after partial hepatectomy (Cairo et al., 2002; Hirose-Kumagai and Akamatsu, 1989). It reflects the iron requirements of the rapidly growing hepatocyte mass and is likely mediated through the IRE/IRP system. Finally, TfR1 gene expression can also be modulated by cytokines, hypoxia, and nitric oxide, and these factors could play a role in TfR1 synthesis, particularly during disease states (Cairo et al., 2002; Trinder et al., 2002).

1.3.1.2 *Transferrin receptor 2 (TfR2) mediated iron uptake*

Many studies suggest that the low-affinity iron uptake process may be mediated by the TfR2, TfR1 homolog (Cairo et al., 2002; Kawabata et al., 1999; Lee et al., 2003; Robb et al.,

2004; Trinder et al., 2002). TfR2, like TfR1, is a plasma membrane Tf-binding protein with 25-fold lower affinity for diferric Tf than that of TfR1, making it a strong candidate for the low-affinity binding site. Expression pattern of TfR2 also differs from TfR1. TfR2 has a much more restricted tissue distribution. High expression levels of TfR2 are present on hepatic parenchymal-

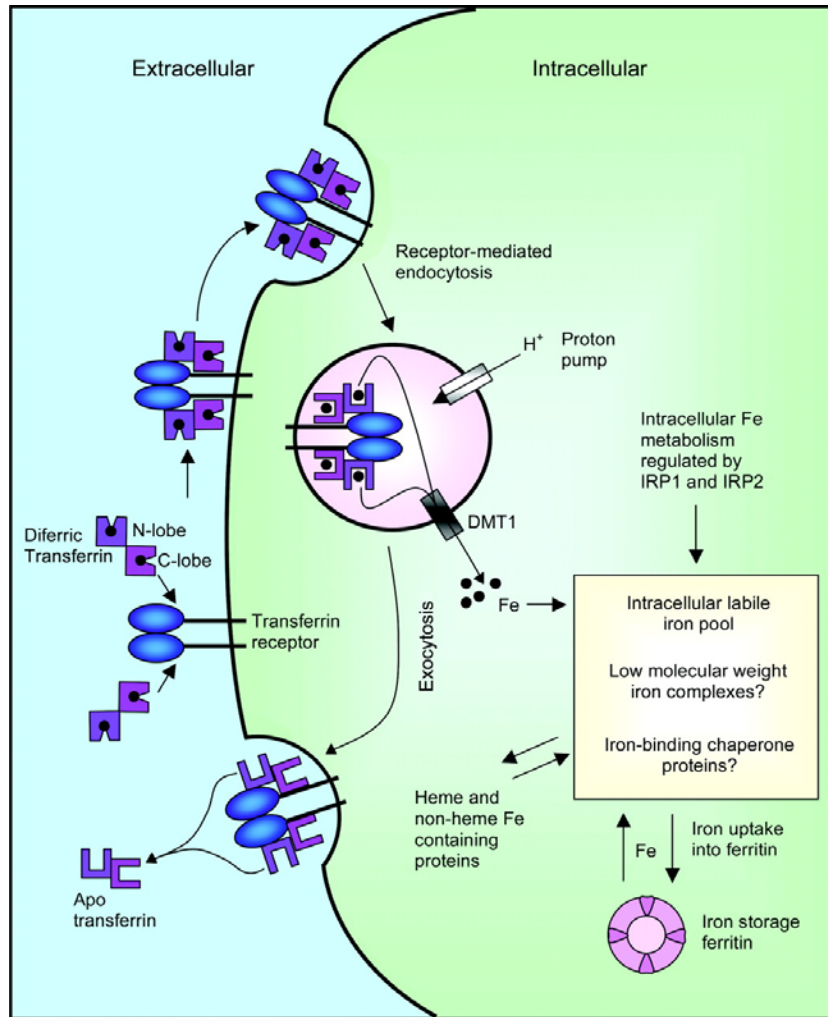**Figure 2:** Schematic diagram illustrating the iron uptake mechanisms. After binding to TfR1 the diferric Tf undergoes endocytosis. Iron is released from Tf by a decrease in pH and is exported out of the endosome by DMT1, where it enters the LIP. Iron in the LIP can subsequently be incorporated into ferritin for iron storage or into iron-containing proteins. Source:Kalinowski and Richardson, 2005.

cells, (Deaglio et al., 2002; Fleming et al., 2002) with lower levels in mature erythroid cells, spleen, lung, skeletal muscle, and prostate. Small amounts of TfR2 mRNA have also been found in Kupffer cells, sinusoidal endothelial cells, and stellate cells (Zhang et al., 2004). Many studies have demonstrated the presence of TfR2 on the cell surface, although a considerable fraction is also located at intracellular sites (Deaglio et al., 2002; Robb et al., 2004). TfR2, unlike TfR1, does not contain any IREs in its mRNA and its expression at transcriptional level is not regulated by cellular iron content (Fleming et al., 2000). However, TfR2 protein levels increase with iron loading and decrease with iron depletion (Robb and Wessling-Resnick, 2004). Similarly, treatment of a hepatoma cell line with diferric Tf leads to an increase in TfR2 protein, apparently by increasing protein half-life (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004). According to different studies, mutations in the TfR2 gene in humans or disruption of the gene in mice lead to systemic body iron loading (Camaschella et al., 2000; Fleming et al., 2002) due to an inability (presumably loss of function) to correctly regulate intestinal iron absorption. Although, it appears that most of Tf-bound iron is taken up by cells via the endocytosis of Tf, there is also evidence that Tf-bound iron can be released at the cell surface. This process has been described for several cell types but is most prominent in hepatocytes (Thorstensen and Romslo, 1990). Membrane impermeant iron chelators can reduce the uptake of iron from Tf, suggesting iron release at the cell surface (Cole and Glass, 1983; Thorstensen and Romslo, 1984), and there is also evidence for a cell surface ferric iron reductase activity that may facilitate iron release from Tf (Thorstensen and Romslo, 1984).

1.3.1.3 *Non-Transferrin bound iron uptake (NTBI)*

Under normal conditions, 30% of plasma Tf is hyposaturated and shows high capacity for iron binding to restrain the accumulation of NTBI (non-transferrin bound iron). On the

contrary, during hereditary haemochromatosis and certain other iron-overload condi-tions, plasma iron levels exceed the saturation capacity of Tf, and NTBI pool builds up contrib-uting significantly to hepatic iron loading (Breuer et al., 2000a). The exact chemical nature of NTBI is still elusive, whereas its redox reactivity and toxicity has been established very well. It may consist of loosely chelated ferric (Fe^{3+}) by albumin or small organic molecules, such as cit-rate (Hider, 2002). The mechanism of NTBI uptake by cells is poorly understood.

Liver can take up NTBI very efficiently. This mechanism is well documented from differ-ent studies including rare instances of congenital Tf deficiency in humans (atransferrinemia) and mice (Bernstein, 1987; Hayashi et al., 1993). Affected individuals, despite lacking Tf, absorb iron from their diet very efficiently and large amounts of iron are deposited in the liver. Lipocalin-2 (LCN-2; human ortholog neutrophil gelatinase-associated lipocalin [NGAL], has been proposed as a mediator of transferrin-independent iron transportation (Yang et al., 2002). It belongs to the lipocalin family which is known to be involved in the regulation of im-mune responses, modulation of cell growth and metabolism, prostaglandin synthesis and iron transport (Yang et al., 2002). In fact, it is a bacteriostatic agent and capable of sequestering iron in the form of siderophores (Flo et al., 2004). A recent study has shown that LCN-2 can transport iron during kidney development through siderophore–iron complex (Yang et al., 2003), inju-ry (Mori et al., 2005) and under inflammatory conditions (Devireddy et al., 2005). Iron-loaded LCN-2 is internalized by the LCN-2 receptors (24p3R and megalin) (Devireddy et al, 2005; Hvidberg et al, 2005) (Fig. 3). Siderophores are low-molecular mass iron-chelating metabolites, synthesized by bacteria and fungi for the acquisition of extracellular iron.
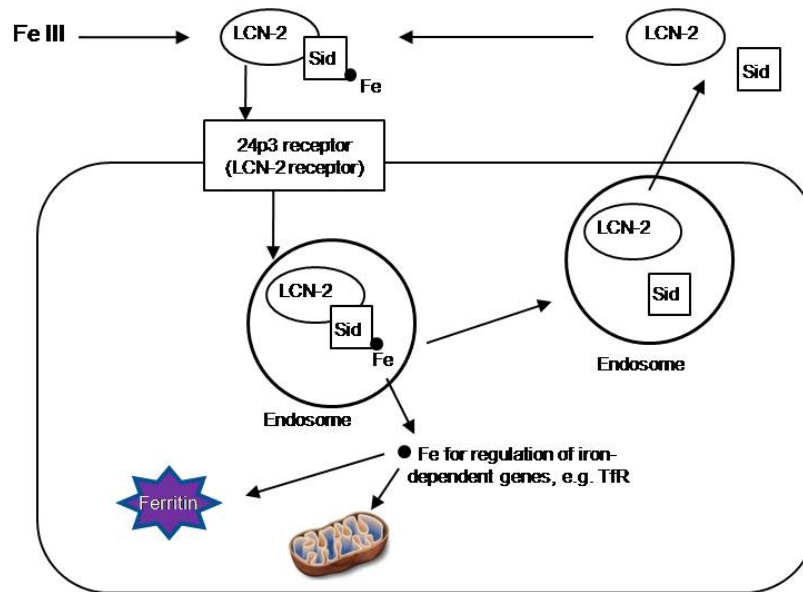
Figure 3: Schematic overview of non-transferrin bound iron uptake through lipocalin-2 (LCN-2). LCN-2 mediates iron uptake through 24p3 receptor (LCN-2 receptor) endocytosis of a LCN-2 siderophore (Sid)-iron complex. Siderophore-iron-associated LCN-2 (holo-LCN-2) delivers iron into the cell. LCN-2 then traffics in acidic endosomes, which promote the release and cytoplasmic accumulation of iron, resulting in regulation of iron-dependent genes. Source:Haase et al., 2010.

It was recently described that mammals synthesize the siderophore dihydroxybenzoic acid (2,5-DHBA), an isomeric of 2,3-DHBA, the iron-binding component of enterobactin (Devireddy et al., 2010). Most importantly by depletion of 2,5-DHBA iron metabolism was deregulated in mammalian cells and zebrafish embryos, showing the biological importance of the LCN-2-dependent mechanisms.

1.4 Iron storage

Liver ferritin has a major role in iron storage and iron is stored mainly as ferritin within the cell (Ganz and Nemeth, 2012). Ferritin consits of L and H subunits that are highly conserved (Arosio et al., 2009) nevertheless, these subunits are genetically separate (Caskey et al., 1983; Worwood et al., 1985) and maintain distinct functions (Sammarco et al., 2008). Iron storage is considered to take place in the cytoplasm only, however is also required for the nuclear func-

tions. L and H subunits of ferritin assemble spontaneously to produce a 24-subunit protein "cage" with a variable H: L ratio. This H: L ratio can vary between different cell types (Arosio et al., 2009; Sammarco et al., 2008). Hepatocytes have a high proportion of L subunits and relatively fewer H subunits (ratio 1:10 to 1:20 in human liver ferritin and 1:4 in rat liver ferritin), whereas cells that are involved in rapid iron turn over, such as macrophages, express more H subunits (Wang et al., 2013). The studies on ferritin proved that the the L gene showed very less tissue-specific regulations whereas the H ferritin gene is activated by multiple factors or conditions (Briat et al., 2010; Ponka et al., 1998) including changes in the cell proliferation, cytokines and heme. A pre-vious study clearly provided evidence of the association between ferritin expression and cell pro-liferation (Cozzi et al., 2004).

All cell types present within the liver can store iron but during normal physiological conditions hepatocytes are the major storage site and site of ferritin synthesis (Ganz and Nemeth, 2012). During iron-loading disorders characterized by elevated iron absorption, most of excess iron is deposited in parenchymal cells, while with transfusional iron overload Kupffer cells can store considerable amount of iron (Harmatz et al., 2000). There are numerous studies showing positive correlation between ferrtin and body iron store and ferritin level within the cell is determined by the iron content (Arosio et al., 2009). Ferritin subunit synthesis is iron-dependent and is controlled predominantly through a post-transcriptional mechanism by the IRE/IRP system (Eisenstein, 2000; Harrison and Arosio, 1996). Unlike TfR1, which has several IREs in its 3´ UTR, the ferritin mRNAs contain only a single IRE in their 5´ UTR. When iron contents remain low, the IRPs bind to this IRE present in ferritin transcripts and translation blocked. However, during iron accumulation conditions within the cell, the ferritin translational block is reversed and synthesis of new ferritin subunits is started (Fig. 4). This is not the only one mechanism for

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regulating ferritin synthesis in response to iron status, transcriptional regulation also plays a role in regulation (Cairo et al., 2002). Under normal conditions iron is the major factor influencing expression of ferritin, but it is also an acute phase protein and its synthesis that is regulated differently by different inflammatory stimuli and various acute phase cytokines e.g. IL-1β, IL-6, TNF-α (Cairo et al., 2002).
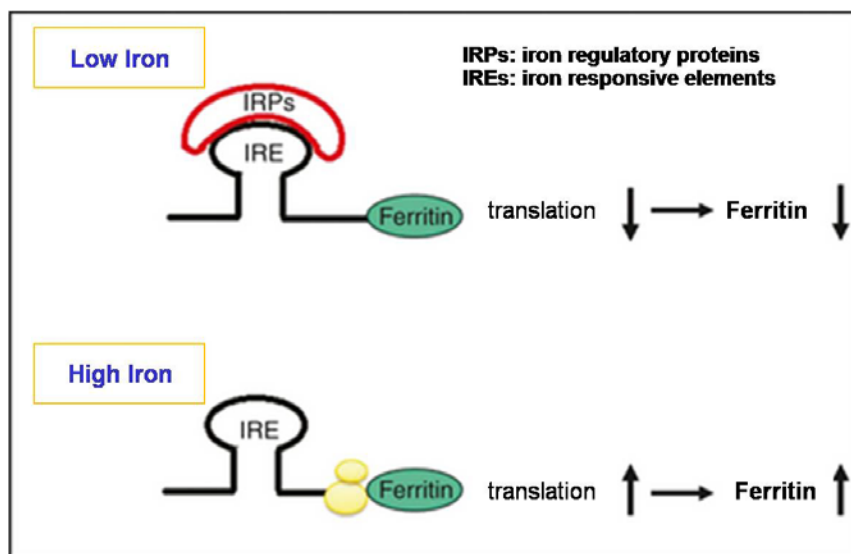
Figure 4: Regulation of ferritin expression by iron at the translational level via the IRE–IRP system. Iron-responsive element (IRE) in ferritin is an mRNA element at the 5′-untranslated region forming a hairpin structure. Iron regulatory proteins (IRPs), including IRP1 and IRP2, bind to IRE and inhibit ferritin mRNA translation. When the iron level is high, IRP2 is degraded, and IRP1 is converted from its active RNA-binding form into an Fe–S cluster-containing aconitase that lacks IRE-binding activity. Thus, IRPs cannot bind to IRE, and ferritin translation increases. Conversely, when the iron level is low, IRPs bind to IRE, and the ferritin level decreases. Source:Wang et al., 2013

1.5   Acute phase response (APR)

The acute phase response (ARP) is a key physiological defense mechanism of the organisms in response to any local or systemic insult caused by infection, tissue injury, trauma or surgery and immunological disorders (Gruys et al., 2005; Koj, 1985). The purpose of generating an acute phase response is to recover the damage caused by the injuring noxae and it also ensures the restoration of homeostasis. Acute phase can be characterized clinically by observing different

signs i.e. fever, drowsiness, weakness, muscular pain, and adynamia. A large group of specific mediators/cytokines including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) others is responsible for mediating acute phase reaction (Ramadori and Christ, 1999).

Acute phase proteins are divided into two groups:

1. Type I acute phase proteins include SAA (serum amyloid A), CRP (C-reactive protein; human), complement C3, haptoglobin (rat), and α1-acid glycoprotein. They are induced by interleukin-6 (IL-6), interleukin-1 (IL-1)-like cytokines which comprise IL-1α, IL-1β, tumour necrosis factor (TNF)-α and TNF- β.

2. Type II acute phase proteins are induced by IL-6 cytokine and its family members LIF (leukaemia inhibitory factor), IL-11, OSM (oncostatin M), CNTF (ciliary neurotrophic factor) and CT-1 (cardiotrophin-1). Type II proteins include fibrinogen, haptoglobin (human), α1-antichymotrypsin, α1-antitrypsin, and α2-macroglobulin (rat).

In general, IL-6-like cytokines synergize with IL-1-like cytokines to induce type I acute phase proteins, whereas IL-1-like cytokines do not affect induction of type II acute phase proteins or even have inhibitory effect on it (Moshage, 1997). After injury these cytokines are released or synthesized by different cell types including endothelial cells, fibroblasts, macrophages, granulocytes and lymphocytes, at the site of injury. The systemic circulation brings these floating cytokines to the liver and in response liver starts regulation of different acute phase proteins, differentiated as positive and negative acute phase proteins (APP). Proteins with an increased expression and plasma concentration are classified as positive acute phase proteins (APP), e.g., α2-macroglobulin and LCN2 in rats (Sultan et al., 2012). These APPs phase proteins

are involved in the process of wound healing to neutralize or recover the tissue damage (Ramadori and Christ, 1999). The other positive APPs include clotting proteins, transport proteins, antiproteases, and complement factors serum amyloid A (SAA) and C-reactive protein (CRP) in humans or in mice its homologue, serum amyloid P component (SAP). Furthermore, the decrease of the serum iron level is also a hallmark of APR (Sheikh et al., 2007). There are some other proteins which are down-regulated and consequently their plasma concentration decreases during APR. These are categorized as negative acute phase proteins and protein such as albumin belongs to this family (Ramadori and Christ, 1999). The major and key source of these APPs is the hepatocytes which become metabolically active during APR (Ramadori et al., 1985; Ramadori and Christ, 1999).

IL-6-like cytokines use the common receptor β-subunit (gp130) for signal transduction which in some cases requires an α-receptor subunit (IL-6, IL-11, CNTF) but in other cases do not (OSM, LIF) (Heinrich et al., 1998). After ligand binding, dimerization of two β-subunits leads to the activation of the receptor associated Janus kinases (JAKs), which in turn, phosphorylate transcription factors from the signal transducer and activator of transcription (STAT) family, namely STAT1, 3, and 5 (Heinrich et al., 2003). After homo- or heterodimerization, activated STATs are translocated to the nucleus, where they bind to target sequences in the promoters of type II APP genes, stimulating synthesis of corresponding proteins (Fig. 5). Among STAT factors, STAT3 is considered to play a pivotal role in the regulation of the APR, since STAT3 binding sites were shown in the promoters of various APP genes induced by IL-6 (Streetz et al., 2001).
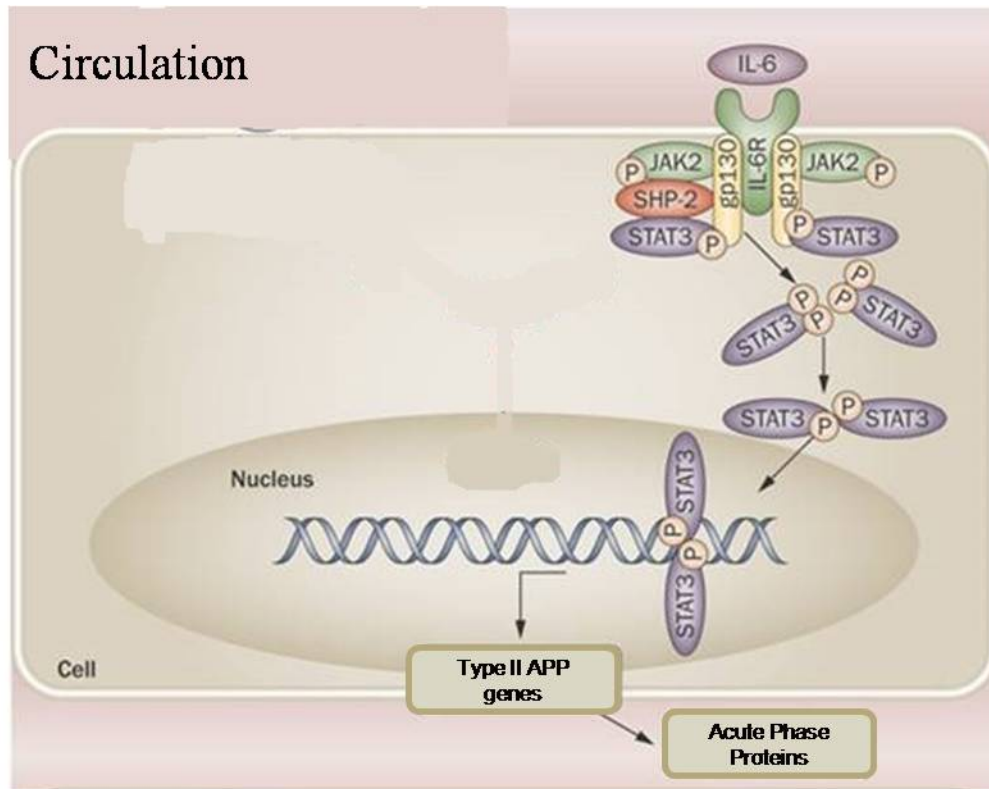
Figure 5: IL-6 stimulation induces the expression of a number of pro-inflammatory genes (Type II APP genes) products via activation of JAK/STAT3 pathway. Modified from:Walters and Griffiths, 2009.

1.6 Experimental model of acute phase study (ARP)

1.6.1 Animal Model of ARP

There are two well established animal models for the experimental study of the APR and acute phase mediated changes in acute phase proteins. Bacterial endotoxin lipopolysaccharide (LPS) administration leads to induction of APR by inducing systemic APR along with liver damage (Boelen et al. 2005;Ramadori et al. 1985). The other know model of APR, turpentine oil (TO) induced sterile muscle abscess without causing any detectable injury to liver and other tissues (Boelen et al. 2005;Ramadori et al. 1990;Tron et al. 2005;Wusteman et al. 1990). Thus, the TO-induced ARP model allows studying the effect of cytokines on liver produced at distant sites (Fig. 6). This model of ARP reproduces changes observed in human disease states (Basso et al,

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2005; Bodet et al, 2006; Boelen et al, 2005; Gabay and Kushner, 1999; Halter et al, 2005; Kim et al, 2002; Stoeck et al, 2006).
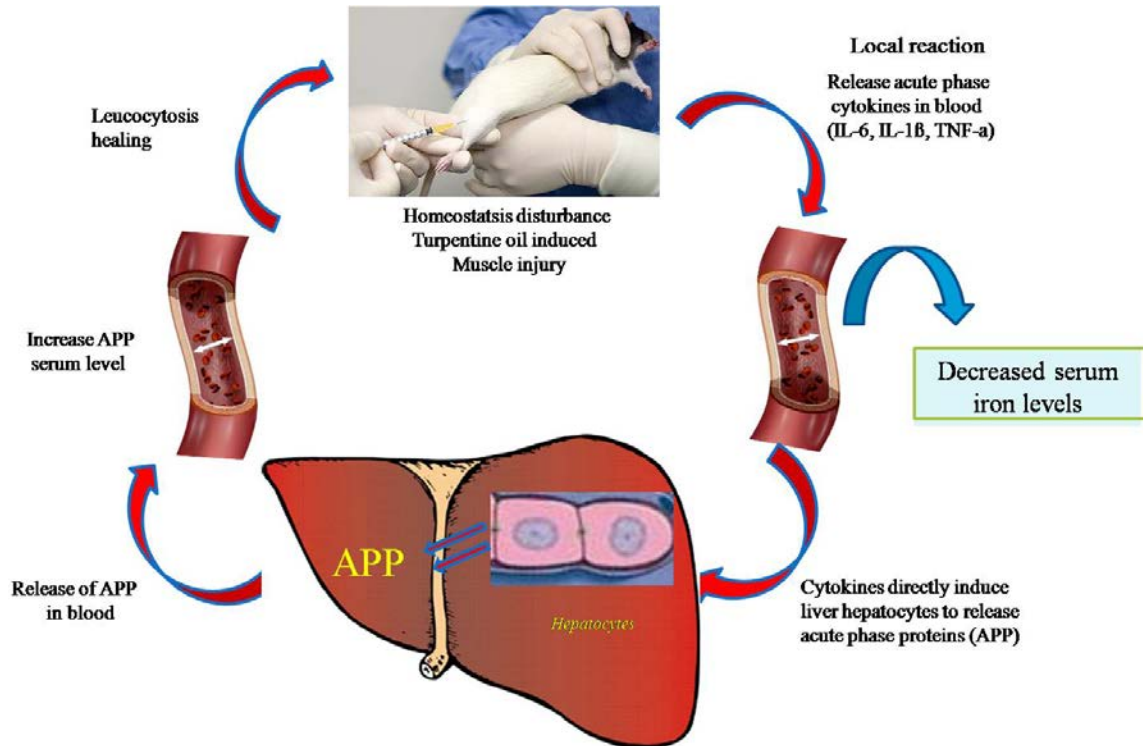
Figure 6: The acute phase model of study. Turpentine injection into the limb muscle results in cytokine production at the site of injury. These cytokines are released into the blood and when they reach the liver, liver becomes metabolically active and release acute phase mediators to trigger the healing process at the site of injury. The decreased iron concentration in serum is the hallmark of APR.

1.6.2   In vitro Model of ARP

When it became evident that liver is a primary target organ for the APR, hepatocytes cell culture was used to investigate a hierarchy of the events triggering the full APR in the liver. Besides the ability to respond to the cytokine, different cell types within the liver also express IL-1β, IL-6 , TNF-α, and other modulatory cytokines of the hepatic APR (Ramadori and Christ, 1999). Kupffer cells are the most active intra-hepatic "amplifiers" of the systemic APR in the liver by liberating a second wave of pro-inflammatory cytokines, promoting autocrine stimula-

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tion and paracrine hepatocyte stimulation (Decker, 1990). Hepatocytes express a great variety of receptors for cytokines, growth factors, and prostaglandins and therefore represent the major target for a multiple set of mediators involved in both systemic and local host defense reactions. Hepatocytes also express and secrete cytokines of the APR, which might further stimulate adjacent hepatocytes (Rowell et al., 1997).

1.7 Aims of study

Change in hepatic iron status is hall mark of APR. Turpentine oil (TO) induced APR model is well known for studying sterile local inflammation. TO injection leads to local inflammation with a subsequent systemic induction of APR mediated by cytokines (Ramadori and Christ, 1999; Ramadori et al, 1985). Sterile inflammatory processes develop through a cascade of events, char-acterized by a local increase of blood supply, small molecules, and proteins, leakage of fluids, and infiltration of inflammatory cells (Roitt and Delves, 2001). TO induces an aseptic local abscess without any damage caused to other organs (Boelen et al, 2005; Ramadori and Meyer zum Buschenfelde, 1990; Tron et al, 2005; Wusteman et al, 1990). This is clearly differ-ent from agents inducing an acute systemic response, like the administration of bacterial endo-toxin (lipopolysaccharide) (Boelen et al, 2005). Thus, the TO-induced acute-phase response model allows studying the effect of cytokines on the liver produced at distant sites.

Based on the literature and our previous findings it is known that sterile muscle abscess can cause a decrease in serum iron level in parallel to increase in hepatic iron content. Accordingly, it has also been reported that the intramuscular administration of TO induced the changes in gene expression of several proteins involved in iron metabolism in hepatic and extra-hepatic organs (Sheikh et al. 2007). However, the mechanisms governing hepatic iron metabolism under acute

phase conditions is poorly understood. In addition, previously we also localized several hepatic iron import and export proteins whereas the differential localization of these hepatic iron regulatory proteins in comparison to extra-hepatic organs has not been reported before.

Therefore, the perspective of this study was to compare sub-cellular localization of iron regulatory proteins in hepatic as well as extra-hepatic organs under physiological and acute-phase conditions. Further, it was also the aim of the current study to analyze the regulation and signaling pathways of these proteins during APR.

Therefore, following approaches were adopted to address the aims of the current study:

1. Investigate and compare the expression changes in iron transport- (TfR1, TfR2 and LCN-2) and iron storage proteins (ferritin) under the influence of acute phase cytokines (IL-6, IL-β and TNF-α) in the presence/absence of iron in primary culture of isolated rat hepatocytes; *in vitro*.

2. Demonstrate role of cytokines in iron uptake using primary cultures of isolated rat hepatocytes stimulated with acute phase cytokines (IL-6, IL-β and TNF-α) in the presence/absence of iron; *in vitro*.

3. Evaluate the role of IL-6, the principle mediator of inflammation, in the regulation of expression pattern of iron transport (TfR1, TfR2 and LCN-2) and iron storage proteins (FTH, FTL) in wild type and IL-6 knock-out mice models of acute phase response.

4. Investigate and compare the cellular localization of iron transport- (TfR1, TfR2 and LCN-2) and iron storage proteins (FTH, FTL) in liver, spleen, and heart in rat model of acute phase response.

2 MATERIALS

2.1 Chemicals

All the chemicals used in the study were of analytical grade and were purchased from commercial sources as indicated below:

TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) for isolation of RNA from the tissues; real-time polymerase chain reaction (PCR) primers, M-MLV reverse transcriptase, reverse transcription buffer and 0.1M DTT, platinum Sybr green qPCRUDG mix from Invitrogen, dNTPs, protector RNase inhibitor, bovine insulin, Klenow enzyme, primer oligo (DT)15 for cDNA synthesis and salmon sperm DNA from Roche (Mannheim, Germany). All other reagents and chemicals were from Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany). Mouse anti-transferrin receptor-1 from Invitrogen (Darmstadt, Germany), Rabbit anti-transferrin receptor-2 from Abcam (Cambridge, UK), Mouse anti-lipocalin-2 from Novus Biologicals, Goat anti-lipocalin-2 from R & D, Rabbit anti-STAT3 from Cell Signaling, Rabbit anti-pSTAT3 from Cell Signaling, FTH from LS Bio and Santa Cruz, Rabbit anti-FTL from Abcam, Mouse anti-beta-actin antibody from Sigma Aldrich (Germany) were used in the study. Among secondary antibodies; Horse-reddish-peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgGs from DAKO (Germany) and among Fluorescent Dye Conjugates Alexa Fluor-488 Donkey anti-goat, Donkey anti-mouse IgG and Alexa Fluor-555 Donkey anti-rabbit and Donkey anti-mouse IgG from Invitrogen (Darmstadt, Germany), were used in the study.

2.2   Animals and experimental models of acute-phase reaction

Male Wistar rats of about 170–200 g body weight were purchased from Harlan Winkel-mann (Brochen, Germany). Adult male B6.129S2-Il6[tm1Kopf] (IL6-knockout) mice and control wild type adult male C57BL/6J mice (25–28g) were purchased from Jackson Laboratory. The animals were kept under standard conditions with 12 h light/dark cycles and had *ad libitum* access to fresh water and food pellets. All animals were cared for according to the University´s guidelines, the German convention for the protection of animals and NIH guidelines.

In rats APR was induced in ether-anesthetized rats by intramuscular administration of 5 ml/kg TO in both right and left hind limbs (n=5). Control animals did not receive any administration (n=4). Injected and non-injected control rats were euthanized 1h, 2h, 4h, 6h, 12h, 24h and after TO administration under pentobarbital anesthesia. Liver, heart and spleen were excised and minced, rinsed with physiological sodium saline, snap frozen in liquid nitrogen and stored at -80°C till further use. Blood samples were collected from the inferior vena cava of the control and treated animals, allowed to clot overnight at 4°C and centrifuged for 20 min at 2000 g. Serum was removed and stored at -80°C.

For the mice experiments, a group of animals (n=3) for each strain, control (C57BL/6J) and IL6-knockout, was injected intramuscularly with 0.1 ml TO in both right and left hind limbs to induce an aseptic acute phase response. Animals were euthanized 2h, 4h, 6h, 12h and 24h after the treatments under pentobarbital anesthesia. Liver tissues were excised and minced, rinsed with physiological sodium saline, snap frozen in liquid nitrogen and stored at -80°C till further use.

3 METHODS

3.1 Methods of cell biology

3.1.1 Isolation of rat liver cells

3.1.1.1 Isolation of rat hepatocytes

Hepatocytes were isolated from male Wistar rats by circulating perfusion with collagenase essentially as described previously (Seglen 1972).

3.1.1.1.1 Liver perfusion

After laparotomy, the vena portae was canulated, vena cava inferior was ligated above the diaphragm to prevent flow of the perfusion media into a whole body circulation. Finally, the vena cava inferior was cut beneath the liver and canulated. The liver was perfused in nonrecirculative mode through the portal vein with 150-200ml CO_2-enriched preperfusion medium at a flow rate of 30ml/min until the liver was free from blood. To break down components of extracellular matrix, the liver was perfused in recirculative mode with collagenase perfusion medium until it started to feel soft (about 7-11 min).

3.1.1.1.2 Preparation of the hepatocytes suspension

After perfusion, the liver was excised and transferred into a sterile glass beaker filled with culture medium M 199 with additives. Glisson's capsule, i.e. collagen tissue around the liver, was carefully removed and discarded. To obtain a cell suspension, the tissue was disrupted mechanically using sterile forceps. Connective tissue and remainder of the liver capsule as well as big cell aggregates were removed by filtration of the primary cell suspension through a nylon mesh (pore-size 79µm). Non-parenchymal cells and cell debris were removed by numerous selective sedimentations (20 g, 2 min, 4°C) in wash medium. After the last centrifugation, hepato-

cytes were suspended in medium M 199 with additives. 50ml of M 199 was added per 1g of wet weight of the sedimented cells; the cell suspension typically had a density of about 106/2.5 ml.

3.1.1.1.3 *Primary culture treatment and harvesting of rat liver cells*

The cultures of rat hepatocytes were performed on 60mm polystyrol dishes and maintained at 37°C in a 95% air/ 5% CO_2 atmosphere and saturated humidity. Rat hepatocytes were processed further for cytokine stimulation.

3.1.1.1.4 *Primary culture of rat hepatocytes*

Immediately after preparation, fetal calf serum (4 ml/100 ml suspension) was added to the hepatocytes suspension in order to make the efficient cell adhesion to the polystyrol dishes. Furthermore, the antibiotics (1 ml of pen/strep stock solution per 100 ml cell suspension) together with 10-7 M dexamethasone and 10-9 M insulin as permissive hormones were added. Rat hepatocytes were plated onto 60-mm plastic dishes at a density of 2×10^6 cells per dish. After the initial 4 h of attachment phase, the medium was changed, and the hepatocytes were further incubated in medium M 199 with the same concentrations of hormones and antibiotics used previously but without fetal calf serum. A volume of 2.5 ml medium per 60mm culture dish was added.

3.1.1.1.5 *Stimulation of hepatocytes with acute phase cytokines and iron*

Primary rat hepatocytes were stimulated with pro-inflammatory cytokines (IL-1β 100ng/ ml, IL-6 500ng/ml, and TNF-α 100ng/ml concentrations) in the presence/absence of iron (0.1mM) or different concentrations of iron ($FeCl_3$:0.01mM, 0.1mM and 0.5mM) alone, on the next day of plating. The medium was changed 6 hours prior to stimulation; the stimuli were diluted to the required concentrations in the culture medium and added directly to the culture dishes by pipetting. An equal volume of the normal culture medium was added to the dishes with

cells which later served as experimental controls. After addition of the stimuli, cells were incubated in the incubator prior to RNA and protein isolation. The cells were harvested at different time points (0, 6, 12 and 24h), washed with phosphate buffered saline, pH 7.4 and frozen at −80°C for subsequent RNA and protein isolation.

3.2 Methods in molecular biology

3.2.1 RNA isolation

During the whole study, total RNA was isolated from liver, brain, spleen and injured muscle tissue samples with TRIzol reagent according to the manufacturer's instructions. Briefly, tissue samples were homogenized in 1 ml TRIzol reagent per 100mg of tissue using a power homogenizer. After 5 minutes incubation at room temperature, 0.2ml chloroform per sample was added and samples were vigorously shaken and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000g for 30 minutes and the acqueous phase so obtained in the supernatants was carefully collected. A volume of 0.5ml of isopropyl alcohol was succevssively added to the collected phase and samples were incubated at room temperature for 10 minutes. The RNA precipitation was favoured centrifuging again at 12,000g for 10 minutes. After a short washing step in ethanol 75%, RNA was shortly air-dried and resuspended in a volume of approximately 50μl of RNase and Dnase free water. The RNA was then quantified by measuring the absorbance at 260nm/280nm.

3.2.2 Reverse transcription (RT)

The cDNA was generated by reverse transcription of 3.0 g of total RNA with 100nM of dNTPs, 50pM of primer oligo(dT)15, 200U of moloney murine leukemia virus reverse transcrip-

tase (M-MLV RT), 16U of protector RNase inhibitor, 1X RT buffer and 2.5 ml of 0.1M DTT for
1 h at 40°C.

3.2.3   Real time quantitative Polymerase chain reaction

The cDNA samples are analyzed by the Real Time PCR using the following ingredients for
each PCR reaction:

Volume per reaction

"X" primer-forward (5mM)   1.5µl

"X" primer-reverse (5mM)   1.5 µl

H_2O 6.5µl

SYBR Green Master Mix Taq Polymerase   13.0 µl

2.5µl of the cDNA sample or 2.5µl of H_2O for the negative control was added to each PCR reac-
tion.

3.2.4   Thermal cycler amplification program

The amplification was performed at 95°C for 20 seconds, 95°C for 3 seconds to 60°C for
30 seconds for 40 thermal cycles in an ABI prism 7000 sequence detection system. All samples
were assayed in duplicate. Expression of different genes was analyzed using Platinum SYBR
Green qPCR mix UDG. The PCR amplification program was followed by dissociation curve
protocol for controlling the specificity of the PCR products. Specific temperature of dissociation
of the PCR product was calculated by the Primer Express software. Curves of amplification were
analyzed to measure the Ct value in the linear range of the amplification. The results were nor-

malized to the house keeping gene and fold change expression was calculated using Ct values by Prism Graph Pad 5 software.

3.2.5   Standard Curve

Serially diluted PCR products, of the gene of interest are amplified by Real Time PCR and Ct values are calculated. The standard curves were obtained graphically by using the following parameters: Ct values and the logarithm of the number of copies. Standard curve were calculated for the gene of interest and the housekeeping genes. Number of copies of gene in the sample was obtained by extrapolation of the measured Ct value to the relative standard curve. Normalization of gene expression in each sample was performed by calculating the ratio of number of copies of the gene of interest with respect to the number of copies of the housekeeping gene.

3.2.6   Primers designing

Primers for different genes were designed using the program "Primer Express" (ABI System) and the gene bank data (http://www.ncbi.nlm.nih.gov).

3.3   Methods of biochemistry

3.3.1   Protein extraction from liver tissue and cultured hepatocytes

3.3.1.1   Preparation of tissue homogenates

All steps were performed at 4°C to prevent proteolytic degradation of the proteins. About 100mg of frozen tissue was homogenized with Ultra-Turrax TP 18/10 model homogenizer 3times for 10sec each in 10 volumes of 50mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM PMSF, 1mM benzamidine, 1µg/ml leupeptin, 10µM chymostatin, 1µg/ml antipain, 1µg/ml pepstatin A. Crude homogenates were passed 5 times through a 22G administration canula connected to a syringe. To pellet the nuclei and particular

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matter, crude homogenates were centrifuged for 5 min at 10,000g (4°C). Protein concentration of supernatants was determined by the bicinchoninic acid (BCA) method (Smith et al. 1985) using the BCA protein assay reagent kit (Pierce, Bonn, Germany). Prepared homogenates were dispensed in aliquots and stored at −20°C until use.

3.3.1.2   *Preparation of cell lysate*

All steps of the procedure were performed at 4°C to prevent proteolytical degradation of the proteins. The cells frozen on the culture dishes were thawed on ice. 1X ice-cold lysis buffer, comprised of 150mM NaCl, 1mM EDTA, 1% Triton X-100, 50mM Tris-HCl, pH 7.4 and supplemented with protease inhibitors, was added to the cells (500μl per 6 cm dish) followed by incubation on ice for 10 min. Afterwards, the cells were scraped with a disposable scraper, transferred to new tubes and passed 5 times through a 22G administration canula connected to a syringe. To pellet the nuclei and particular matter, prepared lysates were centrifuged for 5 min at 10,000g (4°C) and the protein concentration of supernatants was determined by BCA method using the kit from Pierce. Prepared lysates were aliquoted and stored at −20°C until use.

3.3.2   *Proteins isolation and Western Blot*

Fifty micrograms from the total protein lysate were loaded in a 4-12% Nu-PAGE BisTris (Invitrogen) gel and separated after 2 hours electrophoresis at 80V. After the transfer in a semi-dry apparatus at 26V for 1.5h, the membranes were blocked in 5% milk, and blotted with primary antibodies overnight at 4°C. The secondary antibodies were horse reddish peroxidase conjugated goat anti-rabbit, goat anti-mouse and rabbit anti-goat immunoglobulins (DAKO) diluted at 1:2000. Membranes were developed with ECL chemiluminescence Kit (Amersham).
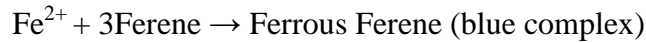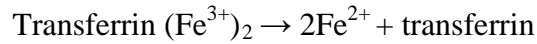
3.3.3 *Immunohistochemistry*

Immunohistochemical analysis was performed on 4µm thin cryostat sections fixed in methanol/acetone to localize the antigens on the tissues. After blocking non-specific binding with a solution of PBS containing 1% bovine serum albumin (Serva, Heidelberg, Germany) and 10% donkey serum (Abcam) for 1 h at room temperature, the primary antibodies (TfR1, TfR2, LCN-2, FTH and FTL) were incubated overnight at 4°C on the sections. The mouse monoclonal (TfR1) and rabbit polyclonal (TfR2) antibodies were detected with an Alexa Fluor-555 conjugated donkey-anti-rabbit/donkey anti-mouse and goat polyclonal (LCN-2), rabbit polyclonal (TfR2) and mouse monoclonal (LCN-2) antibodies with Alexa Fluor-488 conjugated secondary antibody from Invitrogen (Darmstadt, Ger-many). For double-staining, each of the TfR1 and TfR2 primary antibodies was incubated with goat/mouse anti LCN-2 (R & D) (diluted 1:100) overnight at 4°C. A mix of Alexa Flour-555-conjugated donkey-anti-rabbit, donkey-anti-mouse and FITC/ Alexa Fluor-488-conjugated anti-goat Igs from (Invitrogen Darmstadt, Germany) was successively incubated for 1h at room temperature in dark. Sections were counter-stained with DAPI (Molecular Probes, Invitrogen) and observed with an epifluorescence microscope (Axiovert 200M, Zeiss, Germany). In peroxidase staining antigens were visualized using HRP-conjugated secondary antibodies. Negative control immunostainings were performed by omission of the primary antibody, by using isotype of matching control immunoglobulins.

3.4 Methods in clinical chemistry

3.4.1 Iron measurement

3.4.1.1 Principle

Iron bound to transferrin is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence o ascorbic acid. Ferrous iron forms a blue complex with ferene. The absorbance at 595nm is directly proportional to the iron concentration.

$$\text{Transferrin } (Fe^{3+})_2 \rightarrow 2Fe^{2+} + \text{transferrin}$$

$$Fe^{2+} + 3\text{Ferene} \rightarrow \text{Ferrous Ferene (blue complex)}$$

3.4.1.2 Reagents

		Final concentration
R1:	Acetate buffer pH 4.5	800mM/l
	Thiourea	90mM/l
R2:	Ascorbic acid	45mM/l
	Ferene	0.6mM/l
	Thiourea	20mM/l
	Standard	100µg/dl (17.9µM/l)

Should be protected from light, reagents are stable at 2-25°C until the expiry date.

3.4.1.3 Assay Procedure

To determine the hepatocytes iron level, 100µl of the sample along with 1000µl of the reagent1 was taken in the reaction tube. For blank 100µl of dd H_2O was used. The sample and reagent 1 was mixed thoroughly, incubated for 5 minutes and read the absorbance (A1). After that

250µl of the Reagent 2 was added, mixed thoroughly and the absorbance (A2) was read after 10 minutes of incubation.

3.4.1.4 Calculations

$$\Delta A = [(A2 - 0.82\ A1)\ sample/Std.] - [(A2 - 0.82A1)\ blank]$$

The factor 0.82 compensates the decrease of the absorbance by addition of reagent 2. The factor is calculated as follows:

$$(Sample + R1)/Total\ volume.$$

This compensation is necessary as a high sample volume is used.

$$Iron\ [\mu g/dl] = \Delta A\ Sample/\Delta A\ Std/Cal \times Conc.\ Std.\ /Cal\ [\mu g/dl]$$

$$Conversion\ factor\ Iron\ [\mu g/dl] \times 0.1791\ [\mu M/l]$$

3.4.1.5 Measuring range

The test has been developed to determine iron concentrations within a measuring range from 5-1000µg/dl (0.9-179µM/l). When values exceed this value samples should be diluted 1 + 2 with NaCl solution (9g/l) and the results multiplied by 3.

3.4.1.6 Specificity and sensitivity

The test was specific and no interference was observed by conjugated and free bilirubin up to 60 mg/dl, hemoglobin up to 100 mg/dl, lipemia up to 2000 mg/dl triglycerides, and copper up to 200µg/dl. The lower limit of detection for the test is 2µg/dl (0.4µM/l).

3.5 Statistical analysis

The data were analyzed using Prism Graph pad 5 software (San Diego, USA). All experimental errors are shown as SEM. Statistical significance was calculated by Student´s t test and one way ANOVA. Significance was accepted at *P < 0.05 and *P< 0.0125.

4 REGULATION OF IRON UPTAKE IN PRIMARY CULTURE RAT HEPATO-CYTES: THE ROLE OF ACUTE PHASE CYTOKINES

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REGULATION OF IRON UPTAKE IN PRIMARY CULTURE RAT HEPATOCYTES: THE ROLE OF ACUTE-PHASE CYTOKINES

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ABSTRACT—Decreased serum and increased hepatic iron uptake is the hallmark of acute-phase (AP) response. Iron uptake is controlled by iron transport proteins such as transferrin receptors (TfRs) and lipocalin 2 (LCN-2). The current study aimed to understand the regulation of iron uptake in primary culture hepatocytes in the presence/absence of AP mediators. Rat hepatocytes were stimulated with different concentrations of iron alone (0.01, 0.1, 0.5 mM) and AP cytokines (IL-1β, IL-6, TNF-α) in the presence/absence of iron (FeCl₃:0.1 mM). Hepatocytes were harvested at different time points (0, 6, 12, 24 h). Total mRNA and proteins were extracted for RT-PCR and Western blot. A significant iron uptake was detected with 0.1 mM iron administration with a maximum (133.37±4.82 µg/g of protein) at 24 h compared with control and other iron concentrations. This uptake was further enhanced in the presence of AP-cytokines with a maximum iron uptake (481±25.81 µg/g of protein) after concomitant administration of IL-6+iron to cultured hepatocytes. Concomitantly, gene expression of LCN-2 and ferritin subunits (light- and heavy-chain ferritin subunits) was upregulated by iron or/and AP-cytokines with a maximum at 24 h both at mRNA and protein levels. In contrast, a decreased TfR1 level was detected by IL-6 and iron alone, whereas combination of iron and AP-cytokines (mainly IL-6) abrogated the downregulation of TfR1. An increase in LCN-2 release into the supernatant of cultured hepatocytes was observed after addition of iron/AP cytokines into the medium. This increase in secretion was further enhanced by combination of IL-6+iron. In conclusion, iron uptake is tightly controlled by already present iron concentration in the culture. This uptake can be further enhanced by AP-cytokines, mainly by IL-6.

KEYWORDS—Transferrin receptors, lipocalin 2 (LCN-2), acute-phase cytokines (IL-1β, IL-6, TNF-α), FeCl₃

INTRODUCTION

Despite the abundance of iron in nature and in the human body, iron absorption, transport, storage, and excretion are tightly regulated. Within the cell, iron is mainly stored in the form of ferritin (1). In human, ferritin is composed of two subunits: the light-chain ferritin subunit (FTL; with 125 amino acids, 19 KDa) and the heavy-chain ferritin subunit (FTH; 183 amino acids, 21 KDa). Both subunits are highly conserved (2); nevertheless, they are genetically separate (3) and maintain distinct functions (4).

Iron homeostasis is controlled by a large group of iron regulatory proteins including ferroportin 1 (5), transferrin receptors (TfR1, TfR2) (6), hepcidin (7), and hemojuvelin (8). In fact, transferrin (Tf)-bound iron is imported into the reticuloendothelial system, to liver parenchymal cells and to all proliferating cells in the body after binding to TfRs. Interaction of diferric-Tf with TfRs and internalization of the complex by receptor-mediated endocytosis leads to iron uptake into the cells (9). As a result, Tf efficiently transports the majority of iron into the cells (10). However, there has been convincing evidence that, in situations of disrupted iron homeostasis, iron can also be delivered to cells by alternative, Tf-independent mechanisms. This alternative to Tf iron is called non-Tf-bound iron (NTBI)

This pool has been documented in a variety of iron overload syndromes when Tf is saturated (11), including hemochromatosis (69% of patients) and end-stage renal disease (22% of patients) (12); the identification of the components of NTBI, however, remains elusive.

Lipocalin 2 (LCN-2; human ortholog neutrophil gelatinase-associated lipocalin) has been proposed to be a mediator of the Tf-independent iron delivery pathway (13). It belongs to the lipocalin family, which is known to be involved in the regulation of immune responses, modulation of cell growth and metabolism, prostaglandin synthesis, and iron transportation (13). In fact, it is a bacteriostatic agent and capable of sequestering iron in the form of siderophores (14). A recent study has shown that LCN-2 siderophore-iron complexes can transport iron into cells during kidney development (10).

Acute-phase response (APR) is the systemic reaction to tissue injury and inflammation. It is clinically characterized by systemic symptoms such as fever, weakness, anemia, somnolence loss of appetite, and cytokines release (15). In the blood, it results in an increase in the plasma levels of a number of positive acute-phase proteins (APPs), including clotting proteins, transport proteins, antiproteases, and complement factors, with a concomitant decrease in negative APPs such as albumin (16). In addition, a decrease in serum iron levels and consecutive increase in hepatic iron levels are also a hallmark of APR. This reaction is mediated by both interleukin 1 (IL-1)-like cytokines (IL-1, tumor necrosis factor α [TNF-α]) and IL-6-like cytokines (IL-6, oncostatin M, and others), through the activation of different transcription factors.

The liver is a major site of iron storage, and this iron-storage function is achieved by a tight control of bidirectional exchange of liver iron with plasma iron. Although the effect of cytokines on increased iron uptake has already been reported (17), and there have been several reports on iron regulation and metabolism, the mechanism of iron uptake is still poorly understood in the liver.

Previously, we showed *in vivo* that expression of acute-phase (AP) cytokines (IL-1β, IL-6, TNF-α) increased during inflammation, delivered from the site of injury into the blood, can induce changes in expression of iron-regulatory, including iron-storage proteins (18). Furthermore, a decreased serum and increased hepatic iron content during AP conditions were also observed. This sponge (iron uptake) effect of the liver during AP conditions was supposed to be not only due to a change in gene expression of iron regulatory proteins but also due to AP cytokines.

In the current study, we wanted to explore the role of AP cytokines in iron uptake and the changes in gene expression of iron transport and storage proteins in the absence or presence of AP cytokines in primary culture of rat hepatocytes.

MATERIALS AND METHODS

Animals

As sex difference in cytokine secretion has been reported before (19), male Wistar rats (8-12 weeks old, 170-200 g body weight) were purchased from Harlan Winkelmann (Brochen, Germany). The animals were kept under stan- dard conditions with 12:12-h light-dark cycles and were given *ad libitum* access to water and food. All animals were cared for in accordance with the guidelines of the German Convention for the Protection of Animals and the US National Institutes of Health.

Hepatocytes isolation and treatment

For each experiment, two to four rats were killed under pentobarbital sodium (50 mg/kg) anesthesia according to the university's guidelines and German regulations for the protection of animals, and hepatocytes were isolated from these animals as described earlier (20). Isolated cells from different animals were pooled and plated for cytokine and/or iron treatment. Then, these isolated hepatocytes were incubated at 37°C in an atmosphere containing 95% air and 5% CO, Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (PAA, Colbe, Germany),1 nmol/L insulin, and 100nmol/L dexamethasone (Sigma-Aldrich, Munich, Germany) for 48 h to obtain con- fluence and to reduce the stress of isolation procedure according to established protocol of our laboratory (20). Afterward (48 h), hepatocytes were divided into three groups: one was administrated with different AP cytokines, i.e., IL-6 (500 ng/mL), IL-1β (100 ng/mL), TNF-α (100 ng/mL) (PeproTech GmbH, Hamburg, Germany). The second group received different concentrations (0.10.1, 0.5 mM) of FeCl3 (Sigma-Aldrich) in serum-free medium containing 0.2% bovine serum albumin up to 24 h. Then, in the third group, cytokines IL-6 (500 ng/mL), IL-1β (100 ng/mL), and TNF-α (100 ng/mL) were supplemented concomitantly with FeCl3 (0.1 mM) (21,22) to cultured hepatocytes in serumfree medium containing 0.2% bovine serum albumin. Each experiment was repeated four times (n=4) in duplicates, cells wereafter treatment, and duplicates were pooled down in one Eppendorf tube for protein and mRNA isolation.

Measurement of hepatocytes iron contents

The iron contents of the cells at different time points after treatment were measured by colorimetric FerroZine-based assay (23). Iron contents were mea- sured as μg/g of hepatocytes protein and analyzed using Microsoft Excel 2007.

Isolation of total RNA and PCR analysis

Total RNA was isolated from the hepatocytes samples with Trizol reagent according to the manufacturer's instructions. RNA was then measured at an absorbance of 260/280nm. The cDNA was generated by reverse transcription of RNA using the superscript kit (Invitrogen, Carlsbad, Calif). Real-time po- lymerase chain reaction (RT-PCR) was performed at 95-60°C for 40 thermal cycles using the Step One Plus real-time PCR cycler (Applied Biosystems, Darmstadt, Germany). Quantification of cDNA was done by relative quantification βusing SYBR Green UDG reaction master mix (Invitrogen,Darmstadt, Germany). Table 1 shows the list of primers, which have been gene specifically synthesized (Invitrogen). β-Actin mRNA is used as a housekeeping gene with every sample. The results in the form of threshold cycle values were normalized to control values and relative to the expression of β-actin.

Protein extraction and Western blot analysis

Proteins were isolated from the hepatocytes of different time points as de-scribed previously (24). Protein contents were calculated by the Coomassie protein assay (Pierce, Germany). Cell supernatant (culture medium in which hepatocytes were cultured) as whole and cell lysates were used for Western blot analysis. Western blot was performed with sodium dodecyl sulfate polyacryl-amide under reducing conditions as described (25) with β-actin as a loading control. The proteins were then transferred onto Hybond ECL nitrocellulose hybridization transfer membranes as described (26). Immunodetection studies have been performed according to the ECL Western blotting protocol of GE Healthcare (Dornstadt, Germany). The antibodies used are listed in Table 2.

Statistical analysis

The data were analyzed using GraphPad Prism 4 software (San Diego, Calif). All experimental errors are shown as SEM. Statistical significance was calculated by Student t-test. Significance was accepted at $P \leq 0.05$. Bonferroni correction was applied to iron uptake data set obtained from FeCl3 treatment groups to prevent accumulation of and significance was accepted at $P \leq 0.0125$ ($\alpha/4$, n = 4).

RESULTS

Lactate dehydrogenase measurement

The cellular damage of iron on hepatocytes was detected by measuring lactate dehydrogenase in the hepatocytes culture medium at all studied time points. All the treatments (iron and cytokines) showed less than 5% lactate dehydrogenase secretion, indicating no cell damage to control and treated hepatocytes (data not shown).

Iron uptake in hepatocytes

To find the optimal iron concentration for studying iron uptake, different concentrations of iron (0.01, 0.1, and 0.5 mM) were administered to the culture medium. The 0.1mM concentrationwas found to be the most efficient for iron uptake in isolated hepatocytes compared with 0.01 & 0.5mM.

T_{ABLE} 1. Primers used in the study

Primer	Forward 5´→ 3´	Reverse 5´→ 3´
LCN-2	GGA ATA TTC ACA GCT ACC CTC	TTG TTA TCC TTG AGG CCC AG
TfR1	ATA CGT TCC CCG TTG TTG AGG	GGC GGA AAC TGA GTA TGG TTG A
TfR2	AGC TGG GAC GGA GGT GAC TT	TCC AGG CTC ACG TAC ACA ACA G
FTH	GCC CTG AAG AAC TTT GCC AAA T	TGC AGG AAG ATT CGT CCA CCT
FTL	AAC CAC CTG ACC AAC CTC GCT A	TCA GAG TGA GGA GCT CAA AGA G
β-Actin	TGT CAC CAA CTG GGA CGA TA	AAC ACA GCC TGG ATG GCT AC

TABLE 2. Antibodies used in the study

Antibodies	Company	Reference No.	Dilution
LCN-2	R&D	AF3508	1:300
TfR1	Invitrogen	13-6890	1:1,000
FTH	LS Bio	LS-C23537	1:500
FTL	Abcam	ab69090	1:1,000
β-Actin	Sigma	A-2228	1:5,000

Therefore, we used 0.1mM concentration for costimulatory (AP cytokines+iron) experiments. In iron-treated hepatocytes, the maximum iron uptake was measured (133.37±4.82 μg/g of protein) at 24 h at a concentration of 0.1 mM iron, which was approximately two times higher compared with untreated controls. Other concentrations of iron (0.01 and 0.5 mM) also showed a significantly increased iron uptake; however, the magnitude of iron uptake using iron concentration 0.1 mM was the maximum among all alone iron-treated hepatocytes (Fig. 1A).

In cytokine-treated hepatocytes (IL-1β, IL-6, TNF-α), no significant uptake of iron was observed (Fig. 1B). However, a significant uptake of iron in hepatocytes was observed when iron was supplemented to the culture medium together with the AP cytokines. The maximum iron uptake was detected by addition of iron and IL-6 together in the culture medium, which was approximately eight times (481±25.81 μg/g of protein at 24 h) higher than untreated controls. Similar increase was also found in hepatocytes exposed to IL-1β+iron (118.91± 10.58 μg/g of protein) and TNF-α+iron (106.91±6.91 μg/g of protein) at 24 h, whereas the magnitude of iron uptake was higher in hepatocytes where iron was added together with IL-6 (Fig. 1C).

Changes in amount of LCN-2 in cultured rat hepatocytes

Cultured rat hepatocytes were exposed to different iron concentrations (0.01, 0.1, 0.5 mM) to observe the changes in LCN-2 at mRNA and protein levels. Addition of alone iron into the hepatocytes culture medium sign ificantly increased the LCN-2 gene expression at mRNA and protein levels. A significant upregulation for LCN-2 gene expression was detected at 6 h on mRNA and protein levels (Fig. 2, A and D). However, the max- imum induction of LCN-2 gene expression was found at 12 h with an iron concentration of 0.1 mM (1.95±0.16-fold) followed by 0.5 mM (1.71±0.304-fold) and 0.01 mM (1.52±0.10-fold).

Similarly, hepatocytes treated with AP cytokines (IL-1β, IL-6, and TNF-α) showed an increase in LCN-2 transcripts (Fig. 2B). Interleukin 6Ytreated hepatocytes showed an early increase in LCN-2 at 6 h (5.01±0.24-fold), which remained upregulated until 24 h compared with untreated hepatocytes. A late (24 h) but significant induction of LCN-2 was detected after adminis- tration of IL-1α (3.53±0.14-fold), whereas TNF-α treated he- patocytes showed a minor change in gene expression of LCN-2 at mRNA level (Fig. 2B).

In the third group (AP cytokines+iron), the upregulating effect of alone iron or AP cytokines was further enhanced by the addition of AP cytokines together with iron into the culture medium. The most pronounced increase was detected at 24 h by IL-6+iron (11.85±1.01-fold) and IL-1β+iron (12.81±2.13-fold) (Fig. 2, C and D), whereas TNF-α+iron showed the least pronounced change of LCN-2 gene expression in hepatocytes.

RT-PCR results were further con- firmed by Western blot by using a specific antibody against LCN-2. Reflecting the results at transcript level, an increased protein level of LCN-2 was revealed after administration of iron and/or AP cytokines (Fig. 2D).

Changes in the amount of TfRs in cultured rat hepatocytes

Alone iron administration to the hepatocytes culture medium decreased the gene expression of TfR1 at mRNA level. The impact of 0.5 mM concentration of iron was stronger than other concentrations in cultured hepatocytes (Fig. 3A) These data were also confirmed at protein level (Fig. 3D).

In group of AP cytokines, IL-1β+treated hepatocytes showed a significant increase in gene expression of TfR1 at 24 h (7.48 ±0.08-fold) in comparison to untreated controls. In contrast, a reduced expression of TfR1 was detected after IL-6 adminis- tration with a minimum (0.44 ±0.08-fold) at 6 h. TNF-α-treated hepatocytes did not show any considerable change at any studied time point as compared with untreated controls (Fig. 3B). These data were also confirmed at protein level (Fig. 3D).

Contrary to iron treatment alone, the observed downregulating effect of iron on TfR1 transcripts was completely abrogated when iron was administrated together with IL-6 to the culture medium.

However, IL-1β and TNF-α treatment showed a further in- crease in TfR1 transcripts in the presence of iron (Fig. 3C). By using a specific antibody against TfR1, these results were further confirmed at protein level, although the reduction in TfR1 at protein level was observed with a delay compared with mRNA level (Fig. 3D).
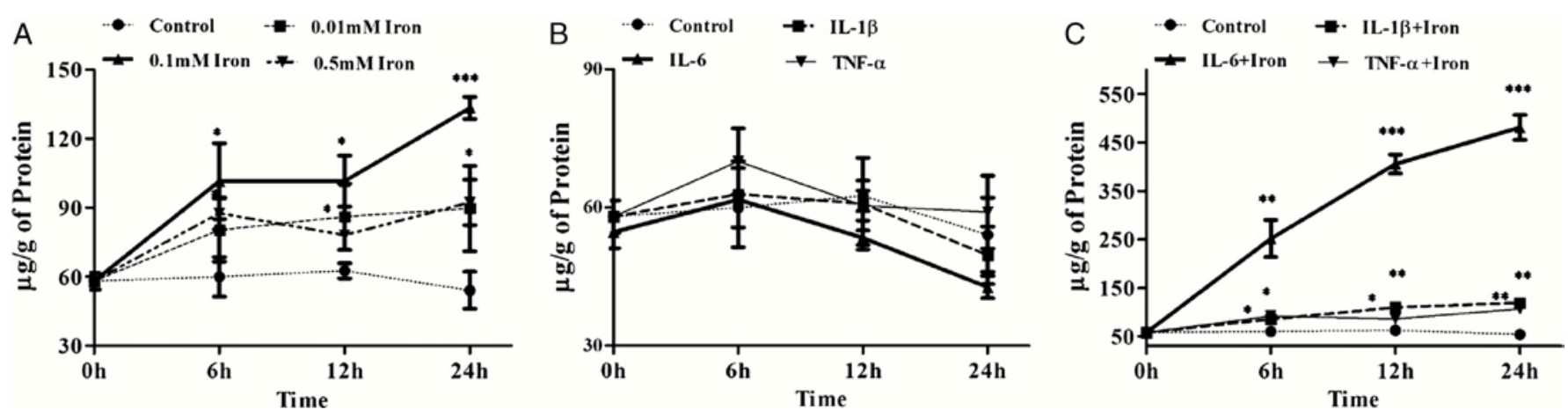
Fig.1. Measurement of iron uptake in hepatocytes: iron-treated hepatocytes (A). Cytokines treated hepatocytes (B). Cytokines+iron-treated hepatocytes (C). Values on y axis represent the intracellular iron contents of hepatocytes compared with nontreated controls. Results representthe mean value±SEM. *P ≤ 0.0125, **P ≤ 0.001, ***P ≤ 0.0001 analyzed by t-test (n = 4).
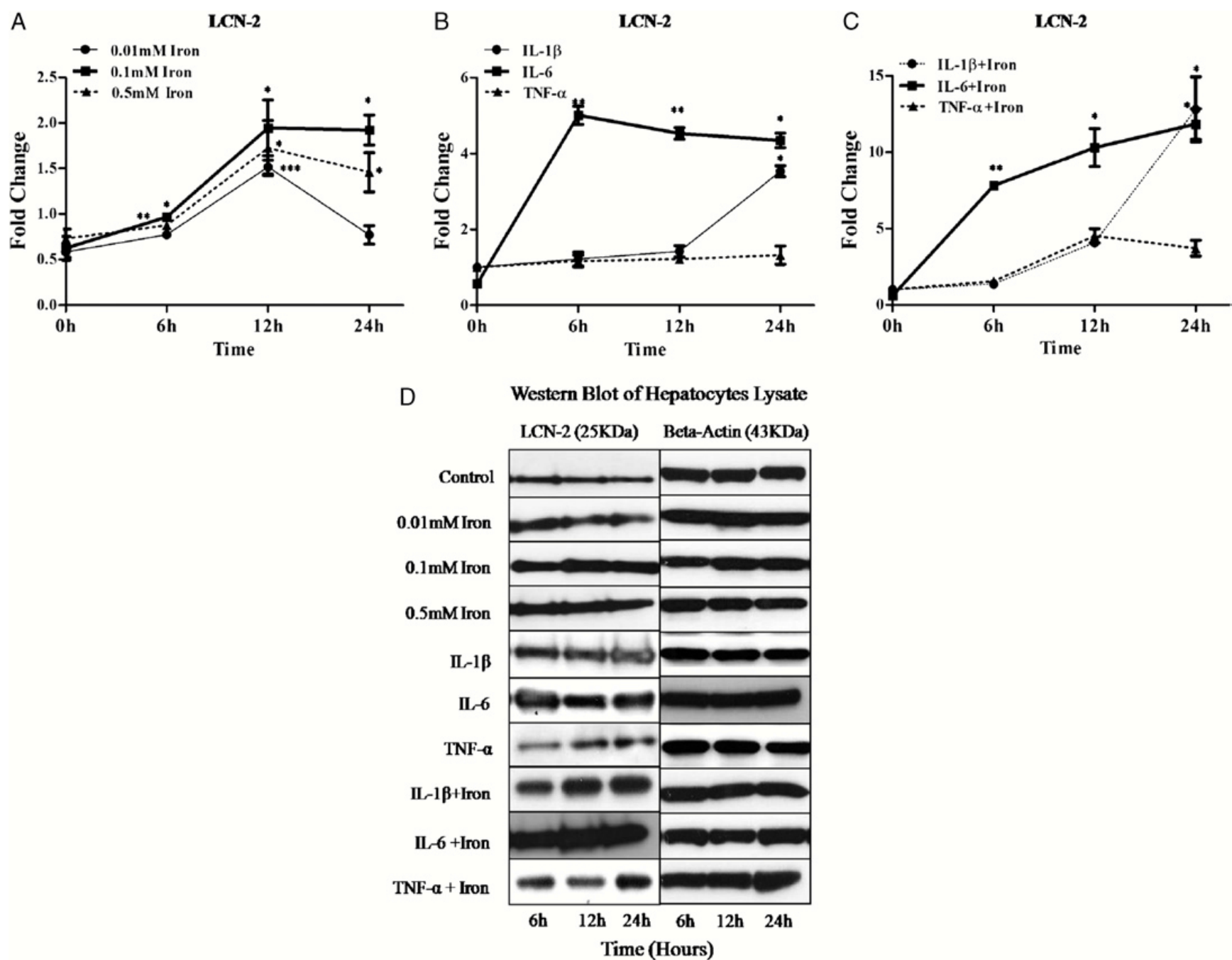
Fig .2. Quantitative RT-PCR analysis of mRNA from rat hepatocytes. Fold change in mRNA expression of LCN-2 gene expression after iron treatment (A), cytokine treatment (B), cytokines+iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value±SEM. *P ≤ 0.0125, **P ≤ 0.001, ***P ≤ 0.0001 analyzed by t-test (n = 4). Western blot analysis of LCN-2 (25 KDa) from total protein of rat hepatocytes. β-Actin (43 KDa) was used as a marker for equal loading (D).

However, IL-1β and TNF-α treatment showed a further increase in TfR1 transcripts in the presence of iron (Fig. 3C). By using a specific antibody against TfR1, these results were further confirmed at protein level, although the reduction in TfR1 at protein level was observed with a delay compared with mRNA level (Fig. 3D).

In contrast to TfR1, TfR2 gene expression was increased by different iron concentrations (Fig. 4A). Interleukin 6 treatment also increased TfR2 gene expression significantly at 6 h, with a maximum expression at 24 h (7.2±1.01-fold), compared with untreated hepatocytes. IL-1β showed a late significant increase (at 24 h), whereas minor changes were observed in TfR2 gene expression after TNF-α treatment in hepatocytes (Fig. 4B).

In hepatocytes treated with a combination of iron and AP cytokines together, IL-6 + iron elicited an early increase in TfR2 transcripts (1.94 ± 0.31-fold) with a maximum at 24 h (7.32 ±0.61-fold), which was followed by TNF-α +iron (7.38±1.5-fold) and IL-1β + iron (4.73±0.1-fold) (Fig. 4C). Treatment of iron and AP cytokine together showed a synergetic effect on TfR2 gene expression as compared with iron or AP cytokine

treatment alone. We could not detect the expression of TfR2 at protein level in control as well as treated hepatocytes. This might be a problem of antibody sensitivity.

Modulation of ferritin subunits in cultured rat hepatocytes after treatment with iron and AP cytokines

In iron-treated hepatocytes, a time-dependent increase in the FTL was detected both at mRNA and protein levels with a peak at 24 h (2.48±0.195-fold) after administration of 0.5 mM iron into the culture medium (Fig. 5A).

The effects of AP cytokines (IL-1β, IL-6, TNF-α) on FTL and FTH were examined in the second group. mRNA analysis of ferritin subunits in rat hepatocytes showed a mild increase in FTL gene expression with a maximum at 24 h upon cytokine (IL-1β, IL-6, TNF-α) treatment (Fig. 5B). This increase was better visible at protein level, where cytokines showed an early increase, which persisted until 24 h compared with un- treated controls. The most pronounced induction was observed by IL-1β (Fig. 5D). Iron-treated hepatocytes showed a higher induction of FTL than administration of cytokines alone. No significant
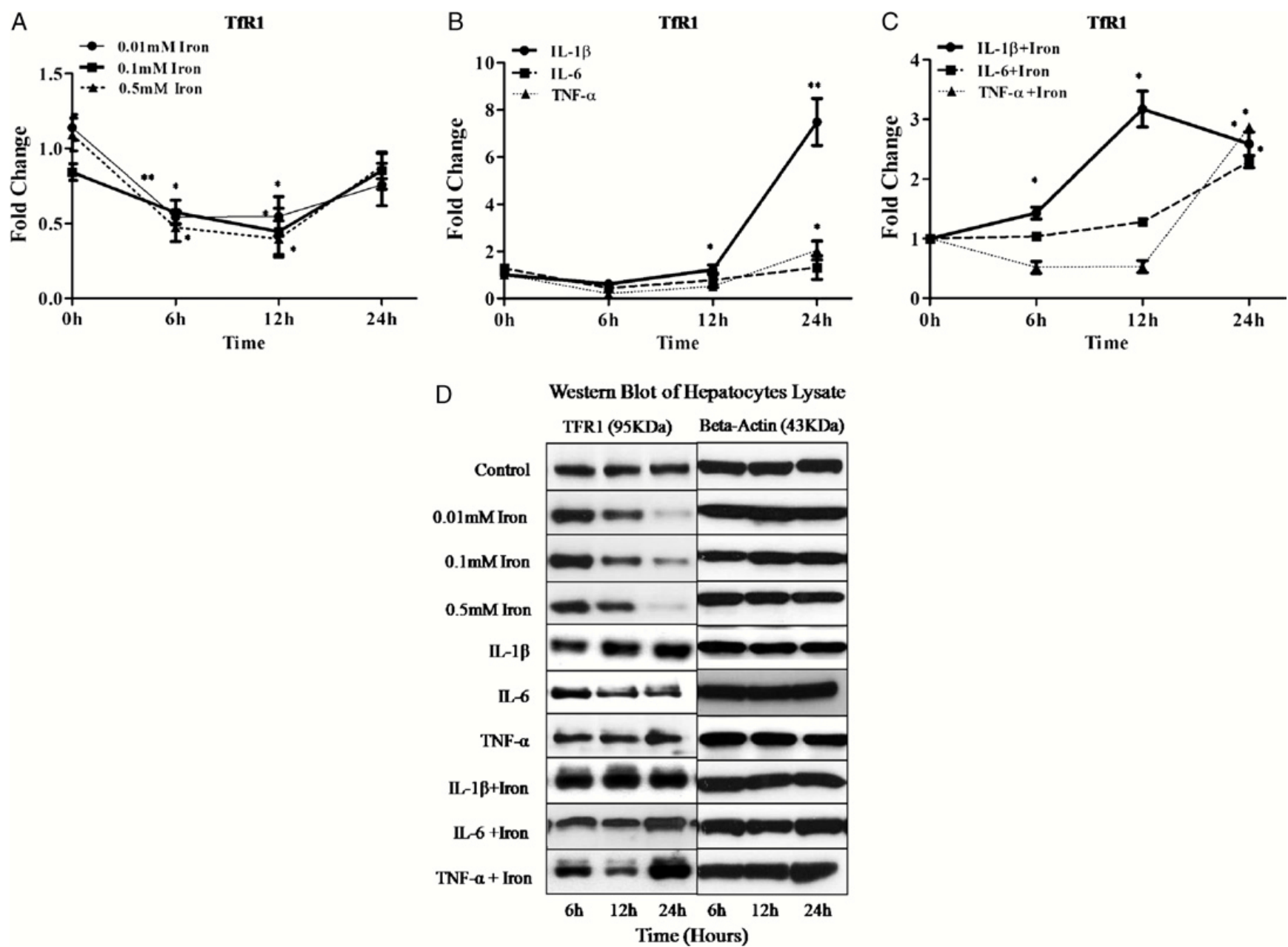
Fig .3. Quantitative RT-PCR analysis of mRNA from rat hepatocytes. Fold change in mRNA expression of TfR1 gene expression after iron treatments (A), cytokine treatment (B), cytokines+iron treatment (C) at different time points related to nont-reated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value ±SEM. *P ≤ 0.05, **P ≤ 0.001 analyzed by t test (n = 4). Western blot analysis of TfR1 (95 KDa) from total protein of rat hepatocytes. β-Actin (43 KDa) was used as a marker for equal loading (D).

difference was visible by combination of AP cytokines and iron in comparison to iron or AP cytokine treatment alone (Fig. 5C).

Similar to FTL, a dramatic induction in the gene expression of FTH was detected after iron treatment to hepatocytes both at mRNA and protein levels (Fig. 6, A and D).

Stimulation of hepatocytes with IL-6 showed an early (6h) increase in FTH both at mRNA and protein levels with a peak at 12 h (3.7±0.26-fold). Similarly, a time-dependent increase in FTH was observed after IL-1β and TNF-α treatment, with a maximum at 24 h (Fig. 6B).

Fig .4. Quantitative RT-PCR analysis of total RNA from rat hepatocytes. Fold change in mRNA expression of TfR2 gene expression after iron treatments (A), cytokine treatment (B), cytokines+iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value ±SEM. *P ≤ 0.0125, **P ≤ 0.001, ***P ≤ 0.0001 analyzed by t-test (n = 4).
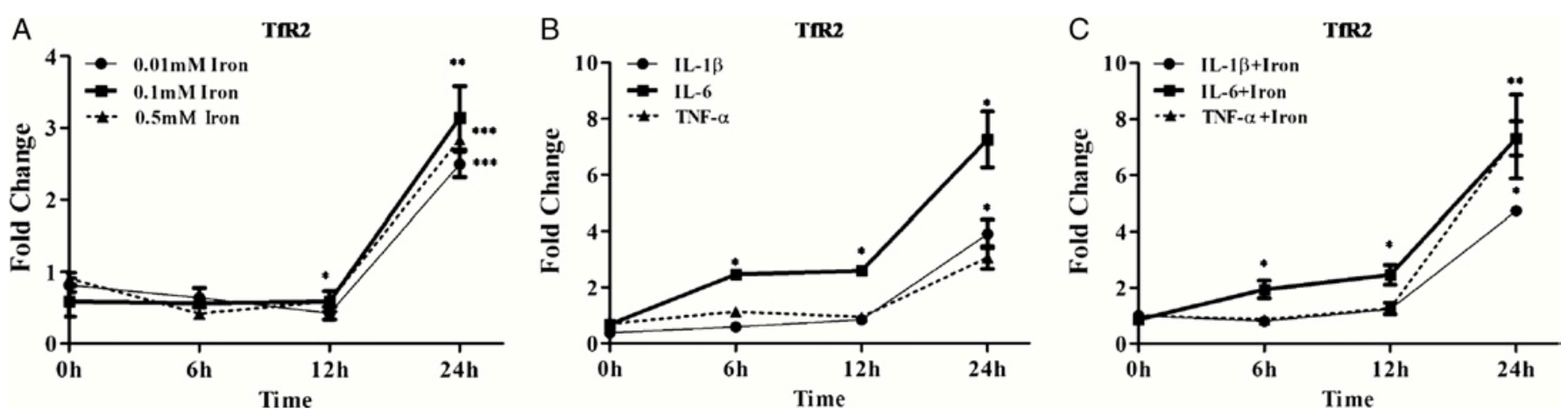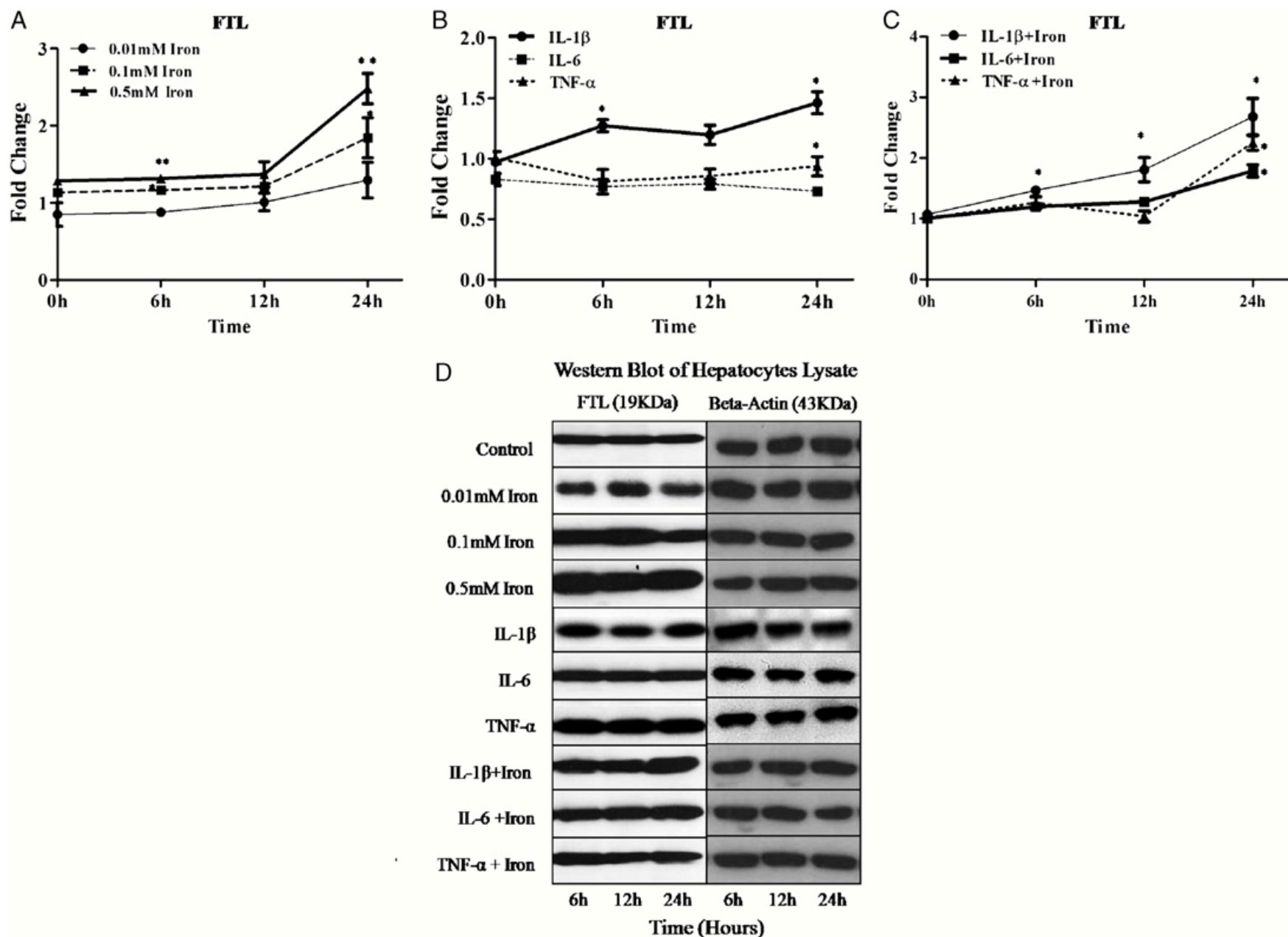
Fig.5. Quantitative RT-PCR analysis of mRNA from rat hepatocytes. Fold change in mRNA expression of FTL gene expression after iron treatments (A), cytokine treatment (B), cytokines+iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin . Results represent mean value ±SEM. *$P \leq 0.05$, **$P \leq 0.001$ analyzed by t-test (n = 4). Western blot analysis of FTL (19 KDa) from total protein of rat hepatocytes. β-Actin (43 KDa) was used as a marker for equal loading (D)

Furthermore, combination of iron and AP cytokines in comparison to cytokines or iron treatment alone did not show a significant difference (Fig. 6C).

Detection of LCN-2 and FTL in culture media of rat hepatocytes after treatment with iron and AP cytokines

Light-chain ferritin subunit is known to be a secretory protein with increased release by iron and cytokines into the culture media of hepatocytes (27). Iron is the main inducer of both ferritin subunits (28). To validate the LCN-2 changes in isolated rat hepatocytes, iron was added into the culture medium of hepatocytes. Western blot analysis of total supernatant from rat hepatocytes demonstrated an increase in LCN-2 protein expression after iron or AP cytokine treatment alone with a peak at 24 h, which was comparable to FTL, a positive secretory APP (27). In AP cytokine group, LCN-2 release was most pronounced with IL-6 followed by IL-1β and TNF-α (Fig. 7) in accordance to hepatocyte lysate.

Moreover, a dramatic synergistic increase in FTL and LCN- 2 at protein level was detected after administration of AP cytokines together with iron in the supernatant of hepatocytes. The protein level of both FTL and LCN-2 remained above t he control level

throughtout the course of the study (Fig.7). Taken together, LCN-2 can be released from hepatocytes into the supernatant. Similar to FTL, this release can be induced by AP cytokines and further enhanced when iron is provided in the cell culture medium.

DISCUSSION

In the present study, an increased iron uptake was detected in cultured hepatocytes by the treatment of iron. This iron uptake was dramatically enhanced when AP cytokines, mainly IL-6, were used together with iro n. In parallel to iron uptake, an increase in gene expression of iron transport (LCN-2 and TfR2) and storage proteins (FTL, FTH) was observed. In contrast to TfR2, the amount of TfR1 was decreased by the addition of IL-6 or iron alone into the culture medium. This reduction was abrogated when IL-6 was used together with iron at the same time. Another striking finding of the current study was the detection of LCN-2 release from hepatocytes into the supernatant after iron administration, similar to what was observed for FTL. This increase in LCN-2 gene expres- sion caused by iron was further enhanced in the presence of AP cytokines, mainly by IL-6.
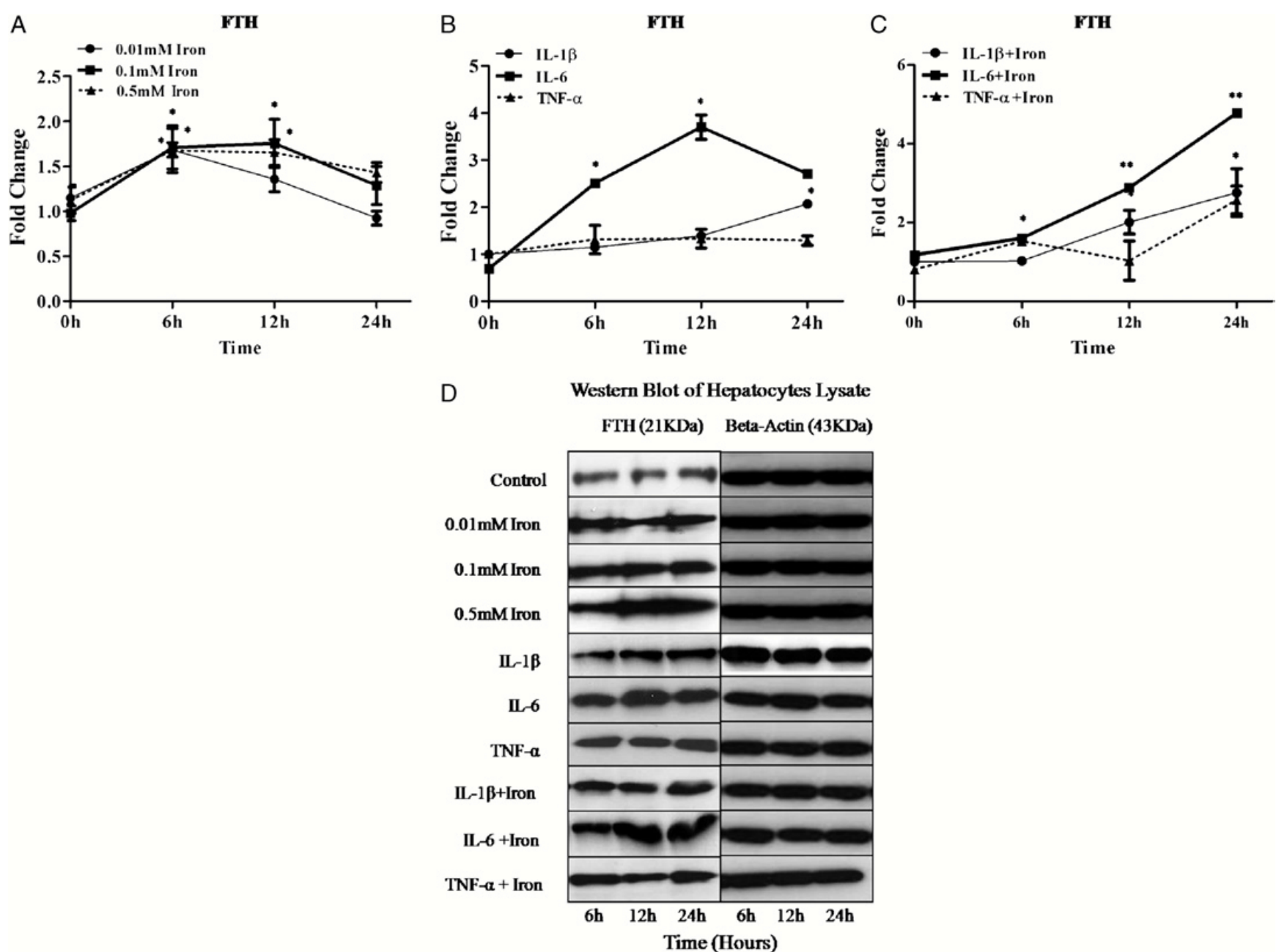
Fig .6. Quantitative RT-PCR analysis of total RNA from rat hepatocytes. Fold change in mRNA expression of FTH gene expression after iron treatments (A), cytokine treatment (B), cytokines+iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value ± SEM. *$P \leq 0.05$, **$P \leq 0.001$ analyzed by t test (n = 4). Western blotanalysis of FTH (21 KDa) from total protein of rat hepatocytes. β-Actin (43 KDa) was used as a marker for equal loading (D).

Indeed, Tf binding maintains iron in a soluble form and serves as a major vehicle of plasma iron delivery into cells via TfRs. TfR2 is positively (29) and TfR1 (30) is known to be negatively regulated by cellular iron status, which is in accordance with our study.

Iron is transported into the cells by two different pathways: Tf-dependent and Tf-independent pathway; the latter is acti- vated in iron overload conditions (31). In addition to Tf-bound iron, NTBI uptake mechanisms have also been described in a variety of cell lines (32), including hepatocytes (31). Recent studies have shown that LCN-2 is responsible for transport of iron through this pathway (33, 34). Based on previous and our current study, one can propose that iron uptake/transportation into hepatocytes could take place by both Tf-dependent and Tf-independent pathways, and the latter could be regulated by LCN-2. However, the role of LCN-2 pathway in iron uptake could be of minor importance compared with the Tf pathway.

An increase in iron level is associated with increased serum levels of AP cyto kines such as IL-6, IL-1β, and TNF-α (35). We and several previous AP studies reported that the liver responded dramatically to elevated levels of IL-6 by releasing APPs (36), which upregulate gene expression of most of iron regulatory proteins in the liver (and isolated hepatocytes). This indicates a strong correlation of these major AP cytokines mainly IL-6 with hepatic expression of iron regulatory proteins. A similar effect can be true in case of LCN-2 in the current study, as our data showed that iron and IL-6 seemed to be the main factors responsible for the dramatically induced LCN-2 gene expression in hepatocytes as has been previously reported (37). Furthermore, a reduced LCN-2 expression was reported in the liver of IL-6 knockout mice during APR (37). In addition, IL-6 is known to cause hypoferremia of inflammation by regulating hepcidin, but a recent study clearly demonstrated hepcidin-independent pathway for hypoferremia (38). In a mouse model of LPS-induced sepsis, LCN-2 has been described to play a key role in causing hypoferremia of inflammation (39). From our experimental data, we can speculate direct increased uptake of iron in hepatocytes stim- ulated by IL-6 can be another mechanism of hypoferremia of inflammation.

Another important aspect of current study showed that LCN-2 is a secretory hepatocellular protein. LCN-2 releases into the supernatants of isolated hepatocytes was detected not only by AP-
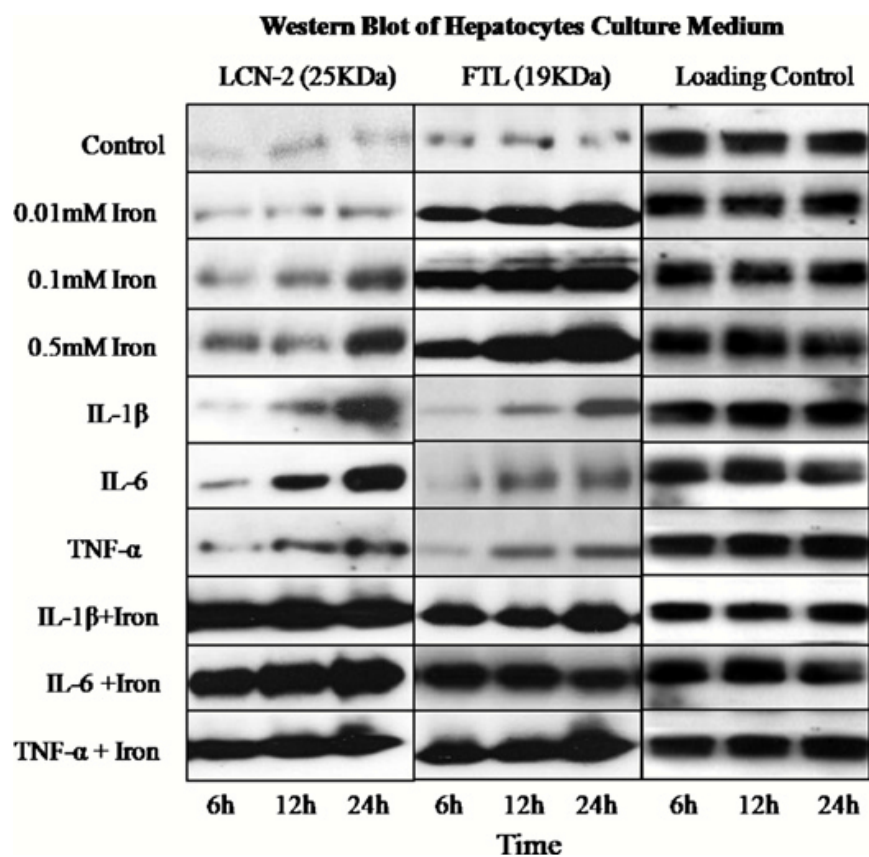
Western Blot of Hepatocytes Culture Medium

Fig .7. Western blot analysis of LCN-2 (25 KDa) and FTL (19 KDa) from total protein of rat hepatocytes supernatant. Ponceau S staining was used for equal loading.

ernatants of isolated hepatocytes was detected not only by AP cytokines (mainly IL-6) administration but also by iron, and this release was comparable to that of FTL (iron-storage and AP secretory protein). This finding indicates that AP cytokines and iron increase the release of LCN-2 probably in a similar way to FTL or another classic AP secretory protein (e.g., α-2M) as has been previously shown in same setting (27).

In fact, hepatocytes are the main source of most of the serum proteins, and they have a pivotal role in iron metabolism (2, 40). This work emphasizes the importance of iron uptake in hepatocytes during AP reaction. Hepatocytes need more iron to respond to the massive increase in protein synthesis under such stress conditions. This information contradicts the usual assumption that iron is just sequestrated in the macrophages to reduce bacterial growth (41). In conclusion, the disappearance of iron from the circulation has a very important functional meaning also in conditions (acute damage) where AP reaction is not caused by bacterial infections.

The results of the current and our previous studies (36) show the change in expression of hepatic iron regulatory genes including LCN-2 is not only due to the increase in hepatic iron concentration but is also due to the effect of AP cytokines (mainly IL-6) produced in hepatocytes during AP conditions.

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5 REGULATION AND LOCALIZATION PATTERN OF TRANSFERRIN RECEP-TORS AND LIPOCALIN-2 IN RAT HEPATIC AND EXTRA-HEPATIC ORGANS UN-DER PHYSIOLOGICAL AND ACUTE PHASE CONDITIONS

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Submitted in Progress in Histochemistry and Cytochemistry

Regulation and localization pattern of transferrin receptors and lipocalin-2 in rat hepatic and extra-hepatic organs under physiological and acute phase conditions

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Abstract

Iron is crucial for tissue functions, DNA synthesis and repair. Iron uptake is controlled by iron transport proteins like transferrin receptors (TfRs) and lipocalin 2 (LCN-2). We previously reported hepatic nuclear detection of iron storage protein subunit, FTH, along with increasing amount of nuclear iron in liver during acute phase response (APR). We now aimed to compare the expression and subcellular localization of iron transport proteins, (TfR1, TfR2 and LCN-2) in the same model of study. Rat, mice wild type (WT) and IL-6 knock-out (KO) mice were injected turpentine oil to generate APR. Total protein was used for Western blot analysis and 4µm thick sections were evaluated with immunohistochemistry. Immunohistochemistry of hepatic and extra hepatic tissues indicated organ dependent localization of TfR1 and TfR2. TfR1 was primarily localized in the membrane and cytoplasm of liver, and spleen cells whereas, in both organs, TfR2 showed nuclear expression mostly. However, in heart, both TfR1 and TfR2 were detected to be surplus in membrane, cytoplasm and nucleus. In all the organs studied, TfR1, TfR2 and LCN-2 were colocalized. Western-blot analysis showed an increased hepatic protein level of TfR1, TfR2 and LCN-2 in rat and WT mice during APR whereas such an increase was not detected for TfR2 and LCN-2 in IL6-KO mice. In parallel to induction of iron transporters, a phosphorylation of STAT3 was also observed in WT mice however such a change was not noticed in IL-6-KO mice. LCN-2 interaction with TfRs suggests its role in iron uptake during APR. Moreover, the differential localization of iron transport proteins in different organs may be due to their diverse role in these organs under physiological and pathological conditions. Furthermore, our results demonstrate that these iron import proteins (TfR2 and LCN-2) mediated mainly but not exclusively by IL-6 induced STAT3 pathway.

Key words: Interleukin 6, lipocalin-2, STAT3, transferrin receptor 1, transferrin receptor 2,

Introduction

Iron is an important co-factor for oxygen transport, heme and nonheme iron proteins, electron transfer, neurotransmitter synthesis, myelin production energy, metabolism and mitochondrial function in the different organs (Wang et al, 2011). Iron homeostasis is controlled by a number of iron-regulatory proteins. After entering enterocytes, iron is used for essential metabolic purposes, stored in ferritin, or translocated to the portal blood by ferroportin-1 (Fpn-1). The plasma protein, transferrin (Tf) binds to diferric iron (Fe_2Tf) and transfers it through portal blood to the liver where either the transferrin receptor-1 (TfR1)(Hentze et al, 2010) or Tf-independent mechanism mediates hepatic uptake of iron (Prus and Fibach, 2011) Interaction of iron bound to Tf with the TfR-1 and internalization of the resultant complex leads to iron uptake in the cells as a result of receptor-mediated endocytosis (Frazer and Anderson, 2005). A second alternative transferrin-mediated route of iron uptake exists via TfR-2. Although the role of TfR-1 in transporting iron across the hepatocyte membrane is well defined, the role of TfR-2 is not yet fully understood. TfR-2 knock-out mice fail to reproduce as a result of embryonic lethality due to severe anemia (Levy et al, 1999). Because TfR-2 mutations result in hepatic iron overload, TfR-2 appears to function, not principally in cellular iron uptake and delivery, but rather in iron storage (Roetto et al, 2010). Several other genes involved in iron homeostasis have been characterized including hepcidin (Pigeon et al, 2001), hemojuvelin [Hjv (Lanzara et al, 2004)] and lipocalin-2 [LCN-2 (Srinivasan et al, 2012)].

LCN-2 (25kDa) also known as NGAL (neutrophil gelatinase associated lipocalin) belongs to lipocalin family which is known to be involved in regulation of immune responses, modulation of cell growth and metabolism, and prostaglandin synthesis (Flower, 1996;Yang et al, 2002). Apart from its function as bacteriostatic agent and capable of sequestering iron in the form of siderophores (Flo et al, 2004), its role in apoptosis, inflammation, angiogenesis, adiposis

has been reported (Borkham-Kamphorst et al, 2011;Devireddy et al, 2001;Zhang et al, 2008). Under physiologic conditions LCN-2 is also known to be involved in iron transportation and iron homeostasis (Bao et al, 2010;Srinivasan et al, 2012). It has also been shown that LCN-2 has a role in stabilizing the labile iron/siderophore complex (Cherayil, 2011;Correnti and Strong, 2012). LCN-2 is an upregulated host protein during the acute phase response, not only during infection but also in sterile inflammation and aseptic diseases (Sultan et al, 2012). In addition, by chelating bacterial siderophores, LCN-2 showed a consistent correlation in driving or dampening an inflammatory response, the known bioactivities of LCN-2 led one to hypothesize that it might play a role in hypoferremia of inflammation and, thereby, the resolution of severe inflammation. Furthermore, several *in vitro* studies have demonstrated that LCN-2 protects against cellular stress and over-expression of LCN-2 allows cells to tolerate super-physiological iron concentrations (Hu et al, 2009;Roudkenar et al, 2011). LCN-2 is known not only to chelate iron limiting iron available to pathogens but also regulates intracellular iron concentration thus loss of LCN-2 may disrupt iron transport/homeostasis (Nairz et al, 2007;Srinivasan et al, 2012). In LCN-2 knockout mice model of LPS-induced sepsis delayed hypoferremia of inflammation has been described (Srinivasan et al, 2012).

A characteristic response to tissue damage is a change in iron metabolism, beginning with an acute decline in serum iron thought to be due to the sequestration of iron in organs of the reticuloendothelial system, the liver and spleen (Cairo et al, 2011). Macrophages sequester iron to prevent its availability to pathogens and its subsequent use for tissue repair and cell regeneration. This acute reduction of serum iron level is not only observed in cases of bacterial infections, but in all conditions associated with tissue damage (Cairo et al, 2011).

Indeed, APR produced by turpentine-oil (TO) induced muscle damage, changes in gene expression of several iron regulatory proteins in hepatic and non-hepatic organs including the brain have already been described by our group in rat model of APR (Malik et al, 2011;Sheikh et al, 2006;Sheikh et al, 2007). Moreover, a significant increase in the hepatic cytoplamic and nuclear content of iron associated with a reduction of the serum concentration was also reported in same model (Naz et al, 2012). In parallel to heaptic iron increase, an elevatation in the proteins involved in serum iron-uptake and transport with a receprocal reduction in iron export proteins (Fpn-1, and hephastin) occurs (Naz et al, 2012). However, the mechanisms governing hepatic iron metabolism under acute phase conditions is poorly understood. In addition, previously we also localized several hepatic iron import and export proteins whereas the differential localization of these hepatic iron regulatory proteins in comparison to extra-hepatic organs has not been reported before.

Therefore, the perspective of this study was to compare sub-cellular localization of iron transport proteins (TfR1, TfR2, LCN-2) in hepatic as well as extra-hepatic organs under physiological and acute-phase conditions. Further, it was also the aim of the current study to analyze the regulation and signaling pathway of these proteins during APR induced by TO-injection.

Materials and Method

Animals

Male Wistar rats of about 170-200 gram body weight were purchased from Harlan-Winkelmann (Brochen, Germany). Adult male B6.129S2-Il6$^{\text{tm1Kopf}}$ (IL-6-KO) mice and control wild-type adult male C57BL/6J mice (25–28g body weight) were purchased from Jackson Laboratory. Animals were kept under standard conditions with 12h light/ dark cycles and had *ad*

libitum access to fresh water and food pellets. All animals were cared according to the University's guidelines, German regulations for the protection of animals, and NIH guidelines.

Materials

All chemicals used, were of analytical grade and purchased from commercial sources as followed: Hybond N nylon membranes were from Amersham Pharmacia Biotech (Germany), 4,6-diamidino-2-phenylindole (DAPI) from Southern Biotech (Germany), donkey serum from Abcam (Germany), Alexa fluor-488 and Alexa fluor-555 from Invitrogen (Germany). All other reagents and chemicals were from Sigma-Aldrich (Germany) or Merck (Germany).

Induction of acute phase and removal of hepatic and extra-hepatic organs

APR was induced by injecting turpentine oil (TO) at a dose of 5 ml/kg body weight of rat and 10 ml/kg body weight of mice. TO was injected into each of the right and left hind limb gluteal muscles of ether-anesthetized animals. Control animals for each time point received a saline injection. All animals were killed at different time points ranging from 1 to 36 hours (rats), 2 to 24 hours (mice) after TO administration under pentobarbital anesthesia (Tron et al, 2005). The liver, spleen and heart were taken, frozen in liquid nitrogen, and stored at −80°C.

Preparation of tissue lysate

About 50mg frozen tissue was homogenized with an Ultra-turrax TP 18/10, three times for 10s each, in 10 vol 50mM TRIS-HCl buffer, pH 7.4, containing 150mM sodium chloride, 1mM EDTA, 1% Triton X-100, 1mM phenylmethane sulfonyl-fluoride (PMSF), 1mM benzamidine, 1mg/ml leupeptin, 10mM chymostatin, 1mg/ml antipain, and 1mg/ml pepstatin A. The entire procedure was carried out at 4°C. Crude homogenates were passed five times through a 22-G needle attached to a syringe and centrifuged for 5 min at 10,000g, at 4°C. The protein concentration was determined in supernatants by using the BCA (bicinchoninic acid) protein

assay reagent kit (Pierce, Bonn, Germany). Aliquots of the homogenates were stored at −20°C until further use for Western blot analysis.

Immunohistochemistry

Liver sections were cut in a cryostat at a thickness of 4µm, air-dried, fixed with acetone (−20°C, 10 min) and used for immunohistochemical studies. The antibodies and respective dilutions used are listed in table. Blocking of non-specific proteins was performed using donkey serum for 1 hour at room temperature in a humidified chamber. After 3 times 5 min washing with phosphate buffer saline (PBS) slides were incubated with primary antibodies diluted in 0.3% Triton-X PBS overnight at 4°C. On the next day slides were rinsed 3 times in PBS for 10 min each. To visualize antigens, slides were incubated in immunoflourescence conjugated secondary antibodies (donkey anti-rabbit/donkey anti-goat/donkey anti-mouse Alexa fluor-488 and donkey anti-rabbit/donkey anti-mouse Alexa Fluor-555) at room temperature for 1 h, and washed 5 times for 5 min in PBS. Finally, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) 5µl/100ml of PBS for 5 min, sections were washed and mounted with Fluoromount-G.

Western blot analysis

Samples of 50µg tissue proteins were applied per well and subjected to polyacrylamide gel electrophoresis using NuPAGE (4%-12% Bis-Tris Gel; Invitrogen) under reducing conditions (Laemmli, 1970). After electrophoresis, the proteins were transferred to Hybond-ECL (enhanced chemiluminescence) nitrocellulose membranes (Towbin et al, 1979). Immunodetection was performed according to the ECL Western blotting protocol. The antibodies used are listed in table.

Results

Hepatic expression and localization of transferrin receptors (TfR1, TfR2)

Immunohistochemical analysis of normal rat liver showed membranous and cytoplasmic expression of TfR1 in hepatic cells which increased (6h) after TO-induced APR. The intensity of expression was found to be increased in hepatocytes. In contrast, TfR2 was mainly localized in the nuclei of hepatic cells and dispersed cytoplasmic dots were also observed in normal rat liver. An increase intensity of nuclear dots (TfR2) was noticed after TO-administration as compared to normal rat liver (Fig: 1A).

Localization of transferrin receptors (TfR1, TfR2) in extra-hepatic organs

Immunodetection of TfR1 in heart tissues showed exclusively membranous and cytoplasmic expression in heart of both normal and of TO-administrated rat. However, in contrast to liver tissue some nuclei of heart cells showed TfR1 positivity. Similar pattern of immuno-expression was observed for TfR2. Both transferrin receptors showed a different expression pattern in heart tissue of normal and of TO-administrated rat compared to liver tissue. The intensity of expression of both transferrin receptors (TfR1, TfR2) increased and a strong immuno-reactivity was also present in cardiac muscle fibers after TO-administration (6h). TfR1 and TfR2 interaction was also noticed in the nuclei and membranes of heart cells (fig. 1B).

In spleen of normal and of TO-administrated rat immunohistochemical analysis of transferrin receptors showed TfR1 localized to membranes of spleen cells and TfR2 in the nuclei mostly but membranous expression was also observed for TfR2. In spleen cells of both red and white pulp area partial colocalization of TfR1 and TfR2 was also observed (fig. 1C).

Lipocalin-2 (LCN-2) interaction with transferrin receptors in hepatic tissue

In the previous few years LCN-2 emerged as a iron transport protein, playing a vital role in iron regulation under different pathological conditions. We and others showed that LCN-2 played a pivotal role in iron transport through transferrin receptor mediated and non-transferrin bound iron (NTBI) pathway. Therefore, we performed double immunostaining of LCN-2 along with TfR1 and TfR2 in control and TO-administrated rat liver. LCN-2 showed no interaction with TfR1 under physiological conditions in hepatic tissue (fig. 2A). However, in control rat hepatic tissues, a slight interaction between LCN-2 and TfR2 was detected (fig. 2B). After TO-administration LCN-2 expression increased and a co-expression of LCN-2 protein with TfR1 in membranes and cytoplasm of hepatic cells was observed (fig. 15A). Parallel to the interaction of LCN-2 and TfR1 proteins, TfR2 was also colocalized with LCN-2 within the cytoplasm and few nuclei of hepatic cells during APR (Fig: 2C).

Lipocalin-2 (LCN-2) interaction with transferrin receptors in extra-hepatic tissues

Immunohistochemical analyses of extra-hepatic tissues showed no interaction of LCN-2 protein with TfR1 and TfR2, in hearts from control rat (fig. 3A, 3B). However after the onset of acute phase LCN-2 was expressed in heart tissue interacting with TfR1 protein in cardiomyocytes and a strong expression of LCN-2 and TfR2 was present in edothelial cells of the myocardial vessel, with partial interaction. In spleen both from control and acute-phase response rats LCN-2 colocalization with TfR1 and TfR2 was detected. The expression of LCN-2 interaction with both transferrin receptors was stronger in red pulp cells of the spleen (fig. 4A, 4B).

Changes in iron transport proteins in rat hepatic tissue during APR

Liver of TO-injected rats demonstrated an increase in both, transferrin receptors (TfR1, TfR2) and LCN-2 with the onset of APR at protein level by Western blot analysis. TfR1 protein was found elevated from 1-24h after TO-injection (Fig. 18). A gradual but less increase in TfR2 protein was observed with a maximum expression at 6h after APR (Fig. 5). Likewise, LCN-2 protein revealed a progressive increase in the liver which became clearly evident at 12h by immunoblotting with a further increase till 36h after TO-injection (Fig. 5).

Modulation of iron transport proteins in mice hepatic tissue during APR

To validate the role of IL-6 in iron regulatory proteins expression, we generated acute phase model in wild type (WT) and IL-6 Knockout (KO) mice. Similar to what was observed in rat liver after TO-injection, Western blot analysis of WT-mice showed an increase in hepatic TfR1, TfR2 and LCN-2 protein level after TO-injection. However such an increase for TfR2 and LCN-2 was not found in IL-6-KO-mice after TO-injection. In contrast, TfR1 showed a time dependent increase at protein level in IL-6 KO mice similar to WT-mice with the onset of APR with a maximum expression at 24h (Fig. 6).

Phosphorylation of STAT3 in mice hepatic tissue during ARP

IL-6 is potent pro-inflammatory cytokine and it activates transcription protein signal transducer and activator of transcription 3 (STAT3). Phosphorylation of STAT3 in turn activates many target downstream genes. By means of Western blot using specific antibody against STAT, it was possible to detect the STAT-3 at protein level both in WT and IL-6-Ko-mice. Phosphorylation of STAT3 was only detectable in WT-mice after TO-injection. The protein expression of p-STAT3 started to increase at 2h with a maximum at 4h with the onset of APR. In

contrast, such an increase was not observed in IL-6-KO mice after TO-injection apart from a slight increase at 12h (fig. 6).

Discussion

In this current study we compared sub-cellular interaction between iron transport proteins (TfR1, TfR2 and LCN-2) and their expression pattern under physiological and acute phase conditions. Immunhistochemical analysis showed no interaction between transferrin receptors (TfR1, TfR2) in hepatic tissue suggesting their own independent iron transfer route in hepatic cells. However, interestingly TfR1 and TfR2 co-expression was detected in extra-hepatic tissues (heart, spleen) suggesting co-transportation of both receptors into the cell through a common route. In nuclei of some cardiomyocytes TfR1 and TfR2 positivity was observed and to some extent co-expression of TfRs can also be seen in these nuclei. In a study colocalization of TfR1 and TfR2 has been reported in HepG2 and Hep3B cell lines (Johnson et al, 2007).

Furthermore, LCN-2 colocalization with TfR1 and TfR2 was detected in liver during physiological and AP-conditions, however after ARP co-expression of LCN-2 and TfRs increased which suggests that, LCN-2 transports iron during acute phase conditions. In extra-hepatic tissues (heart, spleen), LCN-2 interaction with transferrin receptors (TfR1, TfR2) was also observed in spleen of control and of TO-rat, however, in heart LCN-2 expression was detected only after AP reaction, appearance of LCN-2 protein in heart only after the generation of AP reaction can be due to stress conditions. We previously reported LCN-2 expression in heart at mRNA level in the same settings and a different study reported LCN-2 production by isolated neonatal cardiomyocytes (Yndestad et al, 2009). To the best of our knowledge the current study is first of a kind to exclusively report colocalization of TfRs in extra-hepatic tissues. Our this study also suggests that differential localization of transferrin receptors (TfR1,

TfR2) in hepatic and extra-hepatic tissues is due to diverse role of these proteins in different tissues under physiological and pathological conditions (AP-conditions).

Although nuclear expression of iron proteins in different organs has already been reported, we and others showed TfR1 nuclear expression in rat brain (Malik et al, 2011), LCN-2 nuclear expression in mice brain (Ip et al, 2011), FTL (Ahmad et al, 2013a), Fpn1 and DMT-1 nuclear expression in rat liver (Naz et al, 2012), PC12 cells (Roth et al, 2000) and in mice glioblastoma cell lines respectively (Calzolari et al, 2010). However, colocalization and differential localization of iron importer proteins (TfRs, LCN-2) in hepatic and extra-hepatic tissues (heart and spleen) has never been reported before.

These findings support not only the presence of iron transport proteins within the nuclei to transport more iron in the hepatic cell nuclei under stress conditions but suggests that increased level of nuclear iron may be necessary for the increased activity of nuclear enzymes involved in DNA synthesis, repair and the regulation of transcription (Roth et al, 2000).

Western blot analysis and immunohistochemical findings support an intense increase of iron importer proteins (TfRs and LCN2) within liver under AP conditions studied. Under iron overload conditions, level of these proteins increase resulting in cellular iron retention (Canonne-Hergaux et al, 2006). However, the early upregulation of these transporter proteins could be controlled by the acute-phase cytokines (mainly IL-6) which is released at the site of injury resulting in an APR (Sheikh et al, 2007). Moreover, the upregulation of IL-6 gene expression during an APR (Malik et al, 2011;Sheikh et al, 2007) can account for the intense induction of these importer proteins expression, indicating a direct effect of the acute phase cytokine, IL-6, in regulating iron importer proteins.

To answer this question, same APR model was established in wild type (WT) and IL-6-KO mice after TO-injection. Similar to what was observed in rat APR model, a pattern of increase in TfR1, TfR2 and LCN-2 protein level was observed in the liver of WT-mice with the onset of APR, such a increase was not observed for TfR2 and LCN-2 in the liver tissue of IL-6 KO mice. Moreover, IL-6 stimulation leads to the activation and phosphorylation of STAT3 pathway. A striking finding of the current study is to detect STAT3 phosphorylation in WT-mice liver after TO-injection which was not observed in IL-6-KO mice liver, indicating a regulative function of this important acute-phase cytokine on TfR2 and LCN-2 during APR.

In contrast, an increase in TfR1 protein level, similar in both WT and IL-6 KO mice during APR suggest the effects of the other acute phase cytokines such as IL-1β and, to a lesser extent TNF-α, which are produced also at the site of tissue injury to control this protein (Ramadori et al, 2010). In fact, the upregulation of IL-1β and TNF-α gene expression is more intense in the injured muscle of IL-6 KO mice than in their wild type (Ramadori et al, 2010) indicating that in the absence of IL-6 other cytokines may replace its functional role.

By summarizing our previous and current study, the upregulation of DMT-1, Tf, TfR1, TfR2 and LCN-2 at the mRNA and protein levels supports an increased iron uptake by liver cells during the APR. Although TfR1 is thought to be inversely regulated by the cellular iron stores via posttranscriptional IRE-iron regulatory protein mechanism, (Levy et al, 1999) the upregulation of TfR1 in the present study consisting of the effect of an APR might be attributable to either an activation of IRP-1(Caltagirone et al, 2001) or hypoxia-inducible factor-1α (HIF-1α), both of which bind to a conserved binding site within the TfR1 promoter (Tacchini et al, 2002). It should be noted that an induction of hepatic HIF-1α has been reported in the APR model utilized in the present experiments (Ramadori et al, 2010). The upregulation of iron transport molecules

(TfR2 and LCN-2) may be due to an action of the major acute phase cytokine (IL-6) through STAT3 pathway suggested by our current study or the increased iron concentration may induce an upregulation of gene expression of iron regulaotry proteins as has also been shown previously in our *in vitro* study (Ahmad et al, 2013b).

In conclusion, we propose that under normal conditions, portal blood iron is taken up by liver cells mostly through a TfRs and by LCN-2 mediated iron uptake pathway. Furthermore, during APR, IL-6 induced changes in iron transporter genes (TfR2 and LCN-2) could be regulated through STAT3 pathway.

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Legends

Figure 1: Double Immunofluorescence detection of TfR1 and TfR2 positivity in liver, heart and spleen sections (4µm cryostat) of control rats and TO-administrated rats. Sections were stained with TfR1 and TfR2 in liver (A), heart: white arrows showing cytoplasmic and blue arrows indicating nuclear co-localization (B), spleen: white arrows showing cytoplasmic co-localization and blue arrows indicating TfR2 nuclear expression (C) followed by fluorescence immunodetection using Alexa fluor-488 and Alexa fluor-555 (original magnification 200X, Bar 20µm). Nuclear counterstaining was done with DAPI.

Figure 2: Double Immunofluorescence detection of TfR1, TfR2 and LCN2 positivity in liver section (4µm cryostat) of control rats and TO-administrated rats. Sections were stained with antibodies against neutrophil gelatinase associated LCN2 and TfR1, blue arrows indicating LCN-2+TfR1 cytoplasmic interation (A), LCN2 and TfR2, blue arrows indicating cytoplasmic and white arrows showing nuclear co-expression of LCN-2+TfR2 (B) followed by fluorescence immunodetection using Alexa fluor-488 and Alexa fluor-555 (original magnification 200X, Bar 20µm). Nuclear counterstaining was done with DAPI

Figure 3: Double Immunofluorescence detection of TfR1, TfR2 and LCN2 positivity in heart section (4µm cryostat) of control rats and TO-administrated rats. Sections were stained with antibodies against neutrophil gelatinase associated LCN2 and TfR1: yellow arrows indicating TfR1 nuclear positivity, white arrows showing nuclear colocalization of LCN-2 and TfR1 (A), LCN2 and TfR2 (B) followed by fluorescence immunodetection using Alexa fluor-488 and Alexa fluor-555 (original magnification 200X, Bar 20µm). Nuclear counterstaining was done with DAPI.

Figure 4: Double Immunofluorescence detection of TfR1, TfR2 and LCN2 positivity in spleen section (4µm cryostat) of control rats and TO-administrated rats. Sections were stained with antibodies against neutrophil gelatinase associated LCN2 and TfR1 (A), LCN2 and TfR2 (B) followed by fluorescence immunodetection using Alexa fluor-488 and Alexa fluor-555 (original magnification 200X, Bar 20µm). Nuclear counterstaining was done with DAPI. RP (Red Pulp), WP (White pulp)

Figure 5: Western blot analysis of TfR1(˜95kDa), TfR2 (˜88kDa) and LCN-2 (˜25kDa) from total protein of rat liver. β-actin (43kDa) was used as a marker for equal loading.

Figure 6: Western blot analysis of TfR1 (~95kDa), TfR2 (~88kDa), LCN-2 (~25kDa), p-STAT3 (~80kDa), and STAT3 (~80kDa), from total protein of WT and IL-6 KO mice. Beta-actin (43kDa) was used as a marker for equal loading.
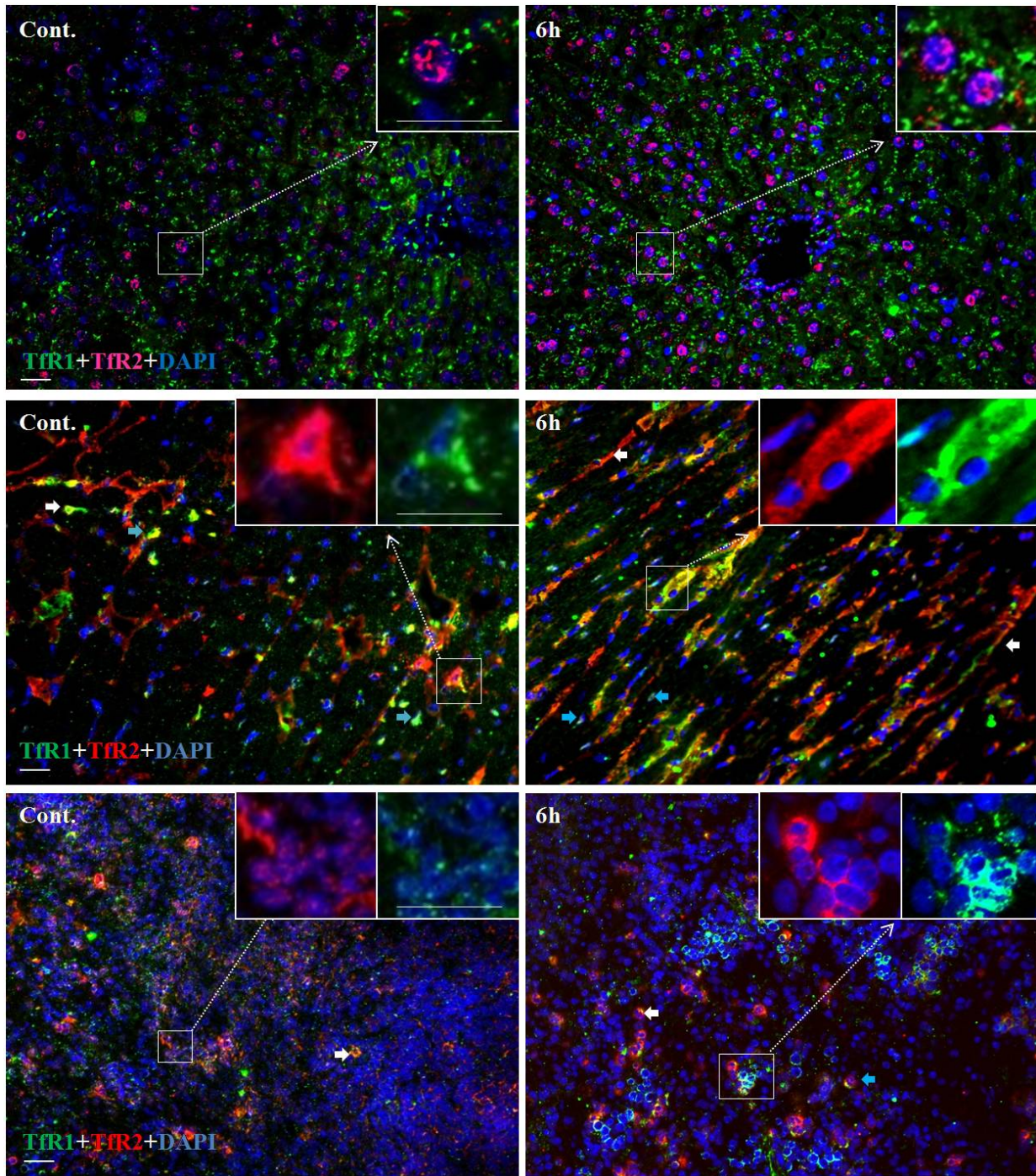
Figure 1

Cont.
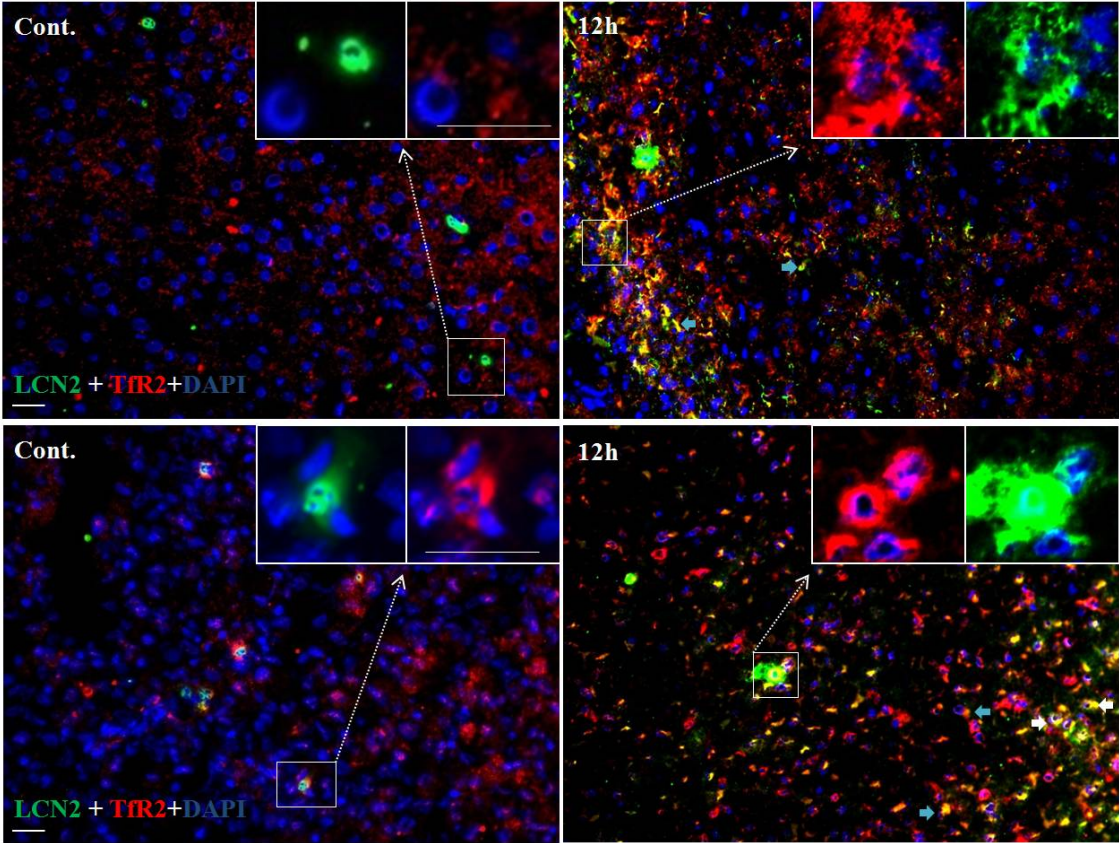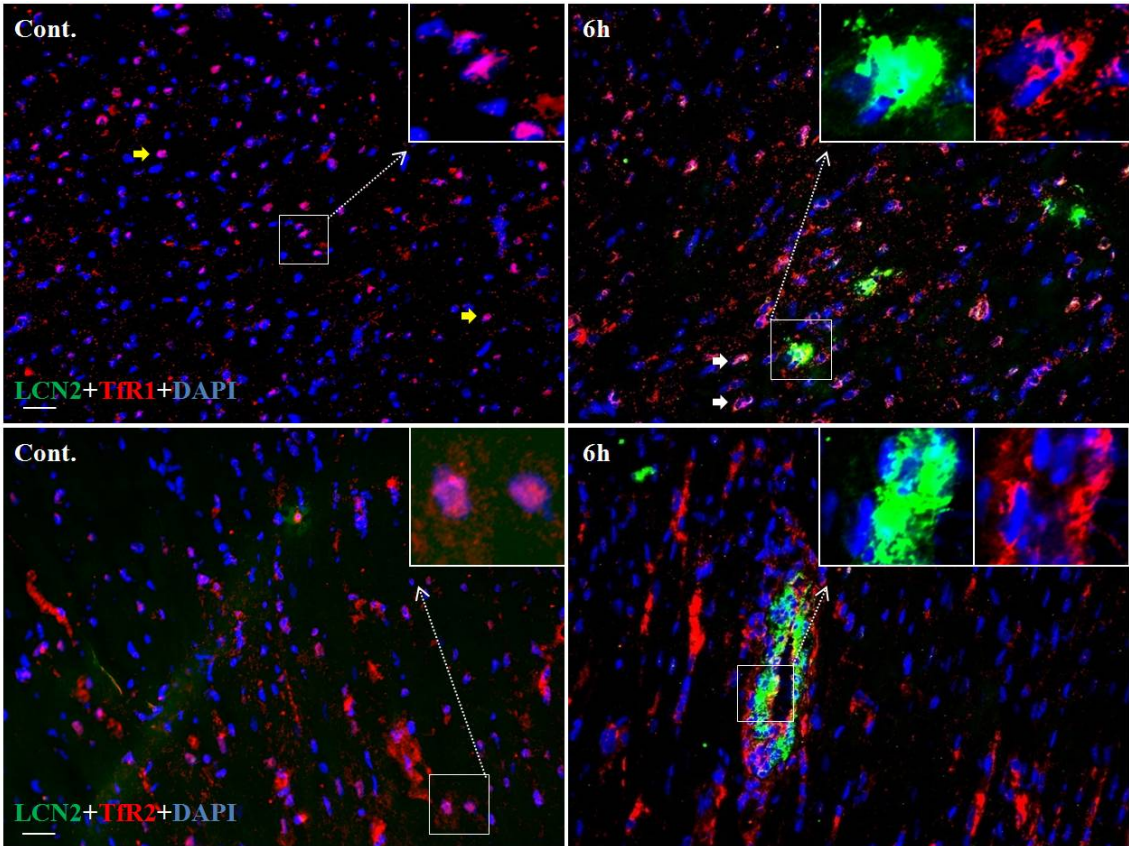TfR1+TfR2+DAPI

6h

Cont.
TfR1+TfR2+DAPI

6h

Cont.
TfR1+TfR2+DAPI

6h

Figure 2

Figure 3

Figue 4

Figure 5

Rat Liver

TfR1								95KDa
TfR2								88KDa
LCN-2								25KDa
ß-actin								43KDa
	Co	1h	2h	4h	6h	12h	24h	36h

Time (hours)

Figure 6

Wild T ype Mice   **IL-6 Knockout Mice**

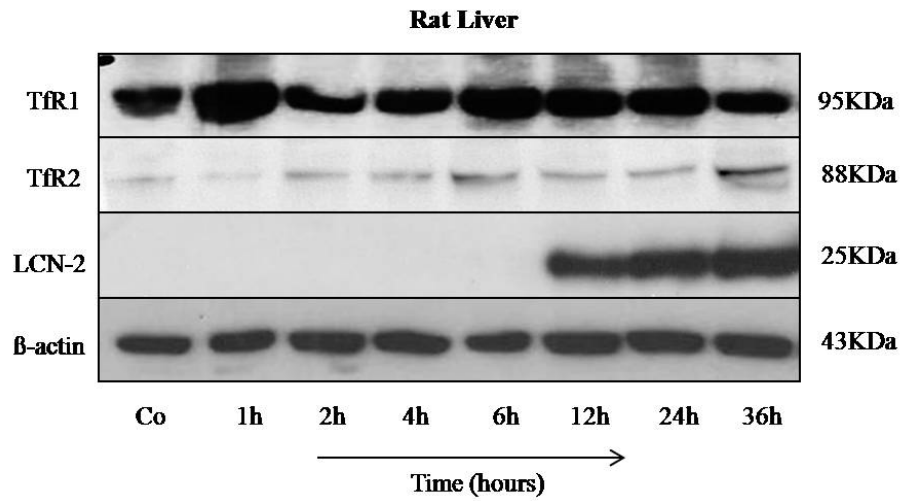|													
TfR-1													95KDa
TfR-2													88KDa
LCN-2													25KDa
p-STAT-3													80kDa
STAT-3													80kDa
ß-actin													43KDa
	Con.	2	4	6	12	24		Con.	2	4	6	12	24

Time (hours)   Time (hours)
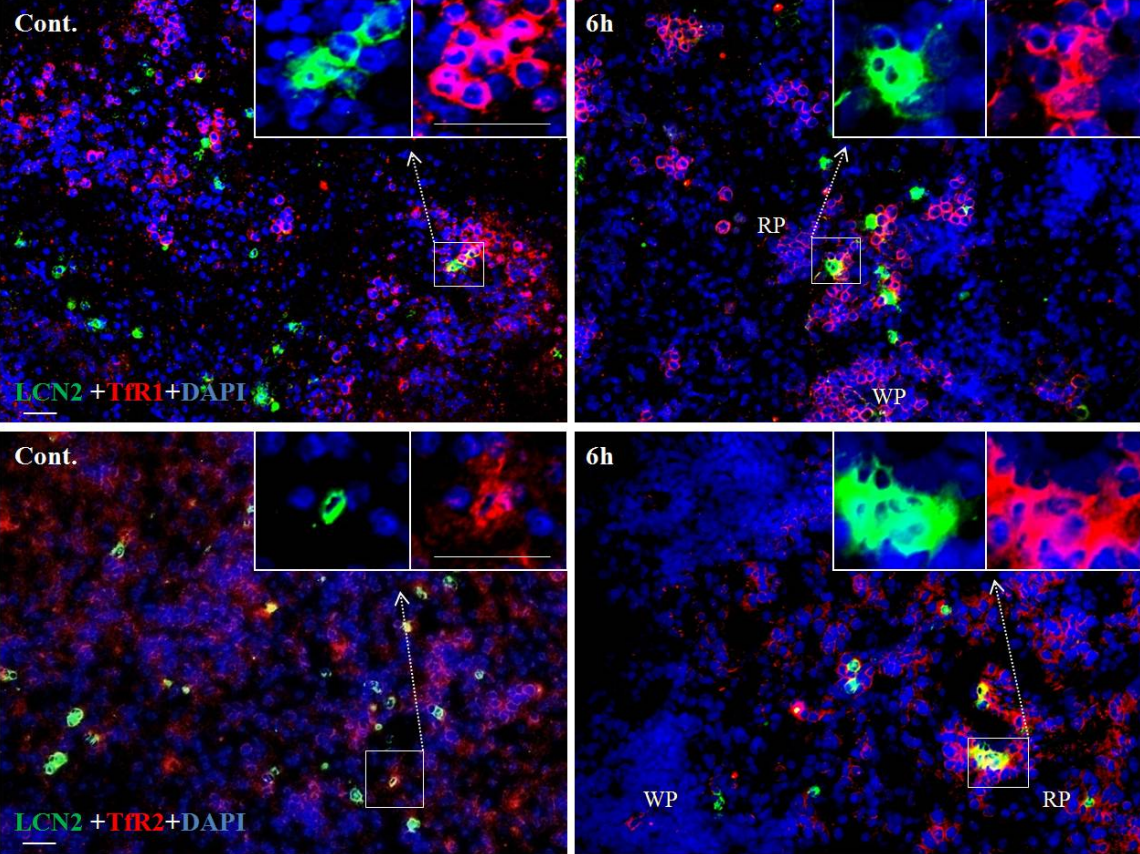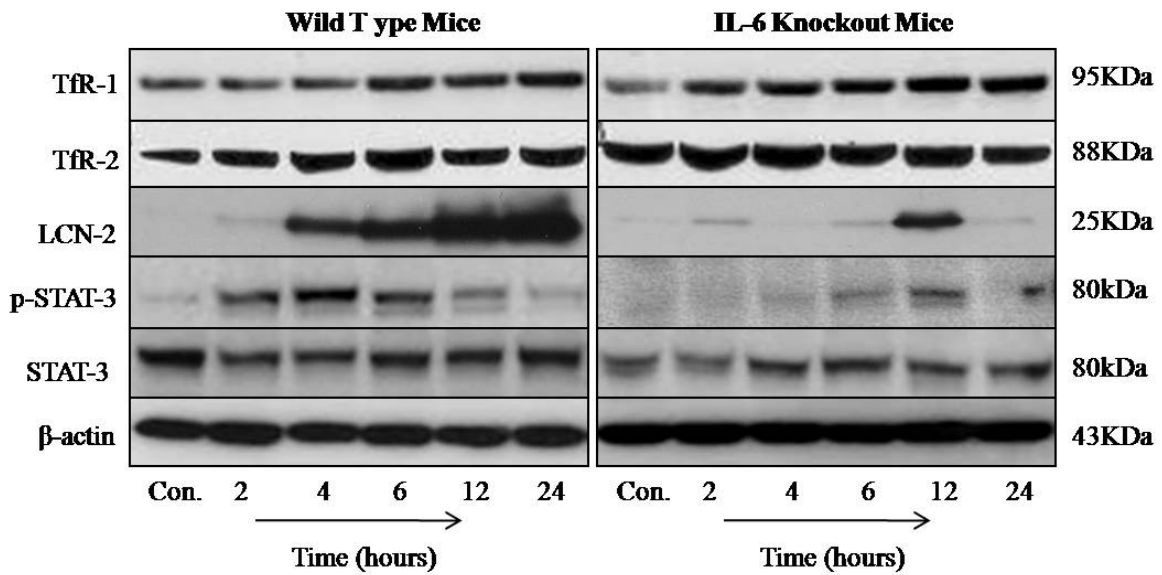
Table: Antibodies used in this study

Antibodies	Company	Reference Number	WB	IHC
Lipocalin-2 (LCN-2) Polyclonal Goat IgG	R & D	AF3508	1:300	1:100
Lipocalin2 (LCN2) Monoclonal Mouse IgG	Novus biological	NBPI-05182	1:100	1:100
Transferrin Receptor1 (TfR1) Monoclonal Mouse IgG	Invitrogen	13-6890	1:1000	1:200
Transferrin Receptor2 (TfR2) Polyclonal Rabbit IgG	Abcam	ab80194	1:1000	1:200
STAT3 Polyclonal Rabbit IgG	Cell Signaling	9132	1:1000	
Phospho-STAT3 Polyclonal Rabbit IgG	Cell Signaling	9131	1:1000	
Beta-actin (β-actin) Monoclonal Mouse IgG	Sigma	A-2228	1:5000	_

6   FERRITIN L AND FERRITIN H ARE DIFFERENTIALLY LOCATED WITHIN HEPATIC AND EXTRA HEPATIC ORGANS UNDER PHYSIOLOGICAL AND ACUTE PHASE CONDITIONS

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PMID: 23573308

Original Article
Ferritin L and ferritin H are differentially located within hepatic and extra hepatic organs under physiological and acute phase conditions

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Abstract: Ferritin L (FTL) and Ferritin H (FTH) subunits are responsible for intercellular iron storage. We previously reported increasing amounts of liver cytoplasmic and nuclear iron content during acute phase response (APR). Aim of the present study is to demonstrate intracellular localization of ferritin subunits in liver compared with extra hepatic organs of rat under physiological and acute phase conditions. Rats were administered turpentine-oil (TO) intramuscularly to induce a sterile abscess (acute-phase-model) and sacrificed at different time points. Immunohistochemistry was performed utilizing horse-reddish-peroxidise conjugated secondary antibody on 4μm thick section. Liver cytoplasmic and nuclear protein were used for Western blot analysis. By means of immunohistology, FTL was detected in cytoplasm while a strong nuclear positivity for FTH was evident in the liver. Similarly, in heart, spleen and brain FTL was detected mainly in the cytoplasm while FTH demonstrated intense nuclear and a weak cytoplasmic expression. Western blot analysis of cytoplasmic and nuclear fractions from liver, heart, spleen and brain further confirmed mainly cytoplasmic expression of FTL in contrast to the nuclear and cytoplasmic expression of FTH. The data presented demonstrate the differential localization of FTL and FTH within hepatic and extra hepatic organs being FTL predominantly in the cytoplasm while FTH predominantly in nucleus.

Keywords: Ferritin, nuclear localization, liver, acute phase, iron regulation

Introduction

Liver is key organ for iron homeostasis and storage under physiological as well as acute phase conditions. Within the cell, iron is stored mainly as ferritin [1]. Ferritin is composed of L and H subunits that are highly conserved [2] nevertheless, genetically separate [3, 4] and maintain distinct functions [5]. The storage of iron is considered to take place in the cytoplasm, however iron is required for the nuclear functions as well. L and H subunits spontaneously assemble in a 24-subunit protein "cage" with a flexible H: L ratio. The H: L ratio can vary between different cell types [2, 5]. The L gene has very little tissue-specific regulations whereas multiple conditions activate H ferritin gene transcription [6, 7] including cell differentiation, changes in the cell proliferation status, oncogenes, cytokines, and heme. Infact, a pre-

vious study has showed an association between ferritin expression and cell proliferation [8].

Acute-phase response (APR) is a major physiological defence reaction of the body aimed to eliminate the injuring noxae and to re-establish homeostasis. Clinically, it is characterized by fever, somnolence, weakness, muscular joint pain, adinamia and increased liver activity. Moreover, decrease of serum iron level is also a hallmark of acute-phase reaction [9, 10]. This decrease is considered to be due to the sequestration of iron by the reticuloendothelial system [11].

In previous work, we demonstrated that under acute phase conditions the liver takes up serum iron [12, 13] and increased hepatic iron level is demonstrable in the nuclear fraction of the liver tissue as well [13]. The increased nuclear iron content was further supported by the nuclear
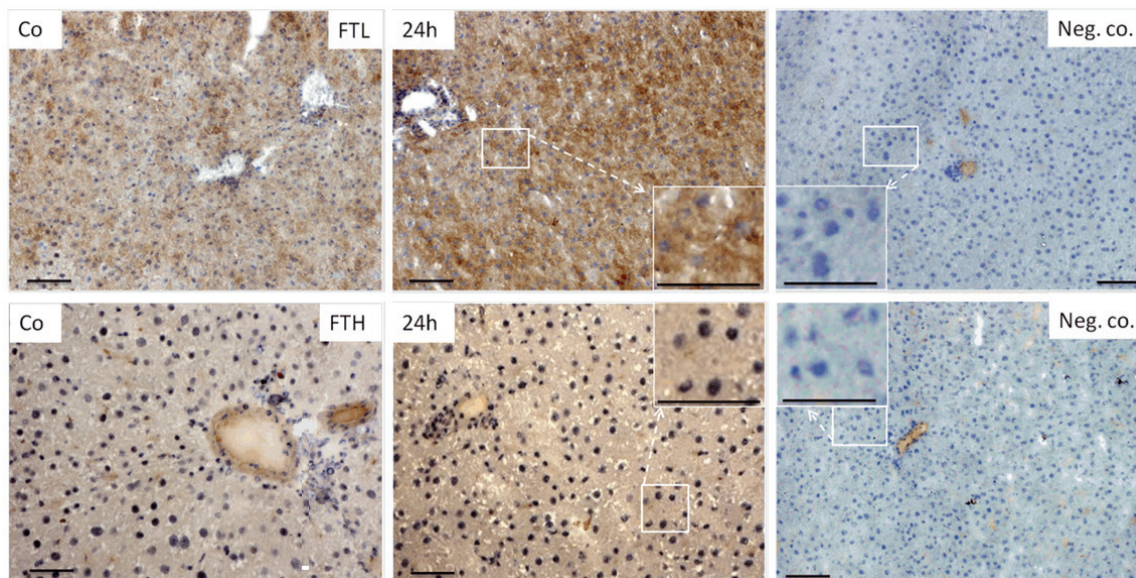
Figure 1. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of rat liver from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Negative control represents immunostaining when primary antibody was omitted. Insets show the enlarged magnification of selected box. Original magnification 200x bar 50μm.

detection of iron import proteins including TfR2 and DMT-1 along with nuclear Fpn-1; the iron export protein under physiological and acute phase conditions [13]. The aim of our prospective study was to determine the intracellular localization of major iron storage proteins; FTH and FTL in hepatic as well as extra hepatic organs.

Methods

Materials

Animals: Rats (170–200 g body weight), were purchased from HarlanWinkelmann (Brochen, Germany). The animals were kept under standard conditions with 12:12-h light dark cycles, and were given ad libitum access to water and food. All animals were cared for in accordance with the guidelines of our institution, the German Convention for the Protection of Animals, and the National Institutes of Health (USA).

Induction of acute phase and harvesting the liver, heart, spleen and brain

APR was induced and organs were removed as described previously [14]. Briefly, tissue damage was induced by injecting 5 ml/kg-TO in both right and left hind limbs of animals. Control animals were treated in the same way for each time point with saline injection. Liver, heart, spleen and brain tissue was harvested, cut into pieces and snapped frozen for further used.

Immunohistochemistry and immunocytology

Four to five micrometer thick cryostat sections (Reichert Jung, Wetzlar, Germany) from rat liver, heart, spleen and brain were used for immunodetection of FTL and FTH. The slides were air-dried, fixed with ice cold methanol (-20°C, 10 min) and ice cold acetone (-20°C, 10 sec) and stored at -20°C. After inhibition of endogenous peroxidase by incubating the slides with phosphate-buffered saline (PBS) containing glucose/glucose oxidase/sodium azide, the sections were treated with FCS for 30 min to minimize nonspecific staining. Peroxidase immunostaining was performed utilizing two different commercially available antibodies for FTL (abcam; UK and Santa Cruz; USA) and FTH (LS Bio and Santa Cruz from USA). The primary antibodies were diluted FTH (1:10), FTL (1:50). Negative controls were incubated with isotype-specific IgGs, instead of the specific primary antibody. After washing, the slides were covered with peroxidase-conjugated anti-rabbit/anti-mouse immunoglobulins pre-absorbed with normal rat serum to avoid cross-reactivity. Slides were washed and incubated with PBS containing 3,3-diaminobenzidine (0.5 mg/ml)
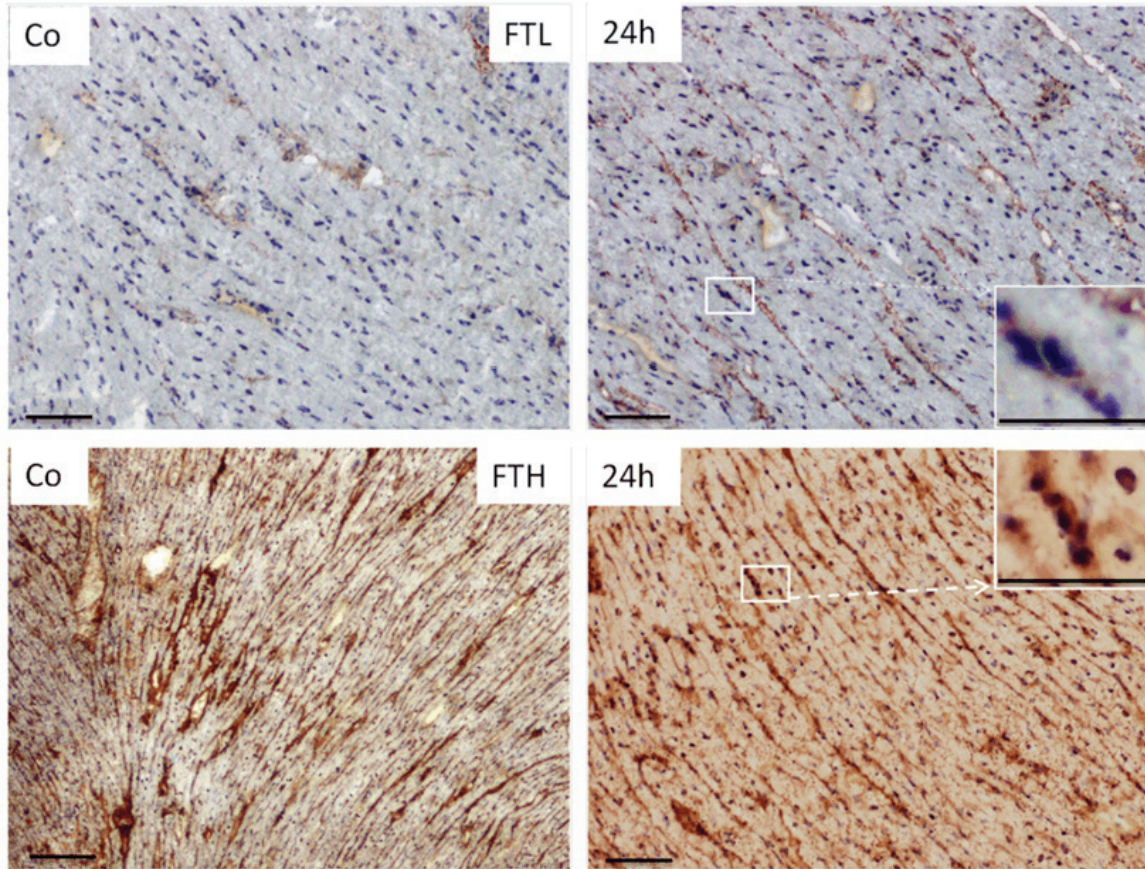
Figure 2. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of rat heart from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25μm.

and H_2O_2 (0.01%) for 10 min to visualize immune complexes. Nuclei were counterstained with Meyer's hemalaun solution before the slides were mounted with cover slips.

Cellular fractionation for protein isolation

Liver, heart, spleen and brain cytoplasmic and nuclear proteins were isolated using NucBuster Protein Extraction kit (Novagen USA) as described by manufacturer, with some modifications. Briefly, 100 mg of tissue sample was homogenized in 300 μl of NucBuster reagent 1 followed by collection of supernatant as cytoplasmic fraction. Pellet was washed thrice with sterile ice-cold PBS and dissolved in 50 μl of NucBuster reagent 2.1μl of 100mMDTT and Protease Inhibitor Cocktail Set I was added to each sample to inhibit proteases activity. Samples were stored at -20°C for further use.

Western blot analysis

30 μg of protein from tissue fraction was applied per well and were subjected to electrophoresis using NuPAGEÒ (4-12% Bis-Tris Gel; Invitrogen) under reducing conditions [15]. After electrophoresis the proteins were transferred to Hybond-ECL nitrocellulose membranes [16]. Immunodetection was performed according to the ECL Western blotting protocol. The anti-Ferritin L (abcam and Santa cruz) and anti-Ferritin H (LSBio, Santa cruz) were used in the study.

Results

Hepatic expression and localization of FTL and FTH

Immunohistochemical analysis of normal liver showed FTL granular positivity mainly in the
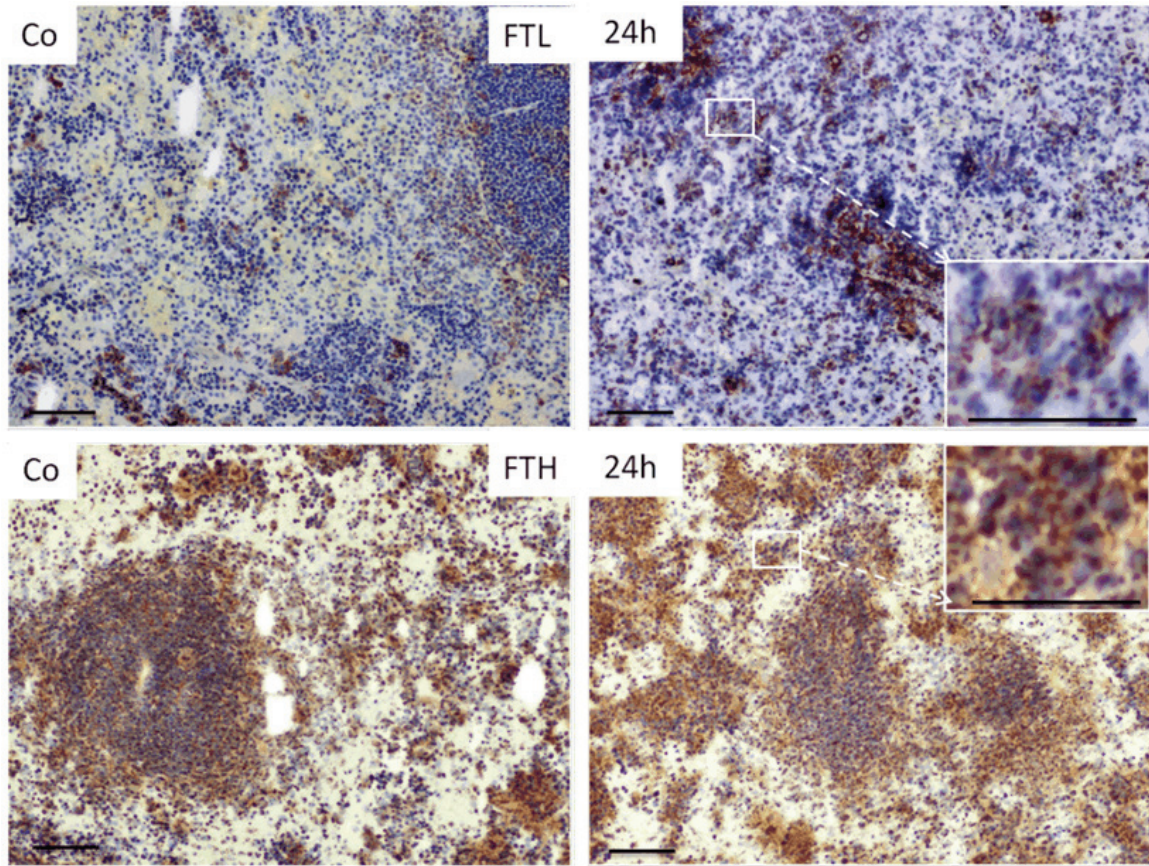
Figure 3. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of Rat spleen from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25μm.

cytoplasm of hepatic cells, which kept on increasing in TO treated rats. Intensity of FTL immunoexpression was found to be stronger after TO-injection with an intense expression in hepatocytes (distinguished on visual morphology; **Figure 1** enlarged insets). The number of cells positive for FTL cytoplasmic expression was highest at 24h after TO-injection. The reaction was negative when the primary antibody was omitted (negative control; **Figure 1**; upper right panel).

In contrast to FTL immunolocalization, peroxidase staining of FTH showed intense granular positivity in the nucleus of hepatic cells of control and TO-injected rats. Compared to negative controls (primary antibody omission) where nuclei were stained clear blue, liver tissue from control and TO-injected rats showed FTH blotching (brown dots) mainly in the large nuclei of hepatocytes (**Figure 1**; lower panel).

Localization of FTL and FTH in extra-hepatic organs

In control heart tissue, the immunodetection of FTL indicated a very weak expression as compared to liver tissue. However, it was localized exclusively within the cytoplasm. The protein expression of FTL showed more intense granular expression in TO-injected animals at 24h **Figure 2**; upper panel). In contrast to FTL expression within the heart tissue, FTH immunodetection was more intense and was spread all over the tissue structure including the cytoplasm and the nuclei of tissue (**Figure 2**; lower panel).

In control spleen tissue, FTL was detected within the cytoplasm of red pulp and white pulp cell population. This expression was more copious in spleen tissue of TO-injected rats (**Figure 3**; upper panel). However, within the spleen tissue, FTH protein showed a dual localization. We
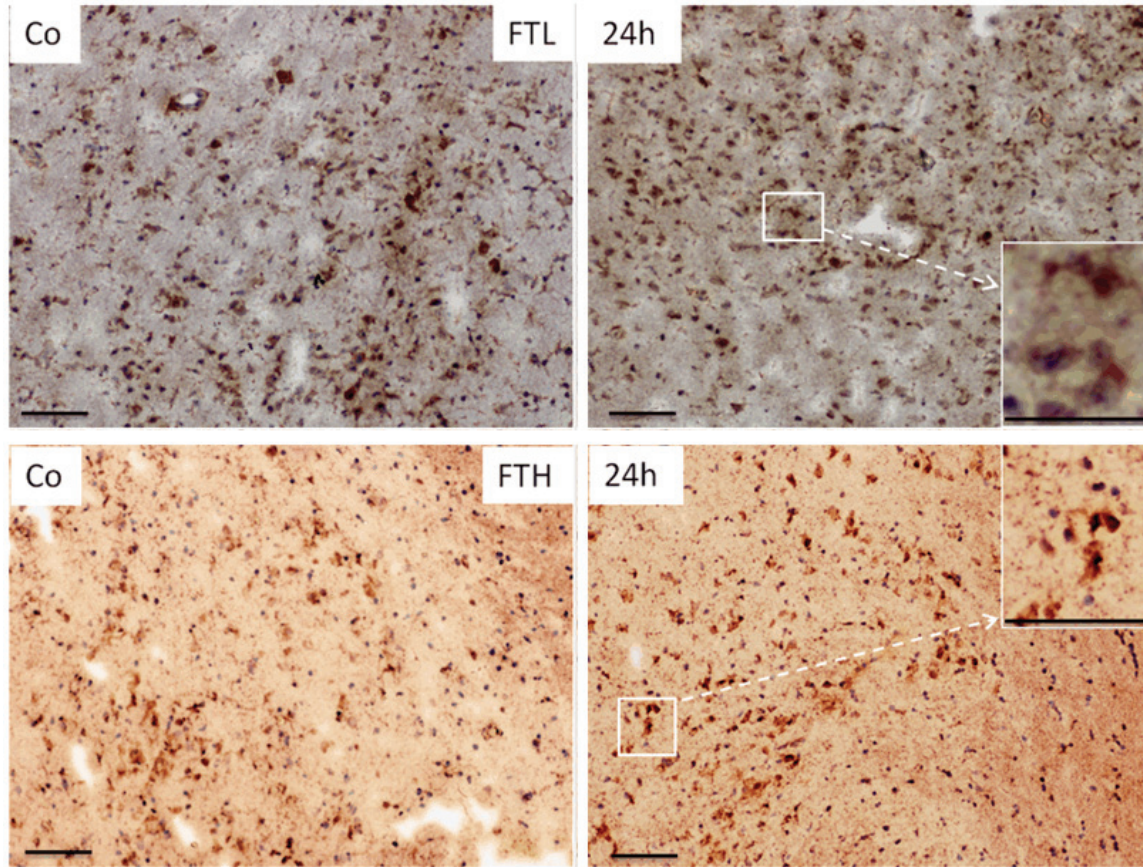
Figure 4. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of Rat brain from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25µm.
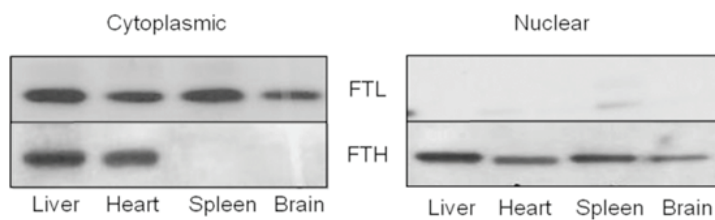

Figure 5. Western blot analysis of FTL and FTH in protein extracted from nuclear and cytoplasmic fractions of different organs of control animals.

could detect a strong nuclear and comparatively weak cytoplasmic expression of FTH within spleen tissue of control and TO-injected rats (**Figure 3**; lower panel).

However, within the brain tissue of both control and TO-injected rats, FTL was plentiful in the cytoplasm and a slight nuclear expression was also detected (**Figure 4**; upper panel). However, in both control and TO-injected animals, FTH was localized strongly within the brain nuclei and weakly in the cytoplasm (**Figure 4**; lower panel).

Western blot analysis of hepatic and extra hepatic protein fractions for FTL and FTH

The immunohistochemical data was further confirmed by means of Western blot analysis of cytoplasmic and nuclear fractions proteins from hepatic and extra hepatic organs of control animals. Western blot analysis demonstrated mainly cytoplasmic expression of FTL in liver, heart, spleen and brain. FTL was found to be more abundant in liver followed by spleen and then heart and brain. While, only a very slight FTL nuclear expression was found in spleen. FTH was detected mainly in the nuclear fraction of liver, heart, spleen and brain. However, it was also detectable in the liver and heart cytoplasmic fraction (**Figure 5**).

Discussion

To our best knowledge, this is the first attempt to determine the predominantly nuclear localization of FTH in contrast to cytoplasmic expression of FTL under physiological and acute phase conditions. Immunodetection protocols and Western blot analysis showed a strong cytoplasmic and very weak nuclear expression of FTL as compared to the strong nuclear and weak cytoplasmic localization of FTH in hepatic and extra hepatic organs of rat including heart, spleen and brain. Moreover, protein expression was found to be elevated for both FTL and FTH by Immunohistology with the onset of APR.

Ferritin has been investigated as a cytosolic iron storage protein [17]. Its localization within the cell however, is controversially debated. So far, nuclear localization of FTH is reported in-vitro in human astrocytoma cell line [18], in corneal epithelial cells [19] and in-vitro in mice hepatocytes under iron overload conditions [20]. Indeed, we showed constitutive nuclear FTH detection not only in the hepatic but also in extra-hepatic organs of rat under physiological and acute phase conditions. As increasing amounts of iron seem to be temporarily needed in the nucleus during the APR, therefore, it is not surprising to detect iron storage proteins not only in cytoplasm, but also in the nucleus of the cells.

Nuclear FTH of our current study suggests iron sequestration not only in cytoplasm but also in nucleus of liver cells indicating an important role of iron for nuclear metabolism. It could also suggest that iron may be necessary for the activity of nuclear enzymes for DNA synthesis and repair and/or to regulate the initiation of transcript [21]. Another possibility could be that under acute-phase conditions, extra iron may be needed to satisfy the increased metabolic work of the liver [10].

We previously reported elevated liver iron stores in the same model [13]. Likewise, our previous and current study showed an increased mRNA and protein expression of FTL and FTH in the liver, in parallel to the increased hepatic uptake of iron during APR [13]. Most of the initial observations reported that the amount of intracellular ferritin could be modified by changes in iron status [17] and accumulation of H-chain [22, 23]. However, our previous study also showed that gene expression of FTL, FTH and of other iron regulatory genes is modulated also by acute phase cytokines [13, 24]. In other words, the increase in hepatic FTL and FTH expression is not only due to the increase in hepatic iron concentration but it is also due to the direct effect of acute-phase cytokines produced during TO-induced APR.

A previous study [25] reported that FTH is the main iron storage protein in the liver. Depletion of FTH in hepatocytes makes these cells more susceptible to the toxic effect of iron [25]. Moreover, it has been shown that in non-hepatic cell lines (K562 cells) the over-expression of FTH resulted in reduced free iron pool [26]. This may indicate that not only ferritin L but also ferritin H subunit could be required to reduce free available iron level in the "stressed" hepatocytes during APR.

FTL shares "the iron storage" function in liver tissue and extra hepatic organs (heart, spleen and brain), however the secretory function of liver makes it unique to extra hepatic organs. We reported FTL as a secretory protein (manuscript submitted) so liver should have more FTL as compared to extra hepatic organs.

An earlier study reported evidence of stainable iron within the nuclei of hepatocytes and Kupffer cells in mouse liver under conditions of iron overload [27]. Our current finding supports not only the presence of iron storage protein within the nuclei of liver cells under non-physiological conditions but suggests that nuclear iron is important in the initiation of defense mechanism.

In summary, FTH is localized not only in the cytoplasm but also in the nucleus of liver, heart, spleen and brain cells. This suggests that iron is not only stored in the nucleus but also that nuclei need to be defended from possible dangerous effects of iron overload on DNA as has been hypothesized previously [25]. This might become more important when the metabolic challenges increase during APR.

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control of the labile iron pool in murine erythro-leukemia cells. J Biol Chem 1998; 273: 15382-6.

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7   DISCUSSION

This study reports the iron uptake in hepatocytes and changes in gene expression of iron transport (TfR1, TfR2 and LCN-2) and iron storage proteins (FTH, FTL) under the influence of acute phase cytokines. Moreover, it identifies induction of TfR2 and LCN-2 by IL-6 through STAT3 pathway. Furthermore, sub-cellular localization of these proteins in liver in comparison to spleen and heart in acute phase response rat model is described.

7.1   Iron uptake and regulation of iron transport and iron storage proteins in primary culture of rat hepatocytes: Role of IL-6

A significant iron uptake was detected in hepatocytes after administration of iron in culture medium. This iron uptake further enhanced when AP-cytokines (mainly IL-6) and iron were administrated concomitantly. There was a parallel increase in iron uptake and gene expression of different iron regulatory proteins i.e. iron import proteins (TfR1, TfR2 LCN-2) and iron storage proteins (FTL, FTH). This up-regulation was further induced when AP-cytokines, mainly IL-6, were administrated together with iron. In contrast to TfR2, the amount of TfR1 was decreased by the addition of IL-6 or iron alone into the culture medium. However, surprisingly this reduction was abrogated when IL-6 was used concomitantly with iron.

Indeed, binding of transferrin to iron keeps iron in a soluble form and acts as major transporter of plasma iron into cells via TfRs. Diferric Tf has a high affinity for the TfRs and this Tf uptake by cells has important implications during physiological conditions (Conner and Schmid, 2003;Frazer and Anderson, 2005;Herbison et al, 2009). Although, it is supposed that TfR1 is inversely regulated by intracellular iron concentration through the posttranscriptional IRE-IRP (iron

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responsive element-iron regulatory protein) mechanism (Levy et al, 1999) but in my study an increase in the TfR1 protein expression was detected in hepatocytes after concomitant administration of AP-cytokines and iron. This TfR1 up-regulation could be due to activation of IRP-1 or hypoxia-inducible factor 1α (HIF-1α) (Caltagirone et al, 2001; Malik et al. 2011) which binds to a conserved binding region in TfR1 promoter site (Tacchini et al, 1999) as induction of hepatic HIF-1α was also reported by us in our model. However TfR2 is known to be positively regulated by cellular iron concentration (Calzolari et al, 2007) which is in accordance with our study.

Furthermore, the existence of non-transferrin-bound iron (NTBI) uptake is now well established (Breuer et al, 2000a). Recent studies have shown that LCN-2 is responsible for transport of iron through this pathway (Kaplan, 2002;Schmidt-Ott et al, 2007). A recent report clearly demonstrated the participation of LCN-2 in iron transportation during kidney damage (Yang et al, 2002). Likewise, a disrupted iron-uptake has been described in LCN-2 knockout mice (Srinivasan et al, 2012).

In fact, this transferrin independent pathway becomes active in iron overload conditions (Chua et al, 2004), because in such conditions plasma iron concentration exceeds the transferrin binding limit. NTBI uptake is known to cause significant iron accumulation in the liver (Breuer et al, 2000a). NTBI uptake mechanisms have been studied in different types of cell lines, (Prus and Fibach, 2011) including hepatocytes also (Chua et al, 2004). Based on experimental observations of current study, it can be proposed that iron transport into hepatocytes can take place by both pathways, the transferrin dependent and the transferrin independent and the latter can be regulated by LCN-2 under the influence of IL-6.

IL-6 is the key regulator of iron metabolism under different inflammatory conditions resulting in iron accumulation. IL-6 causes hypoferremia of inflammation by hepcidin regulatory

pathway but in a recent study hepcidin independent pathway has been described clearly (Deschemin and Vaulont, 2013). In a mouse of LPS-induced sepsis, LCN-2 played a pivotal role in causing hypoferremia of inflammation (Srinivasan et al, 2012). From experimental data of current study it is speculated that increased uptake of iron in IL-6 stimulated hepatocytes could be another mechanism of hypoferremia of inflammation. Few previous studies clearly reported that amount of intracellular ferritin was regulated by change in iron status (Meyron-Holtz et al, 2011). These observations clearly indicate that not only FTL but also FTH is needed for reduction of free available iron in the stressed hepatocytes during APR. FTL shares "the iron storage" function in hepatic and extra-hepatic tissues (heart, spleen and brain) however, liver is a unique organ due to its secretory function when compared to extra hepatic organs.

Even though human plasma ferritin levels are used to estimate body iron stores (Goralska et al, 2001), this seems not always applicable in humans and animals (Kanra et al, 2006). An increase of iron level is associated with increased serum levels of AP-cytokines such as IL-6, IL-1β and TNF-α (Sheikh et al, 2006). Indeed, we and several previous acute phase studies reported that the liver responded dramatically to elevated levels of IL-6 by releasing acute phase proteins (Naz et al, 2012) which up-regulate gene expression of most of iron regulatory proteins in the liver (and isolated hepatocytes). This indicates a strong correlation of these major AP-cytokines with hepatic expression of iron regulatory proteins. A similar effect can be true in case of LCN-2 in the current study, as the data showed that iron and IL-6 seemed to be the main factors responsible for the dramatically induced LCN-2 gene expression in hepatocytes as has been previously reported (Sultan et al, 2012). Furthermore, a reduced LCN-2 expression was reported in the liver of IL-6 knockout mice during APR (Sultan et al, 2012).

In fact, most of the serum proteins are synthesized by hepatocytes which play a pivotal role in regulation of in iron metabolism (Arosio et al, 2009;Tran et al, 1997). This study showed that LCN-2 is a secretory hepatocellular protein. A release of LCN-2 into the supernatants of isolated hepatocytes was detected not only by AP-cytokines (mainly IL-6) but also by iron and this release was comparable to that of FTL (iron storage and acute-phase secretory protein). This finding indicates that acute phase cytokines and iron increase the release of LCN-2 probably in a similar way to FTL.

According to the data of current study, IL-6 is the key player of iron uptake in hepatocytes during AP-conditions and LCN-2 behaves as an alternative iron transport protein in addition/ compared to classical iron transport proteins (like TfRs) during AP-conditions whose gene expression is increased by iron and/or AP-cytokines. In addition, LCN-2 is also a secretory acute phase protein whose release from hepatocytes (major cell population of the liver) into the supernatant is controlled by iron and AP-cytokines (mainly IL-6). The results of current study show that change in expression of hepatic iron regulatory genes including LCN-2 is not only because of increase in iron contents of hepatocytes but is also due to the effect of AP-cytokines produced in hepatocytes during acute-phase conditions.

7.2   Kinetic of acute phase cytokines after turpentine oil induced acute phase response

In the current study, TO injection in animals was used as a means to induce an APR. After turpentine oil (TO)-administration, a dramatic increase in IL-6 gene expression and a significant up-regulation of IL-1β was detected in the injured muscle. However, the kinetics of IL-1β was quite less than that of IL-6. Furthermore, serum analysis showed a strong up-regulation of IL-6 and IL-1β whereas expression of IL-6 was 9-times higher than that of IL-1β (Sheikh et al. 2007),

which clearly indicates IL-6 as a major cytokine induced in this study model. This model is of sterile inflammation in which the inflammatory agent (TO), was restricted to a specific area i.e. the muscle. The local inflammation caused by this insult recruits the inflammatory cells at the site of injury (damaged muscle) and up-regulation of IL-6 is also recorded in the injured muscle.

The inflammatory stimulus is localized to specific area so it could not approach the liver to cause the IL-6 up-regulation. TO is a lipophilic substance which readily accumulates in fatty tissues and cannot reach the hepatic tissue. In different studies of TO administration to rats maximum concentration of turpentine was found in perinephric fat and brain (Savolainen and Pfaffli, 1978) which might be a possible cause of local production of acute phase cytokines.

7.3 Regulation of iron transport proteins after TO-induced acute phase response in rat and mice hepatic tissue

According to an increase in liver iron level (Naz et al, 2012), an intense increase of iron importer proteins (TfRs and LCN2) within liver under AP conditions was studied. Under iron overload conditions, level of these proteins increase resulting in cellular iron retention (Canonne-Hergaux et al, 2006). However, the early up-regulation of these transporter proteins could be controlled by the acute-phase cytokines (mainly IL-6) which is released at the site of injury resulting in an APR (Sheikh et al, 2007) confirming the *in vitro* results which showed a regulative effect of the acute phase cytokine, IL-6, on iron importer proteins.

It further strengthens our hypothesis that liver (and hepatocytes) need iron to respond to AP-conditions. In order to confirm the direct effect of IL-6 on iron importer proteins and to find out the signaling pathway responsible for these changes during APR, same APR model was established in wild type (WT) and IL-6-KO mice after TO-injection. Similar to what was observed in

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rat APR model, a pattern of increase in TfR1, TfR2 and LCN-2 protein level was observed in the liver of WT-mice with the onset of APR , such an increase was not observed for TfR2 and LCN-2 in the liver tissue of IL-6 KO mice. Moreover, IL-6 stimulation leads to the activation and phosphorylation of STAT3 pathway (Taub, 2003). A striking finding of the current study is to detect STAT3 phosphorylation in WT-mice liver after TO-injection which was not observed in IL-6-KO mice liver, indicating a regulative function of this acute-phase cytokine on TfR2 and LCN-2 during APR.

In contrast, an increase in TfR1 protein level, similar in both WT and IL-6 KO mice during APR may suggest the effects of the other acute phase cytokines such as IL-1β and, to a lesser extent TNF-α, which are produced also at the site of tissue injury to control this protein. In fact, an intense up-regulation of IL-1β and TNF-α gene expression is recorded in the injured muscle of IL-6-KO mice than their wild type (Ramadori et al, 2010) indicating that in the absence of IL-6 other cytokines may replace its functional role.

7.4   The cellular localization of iron transport proteins in rat hepatic and extra-hepatic tissues

To elucidate which cell types are responsible for the TO-induced iron importer proteins expression in the liver and extra hepatic organs, immunohistochemical analysis of these organs was performed. By means of double immunohistochemical analysis TfR1 showed membranous and cytoplasmic expression whereas TfR2 positivity was mostly in the nuclei of hepatic and spleen cells. In spleen a partial co-expression of TfR1 and TfR2 was also observed, both in spleens of control rat and after AP-reaction. A nuclear expression of TfR1 was detected in heart tissue as compared to the liver and spleen where a membranous and cytoplasmic expression was observed. Moreover,

in rat liver and spleen we clearly showed that TfR2 is exclusively located in the nuclei of the cells whereas, in heart tissues TfR2 showed nuclear positivity mostly with partial membranous and cytoplasmic expression. Transferrin receptors (TfR1 & TfR2) also expressed a colocalization in heart tissues both in control and TO-administrated rats. In nuclei of some cardiomyocytes, to some extent, co-expression of TfRs was also present. The expression of transferrin receptors (TfR1, TfR2) increased after TO-administration in liver and heart tissues but decreased in spleen. These observations suggest that iron is not only being sequestrated into cytoplasm but also in nuclei of cells as was observed by the increased cytoplasmic and nuclear iron concentrations in liver (Naz et al, 2012). A previous study reported the evidence of stainable iron in the hepatocytes nuclei and kupffer cells of mice liver under iron overload conditions. This finding support not only the presence of iron transport proteins in the nucleus of liver cells under pathological conditions as a defence mechanism (Magens et al. 2005) but also the need of iron for nuclear metabolism. TfR2 nuclear expression has been demonstrated in rat PC12 cells (Roth et al. 2000) and in mice glioblastomas cancer cell lines respectively (Calzolari et al. 2010). However, in contrast to liver and spleen membranous expression, being an iron importer, the nuclear localization of TfR1 in heart cells needs further insights.

Furthermore, LCN-2 colocalization with TfR1 and TfR2 was detected in liver during physiological and AP-conditions, however after ARP co-expression of LCN-2 and TfRs increased strengthening the hypothesis, LCN-2 transports iron during acute phase conditions. In extra-hepatic tissues (heart, spleen), LCN-2 interaction with transferrin receptors (TfR1, TfR2) was also observed in spleen of control and of TO-rat, however, in heart LCN-2 expression was detected only after AP reaction, appearance of LCN-2 protein in heart only after the generation of AP reaction can be due to stress conditions. We previously reported LCN-2 expression in heart at mRNA

level in the same settings and a different also study reported LCN-2 production by isolated neonatal cardiomyocytes (Yndestad et al, 2009). To the best of my knowledge the current study is first of a kind to exclusively report colocalization of TfRs in extra-hepatic tissues. My this study also suggests that differential localization of transferrin receptors (TfR1, TfR2) in hepatic and extra-hepatic tissues is due to diverse role of these proteins in different tissues under physiological and pathological conditions (AP-conditions).

Although nuclear expression of iron proteins in different organs has already been reported, previously, we and others showed TfR1 nuclear expression in rat brain (Malik et al, 2011), LCN-2 nuclear expression in mice brain (Ip et al, 2011), FTH (Ahmad et al, 2013), Fpn1 and DMT-1 nuclear expression in rat liver (Naz et al, 2012), in PC12 cells (Roth et al, 2000) and in mice glioblastoma cell lines respectively (Calzolari et al, 2010). However, colocalization and differential localization of iron importer proteins (TfRs, LCN-2) in hepatic and extra-hepatic tissues (heart and spleen) has never been reported before.

These findings support not only the presence of iron transport proteins within the nuclei to transport more iron in the hepatic cell nuclei under stress conditions but suggests that increased level of nuclear iron may be necessary for the increased activity of nuclear enzymes involved in DNA synthesis, repair and regulation of transcription (Roth et al, 2000).

7.5 The cellular localization of iron storage proteins (FTH, FTL) in rat hepatic and extra-hepatic tissues

Similar to iron importer proteins, protein expression of iron storage proteins (FTH and FTL) was also observed by immunohistochemistry. Immunofluorescence staining showed FTH

localized in nucleus while FTL mostly in cytoplasm under physiological and acute phase conditions. Furthermore, Western blot analysis revealed a FTL expression was strong in cytoplasm and week in the nuclei of cells. In contrast to FTL, FTH expression was stronger in nucleus and weaker in cytoplasm in hepatic and extra-hepatic organs (heart, spleen and brain). After the onset of ARP, protein expression of both ferritin subunits (FTH, FTL) increased. Ferritin is always reported as iron storage protein of cytosol (Meyron-Holtz et al, 2011) and its subcellular localization is controversial. Some previous studies reported presence of FTH in nucleus of human astrocytoma cell line (Surguladze et al, 2005), in corneal epithelial cells (Cai and Linsenmayer, 2001) and in mice hepatocytes during iron overload states (Smith et al, 1990). According to my study, FTH is constitutively expressed in hepatic and extra-hepatic organs during physiological and acute-phase conditions. During ARP, nuclear iron contents increased (Naz et al, 2012) therefore, detection of iron storage proteins in the nucleus of the cells is not surprising. Nuclear expression of FTH like TfR2 further confirms these findings that iron is sequestrated in both cytoplasm and nucleus of hepatic cells for nuclear metabolism. There could be another possibility that during acute-phase conditions, liver needs more iron to combat the increased metabolic work load (Ramadori and Christ, 1999).

Similarly, results of our previous and current study showed a parallel increase in mRNA and protein expression of ferritin subunits (FTH, FTL) and hepatic iron uptake during APR (Naz et al, 2012). Few previous studies clearly reported that amount of intracellular ferritin was regulated by change in iron status (Meyron-Holtz et al, 2011) and accumulation of H-chain (Goralska et al, 2001;Hentze et al, 1987). However, our previous studies reported that modulation in FTH, FTL and other iron metabolism proteins is also due to acute-phase cytokines (Naz et al, 2012;Sheikh et al, 2006). In other words, acute-phase cytokines synthesized during TO-induced

APR have direct effect on hepatic FTL and FTH up-regulation and this increase in their expression is not only due to the hepatic iron uptake.

A previous study (Darshan et al, 2009) reported FTH as main hepatic iron storage protein and hepatocytes become more susceptible to toxic effects of iron when FTH was depleted in these cells (Darshan et al, 2009). Moreover, over-expression of FTH in K562 cells resulted in reduction of free available iron pool in these cells (Picard et al, 1998). These observations clearly indicate that not only FTL but also FTH is needed for reduction of free available iron in the stressed hepatocytes during APR. FTL shares "the iron storage" function in hepatic and extra-hepatic tissues (heart, spleen and brain) however, liver is a unique organ due to its secretory function when compared to extra hepatic organs.

The nuclear expression of FTH in liver, heart, spleen and brain cells suggests that there is not only iron storage in the nuclei but also nuclei need to be protected from toxic effects of iron on DNA due to iron-overload (Darshan et al, 2009). This function might be more important during APR due to increased metabolic challenges.

8   CONCLUSION

In conclusion, I propose that under normal conditions, transferrin bound iron of portal blood is taken up by liver cells through TfRs mediated and non-trasnferrin bound iron uptake by LCN-2 under the influence of IL-6. Once in the cell, it is in part delivered not only to cytoplasmic organelles like mitochondria (Richardson et al, 2010) but also to the nucleus to meet the cellular functional requirements including DNA synthesis and RNA metabolism. As iron is transported into the nucleus, surplus iron in the nucleus is stored within FTH to protect nucleus from oxidative damage.

Under acute phase conditions, liver behaves as a "sponge" for iron as the decrease in serum iron levels is most probably achieved by increased hepatic uptake of transferrin bound iron by TfR1 mediated iron transport into the hepatocytes; increased LCN-2 also serves to transport iron into the cell. Furthermore, differential localization pattern of iron regulatory proteins in hepatic and extra-hepatic tissues indicates their diverse role in these tissues during physiological and acute phase conditions. The findings of current suggest that IL-6 causes hypoferremia of inflammation by inducing TfRs (exclusively TfR2) and LCN-2 through STAT3 pathway activation. These findings will help in the implications for further understanding the importance of iron metabolism in iron related disorders.

9 SUMMARY

Acute phase response (APR) is the systemic reaction to tissue injury and inflammation. It is clinically characterized by certain symptoms such as fever, weakness, and anemia, somnolence loss of appetite and cytokines release. In the blood, it results in an increase of positive acute phase proteins (APPs) in plasma, including clotting proteins, transport proteins, anti-proteases, and complement factors, with a concomitant decrease in negative APPs such as albumin. In addition, a decrease of serum iron-levels and consecutive increase of hepatic iron levels is also a hallmark of APR. This reaction is mediated by acute phase cytokines (APC) such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6 cytokines through the activation of different transcription factors. The mechanism of liver iron uptake during APR is poorly understood. Therefore, current study aimed to investigate the exact mechanism of iron uptake with relation to iron regulatory proteins and their signaling pathway under the influence of APC in liver and liver cells. Furthermore, sub-cellular localization of these proteins in hepatic and extra-hepatic organs during APR is also examined.

In vitro: primary cultures of rat isolated hepatocytes were stimulated with APC (IL-1β, IL-6 and TNF-α) in the presence/absence of different concentrations of iron (FeCl$_3$: 0.01mM, 0.1mM). APR was induced by injecting turpentine-oil (TO) in hind limbs of rat and mice (wild type and IL-6 knock-out). Hepatocytes cell lysates and cell supernatants were prepared for iron measurement, RT-PCR and Western blotting. Tissue samples (liver, spleen and heart) were removed to use for immunobloting and immunohistochemical analysis.

In hepatocytes treated with iron in the presence/absence of cytokines, a significant iron uptake was observed with 0.1mM iron administration with a maximum at 24h (133μg/g protein) in comparison to control and other iron concentrations administrated. This iron uptake was further

enhanced in the presence of acute phase cytokines with a maximum iron uptake (481µg/g of protein) after IL-6 and 0.1mM iron concomitant administration. LCN-2 and ferritin subunits (FTH, FTL) gene expression was up-regulated by iron with further induction by acute phase cytokines both, at mRNA and protein level. The maximum increase was seen for the combination of IL-6 with iron. TfR2 was increased by IL-6 and iron alone, however TfR1 was decreased. Combination of iron and APC abrogated the down-regulation of TfR1. Iron as well as APC stimulation led to LCN-2 release into supernatant, with increased release in the presence of iron and APC (IL-6 mainly) combined.

Similar to *in vitro* data, liver tissue of rat and wild type mice also showed an increase in iron transport (TfR1, TfR2 and LCN-2) and storage proteins (FTH, FTL) during APR whereas such an increase was not detected for TfR2, LCN-2 and FTH in IL6-KO mice. In parallel to induction of iron transporters, a phosphorylation of STAT3 was also observed in WT-mice however such a change was not noticed in IL-6 KO mice.

Immunohistochemistry of hepatic and extra hepatic tissues indicated organ dependent localization of TfR1 and TfR2. TfR1 was primarily localized in the membrane and cytoplasm of liver, and spleen cells whereas, in both organs, TfR2 showed nuclear expression mostly. However, in heart, both TfR1 and TfR2 were detected to be surplus in membrane, cytoplasm and nucleus. In all the organs studied, TfR1, TfR2 and LCN-2 were colocalized. The iron storage protein FTL was localized in cytoplasm while a strong FTH positivity was observed in the nucleus of liver cells. Similarly, in spleen and heart FTL was mostly localized in the cytoplasm however, an intense nuclear and a weak cytoplasmic expression was evident for FTH.

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By summarizing, results of current study demonstrate that liver behaves as a "sponge" for iron during APR. Iron uptake in hepatocytes is tightly controlled by already present iron and this uptake can be further enhanced by APC, mainly by IL-6. Hepatocytes need more iron to respond the massive increase of protein synthesis under such stress conditions. These changes could explain iron retention in hepatocytes during APR. Furthermore, portal blood iron is taken up by liver cells mostly through importer proteins (TfRs and LCN-2) and these proteins are regulated mainly through IL-6 activated STAT3 pathway. The differential localization pattern of iron regulatory proteins in hepatic and extra-hepatic organs suggests their organ specific diverse role during APR.

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11   ACKNOWLEDGEMENTS

Dedication

I dedicate my thesis to my elder brother
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Publications

1. **Ahmad S**, Sultan S, Naz N, *et al;* 2013. Regulation of iron uptake in primary culture rat hepatocytes: The role of acute phase cytokines*: Shock.*

2. **Ahmad S**, Moriconi F, Naz N, *et al;* 2013. Ferritin L and Ferritin H are differentially locat-ed within hepatic and extra hepatic organs under physiological and acute phase conditions*: Int J Clin Exp Pathol.* 6(4):622-9.

3. N. Naz, **S. Ahmad**, S. Cameron, *et al;* 2013. Differential regulation of ferritin subunits and other iron regulatory proteins - an effect of targeted hepatic X-irradiation: *Biomed. Res. Int.*

4. Alwahsh SM, Xu M, Seyhan HA, Schaefer I, **Ahmad S**, *et al;* 2013. Diet high in fructose leads to an overexpression of lipocalin-2 in rat fatty liver model: *World J Gastroenterol.*

5. Birlea SA, Ahmad FJ, Uddin RM, **Ahmad S**, *et al;* 2013. Association of Generalized Vitili-go with MHC Class II Loci in Patients from the Indian Subcontinent: *J Invest Dermatol.* 133(5):1369-72.

6. Uddin RM, Babar ME, Nadeem A, Hussain T, **Ahmad S**, Munir S, Ahmad FA. 2013. Ge-netic Analysis of the Prolactin gene in Pakistani cattle: *Mole Biol Rep.*

7. Rave-Fränk M, Malik IA, Christiansen H, Naz N, Sultan S, Amanzada A, Blaschke M, Cameron S, **Ahmad S**, Hess CF, Ramadori G, Moriconi F. 2013. Rat model of fractionat-ed (2 Gy/day) 60 Gy irradiation of the liver: long-term effects: *Radiat Environ Biophys.* 52(3):321-38.

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9. N. Naz, IA. Malik, N. Sheikh, **S. Ahmad**, *et al;* 2012. Ferroportin-1 is a 'nuclear'-negative acute-phase protein in rat liver: a comparison with other iron-transport proteins: *Lab Invest.* 92(6):842-56.

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11. Sultan, S**.**, M. Pascucci, **S. Ahmad**, *et al;* 2012. LIPOCALIN-2 is a major acute-phase protein in a rat and mouse model of Sterile Abscess: *Shock.* 37(2):191-6.

Abstracts and Posters presented

1. **S. Ahmad,** N. Naz, F. Moriconi, IA. Malik. Differential localization of transferrin receptors and lipocalin-2 in rat hepatic and extra-hepatic organs during acute phase response. J Gastro-enterol 2014; 52 - P_3_07 (DOI: 10.1055/s-0033-1360926)

2. **S. Ahmad,** N. Naz IA. Malik, P. Ramadori, A. Amanzada, G. Ramadori, F. Moriconi. Role of the hypoxia inducible factors during hepatic repair in a rat model of thioacetamide (TAA)-induced chronic liver damage and cholangiocarcinoma. J Gastroenterol 2014; 52 - P_1_34 (DOI: 10.1055/S-0033-1360878)

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6. N. Naz, **S. Ahmad,** N. Sheikh and Malik IA. Ferritin L and Ferritin H are differentially located within hepatic and extra hepatic organs under physiological and acute phase conditions: APASL-2013, Singapore

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8. S. Sultan, **S. Ahmad** and S. Cameron. Expression of Lipocalin2 in upper and lower parts of the liver after lung irradiation. Falk Workshop, Targeted Therapies in Hepatology, January 24-25, 2013 Hannover Germany

9. **Ahmad S**, Sultan S, Naz N, Malik IA and Ramadori G. Lipocalin-2 role in transferrin receptor 1 dependent and independent iron uptake in cultured rat primary hepatocytes. 2nd World Congress on Cell Science & Stem Cell Research, J Cell Sci Ther 2012, 3:8, November 12-14, 2012 San Antonio, USA (DOI:10.4172/2157-7013.S1.021)

10. Naz N., **Ahmad S**., Sheikh N., Mihm S., Ramadori G. and Malik I.A. Ferritin L is the sole serum ferritin constituent and a positive hepatic acute phase protein. 2nd World Congress on Cell Science & Stem Cell Research, J Cell Sci Ther 2012, 3:8, November 12-14, 2012 San Antonio, USA (DOI:10.4172/2157-7013.S1.021)

11. Sultan S, **Ahmad S**, Pascucci M, Ramadori G. Changes of LCN-2 gene expression in different organs in a rat model of tissue damage. GASL-Conference 2012, Hamburg, Germany

12. Sultan S**,** Cameron S, **Ahmad S**, Malik IA, Ramadori G. Serum Lipocalin-2 (LCN-2) is a potential Biomarker of Liver irradiation. ASCO-annual meeting, J Clin Oncol 30, 2012 (suppl; abstr e13039), Chicago, Illinois, USA

Computer and Software Skills:

Microsoft office, Operation of Windows XP, Vista, 7, 8 and 8.1, Graphpad prism 4 and 5, Axio-vision 4 software, ABI Prism 7000 system, Step one software, Reference manager, Adobe pho-toshop, Adobe acrobat professional, 32 Karat Software