

Original Article

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

Shakil Ahmad*, Federico Moriconi*, Naila Naz, Sadaf Sultan, Nadeem Sheikh, Giuliano Ramadori, Ihtaz Ahmed Malik

*Department of Internal Medicine, Division of Gastroenterology and Endocrinology, University Hospital, Georg-August-University, Robert-Koch-Str. 40, 37075 Göttingen, Germany. *These author contributed equally in the work.*

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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-peroxidase 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

Differential localization of ferritin subunits during APR

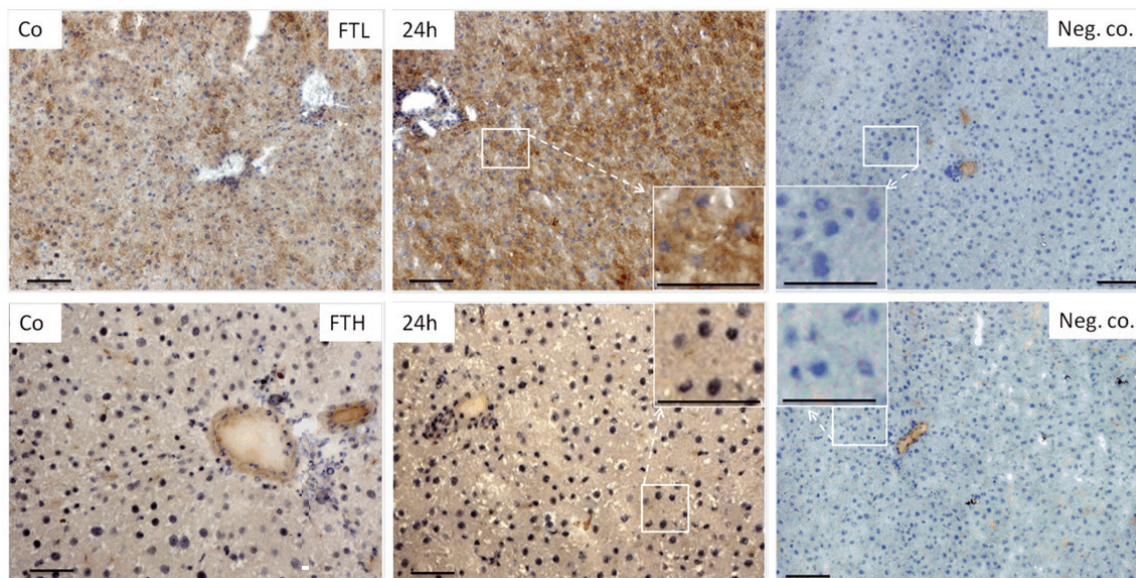


Figure 1. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of rat liver from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Negative control represents immunostaining when primary antibody was omitted. Insets show the enlarged magnification of selected box. Original magnification 200x bar 50 μ m.

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 immunocytochemistry

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)

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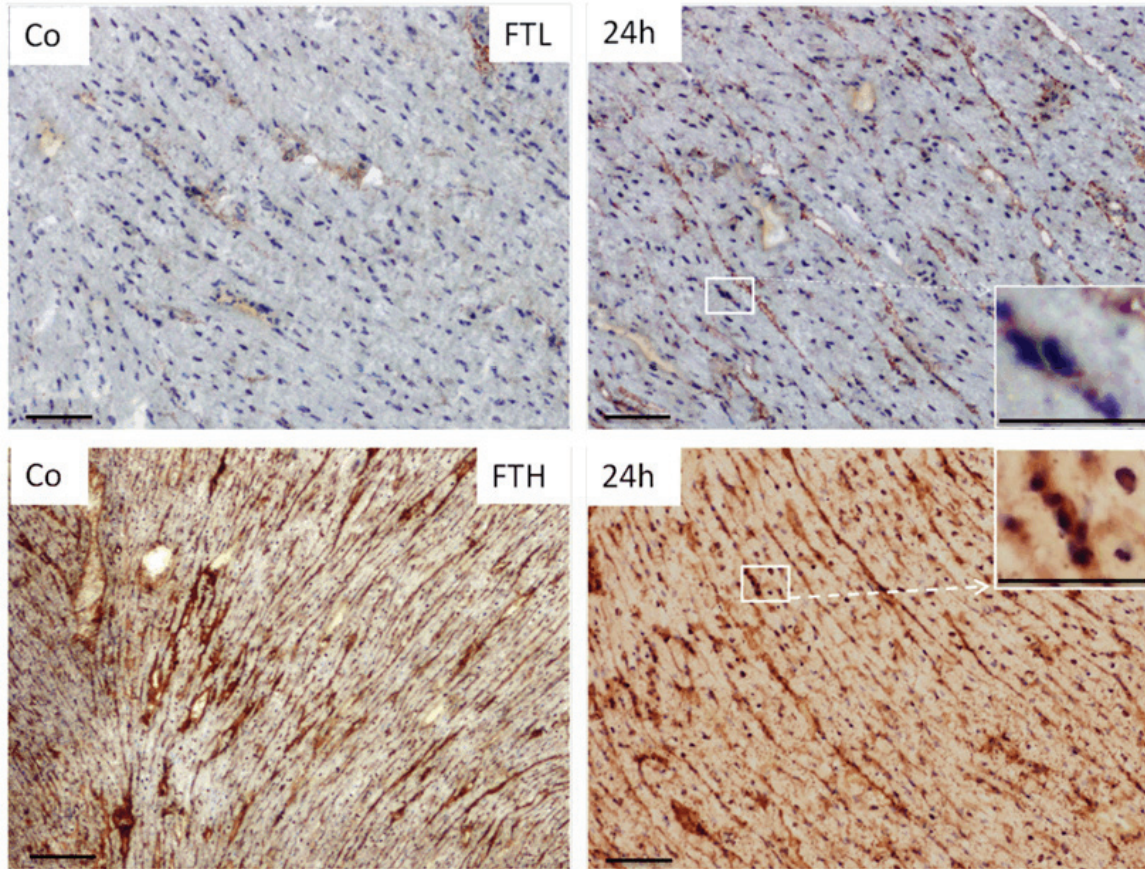


Figure 2. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of rat heart from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25 μ m.

and H₂O₂ (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 μ l 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 μ l of NucBuster reagent 2. 1 μ l 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 μ g of protein from tissue fraction was applied per well and were subjected to electrophoresis using NuPAGE[®] (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

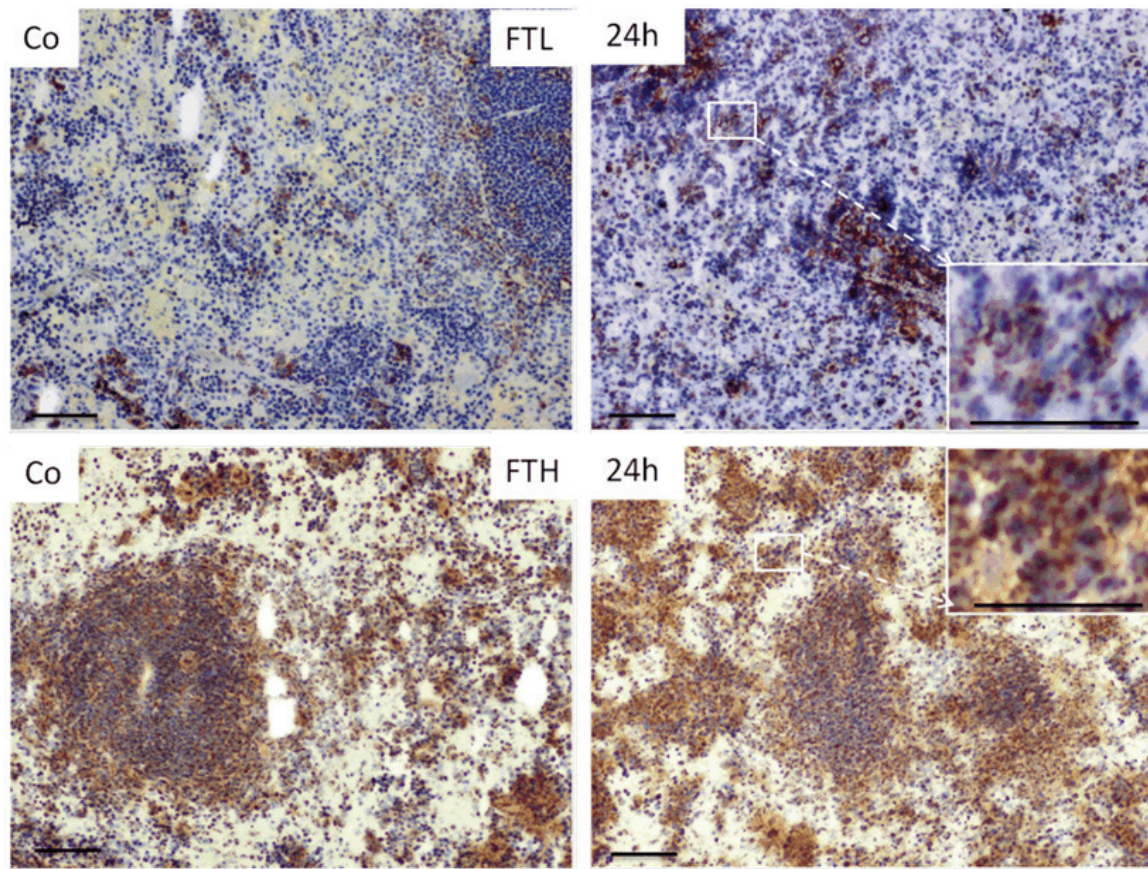


Figure 3. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of Rat spleen from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25µm.

cytoplasm of hepatic cells, which kept on increasing in TO treated rats. Intensity of FTL immunorexpression 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

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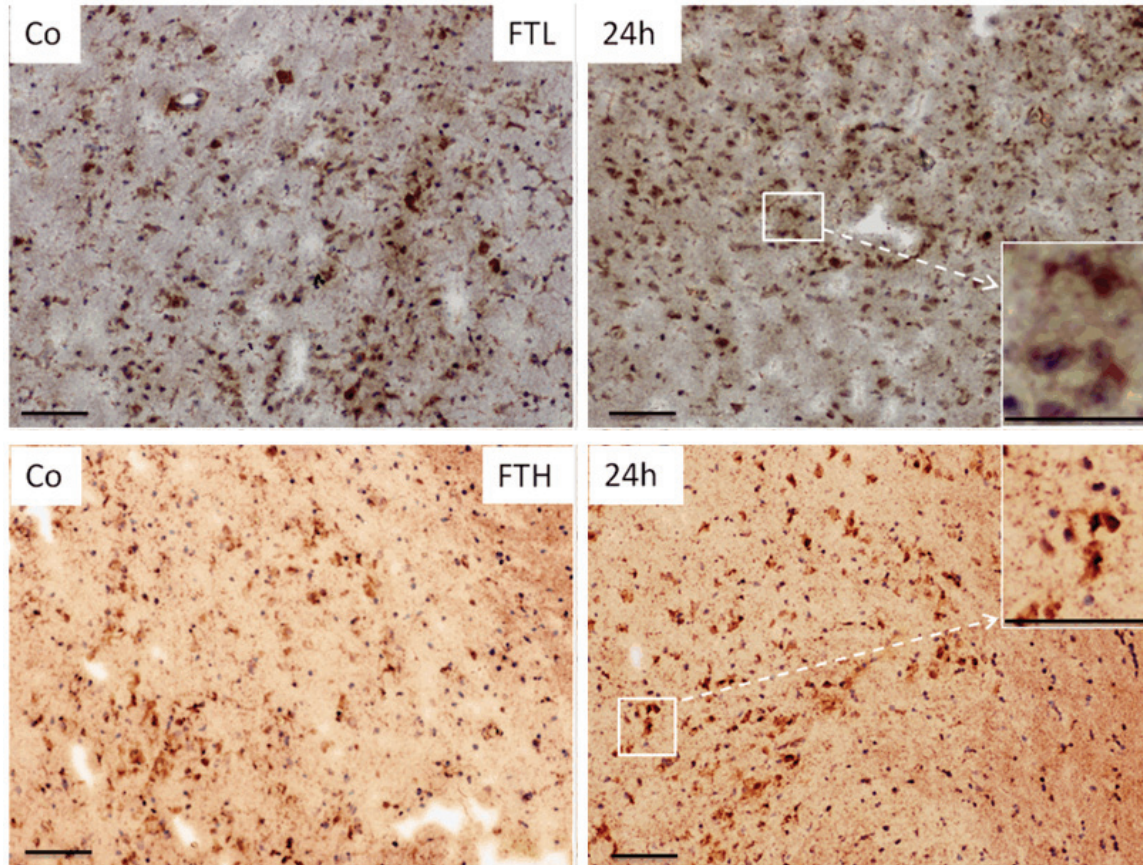


Figure 4. Immunodetection of FTL (upper panel) and FTH (lower panel) on cryostat sections of Rat brain from control and TO-injected animals utilizing horse reddish peroxidase conjugated secondary antibody. Insets show the enlarged magnification of selected box. Original magnification 100x bar 25µm.

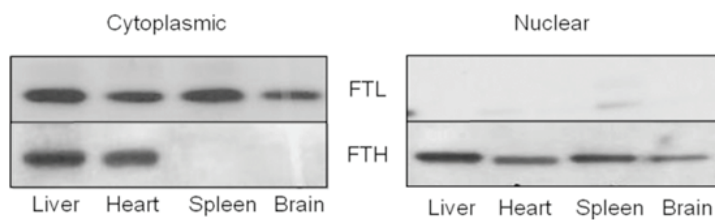


Figure 5. Western blot analysis of FTL and FTH in protein extracted from nuclear and cytoplasmic fractions of different organs of control animals.

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 of 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 previ-

ous 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.

Address correspondence to: Dr. Ihtaz Ahmed Malik, Department of Internal Medicine, University Hospital Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany. Phone: 0049 (551) 398902; Fax: 0049 (551) 396921; E-mail: i.malik@med.uni-goettingen.de

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