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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 (interleukin 6 [IL-6], IL-1β, tumor necrosis factor α) in the presence/absence of iron ($FeCl_3$: 0.1 mM). Hepatocytes were harvested at different time points (0, 6, 12, 24 h). Total mRNA and proteins were extracted for reverse transcriptase–polymerase chain reaction (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), $FeCl_3$

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.

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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 not only be 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 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 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_2. Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (PAA, Cölbe, Germany), 1 nmol/L insulin, and 100nmol/L dexamethasone (Sigma-Aldrich, Munich, Germany) for 48 h to obtain confluence 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 (0.01, 0.1, 0.5 mM) of $FeCl_3$ (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 $FeCl_3$ (0.1 mM) (21, 22) to cultured hepatocytes in serum-free medium containing 0.2% bovine serum albumin. Each experiment was repeated four times (n = 4) in duplicates, cells were harvested at 0, 6, 12, and 24 h after 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 measured as μg/g of hepatocytes protein and analyzed using Microsoft Excel 2007.

Isolation of total RNA and polymerase chain reaction 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 polymerase chain reaction (PCR) was performed at 95°C to 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 using 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 described previously (24). Protein contents were calculated by the Coomassie protein assay (Pierce, Bonn, Germany). Cell supernatant (culture medium in which hepatocytes were cultured) as whole and cell lysates was used for Western blot analysis. Western blot was performed with sodium dodecyl sulfate polyacrylamide 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 $P < 0.05$. Bonferroni correction was applied to iron uptake data set obtained from $FeCl_3$ treatment groups to prevent accumulation of α, and significance was accepted at $P < 0.0125$ (α/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 0.1 mM concentration was found to be the most efficient for iron uptake in isolated hepatocytes compared with 0.01 and 0.5 mM. Therefore, we used

TABLE 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 (Wiesbaden, Nordenstadt, Germany)	AF3508	1:300
TfR1	Invitrogen (Darmstadt, Germany)	13-6890	1:1,000
FTH	LS Bio (Eching, Germany)	LS-C23537	1:500
FTL	Abcam (Cambridge, UK)	ab69090	1:1,000
β-Actin	Sigma (Munich, Germany)	A-2228	1:5,000

0.1 mM 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 (0.01and 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 significantly 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 maximum 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). IL-6–treated 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 administration of IL-1β (3.53 ± 0.14-fold), whereas TNF-α–treated hepatocytes 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.

Reverse transcriptase (RT)–PCR results were further confirmed 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 administration 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.

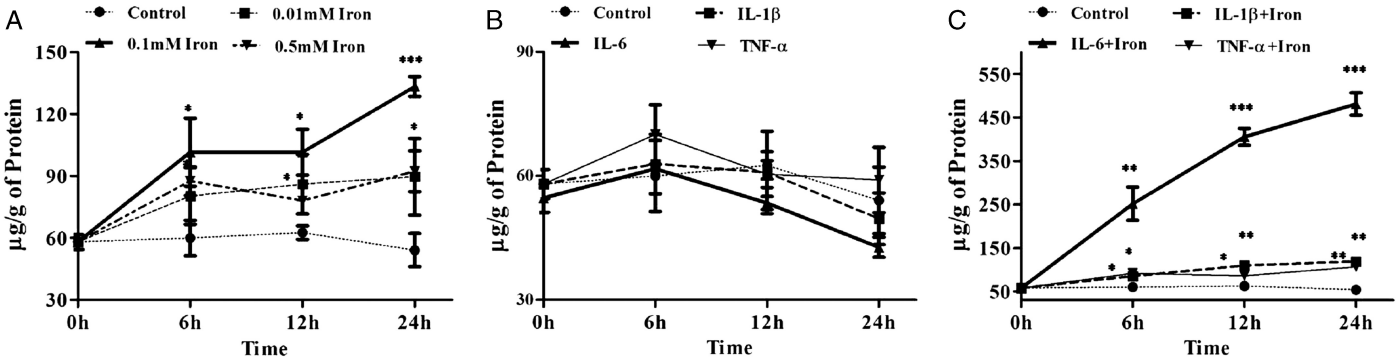

Fig. 1. **Measurement of iron uptake in hepatocytes: iron-treated hepatocytes (A)**. Cytokines treated hepatocytes (B). Cytokines + iron-treated hepatocytes (C). Values on *y* axis represent the intracellular iron contents of hepatocytes compared with nontreated controls. Results represent the mean value ± SEM. **P* < 0.0125, ***P* < 0.001, ****P* < 0.0001 analyzed by *t* test (n = 4).

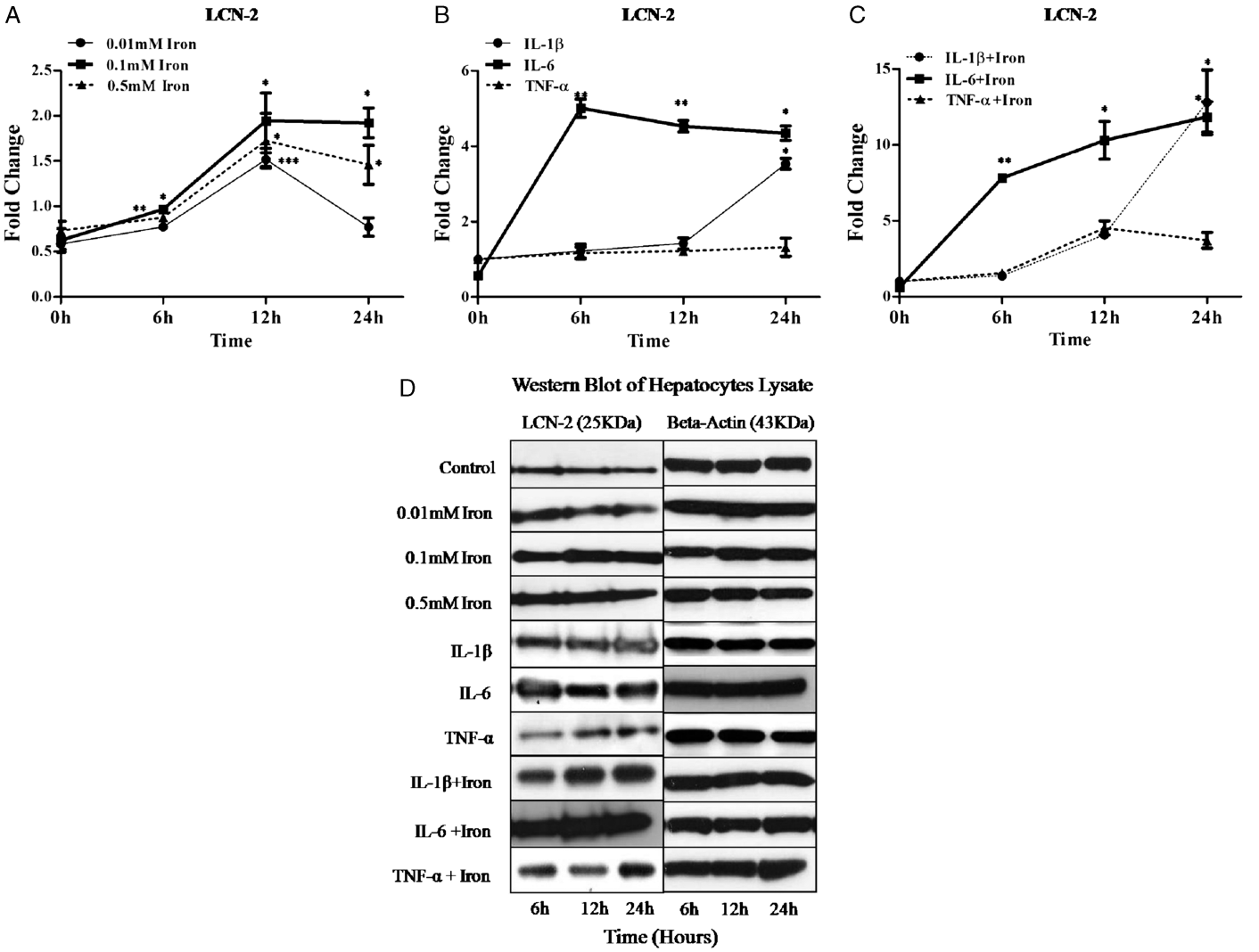

FIG. 2. **Quantitative RT-PCR analysis of total mRNA from rat hepatocytes**. Fold change in mRNA expression of LCN-2 gene expression after iron treatment (A), cytokine treatment (B), cytokines + iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCRwas 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 untreated controls. The most pronounced induction was observed by IL-1β (Fig. 5D). Iron-treated hepatocytes showed a higher

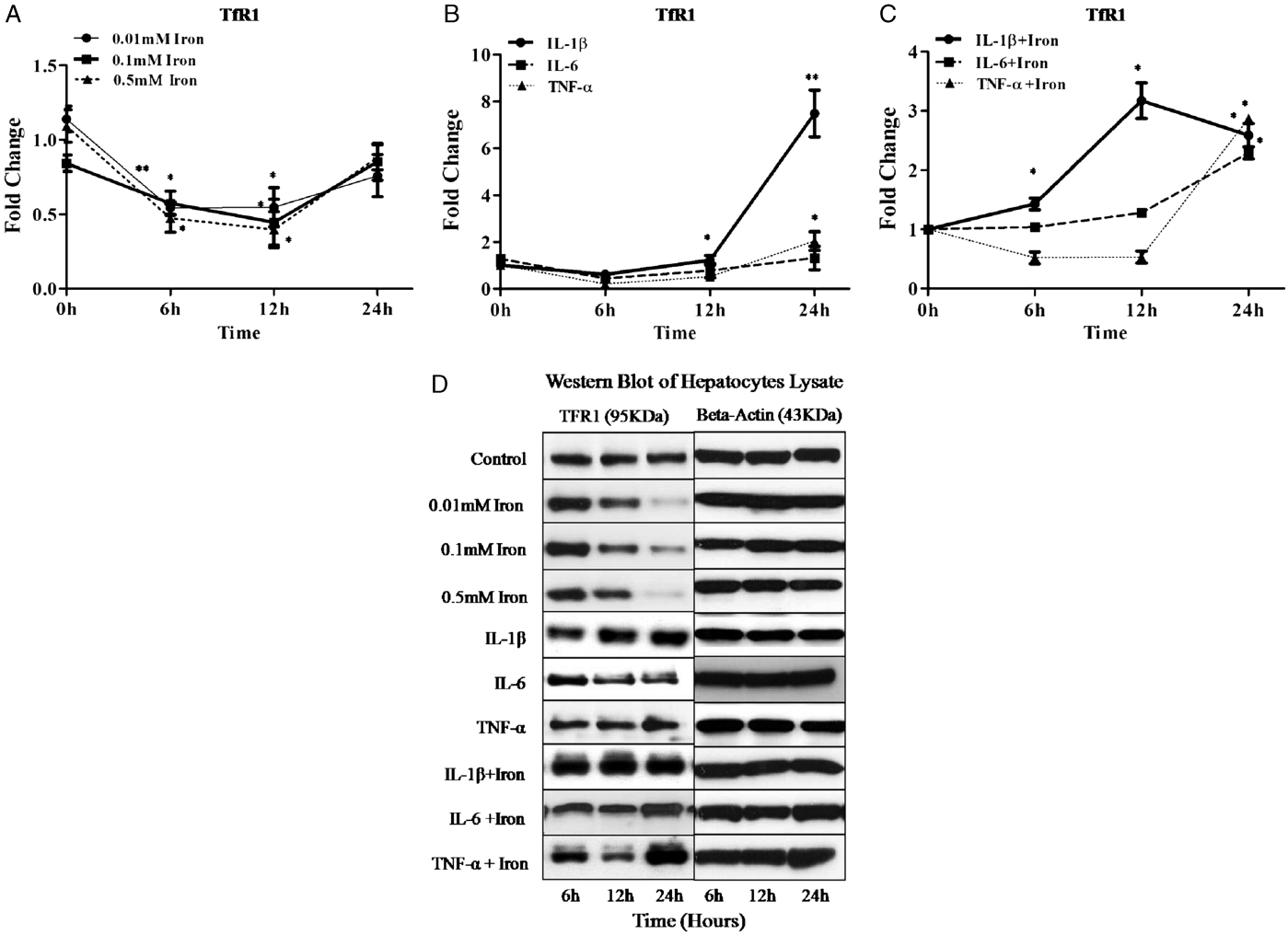

Fig. 3. **Quantitative RT-PCR analysis of total mRNA from rat hepatocytes**. Fold change in mRNA expression of TfR1 gene expression after iron treatments (A), cytokine treatment (B), cytokines + iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative 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).

induction of FTL than administration of cytokines alone. No significant 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 (6 h) 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).

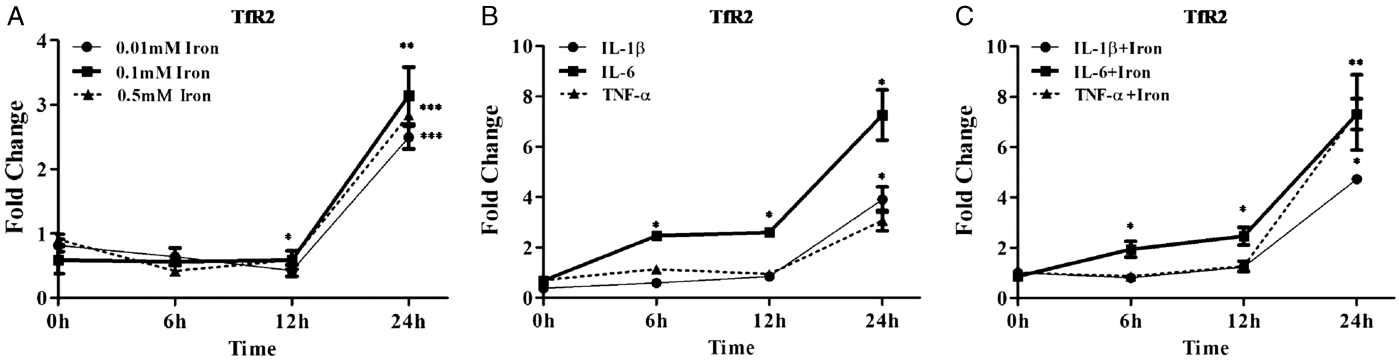

Fig. 4. **Quantitative RT-PCR analysis of total mRNA from rat hepatocytes**. Fold change in mRNA expression of TfR2 gene expression after iron treatments (A), cytokine treatment (B), cytokines + iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative 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).

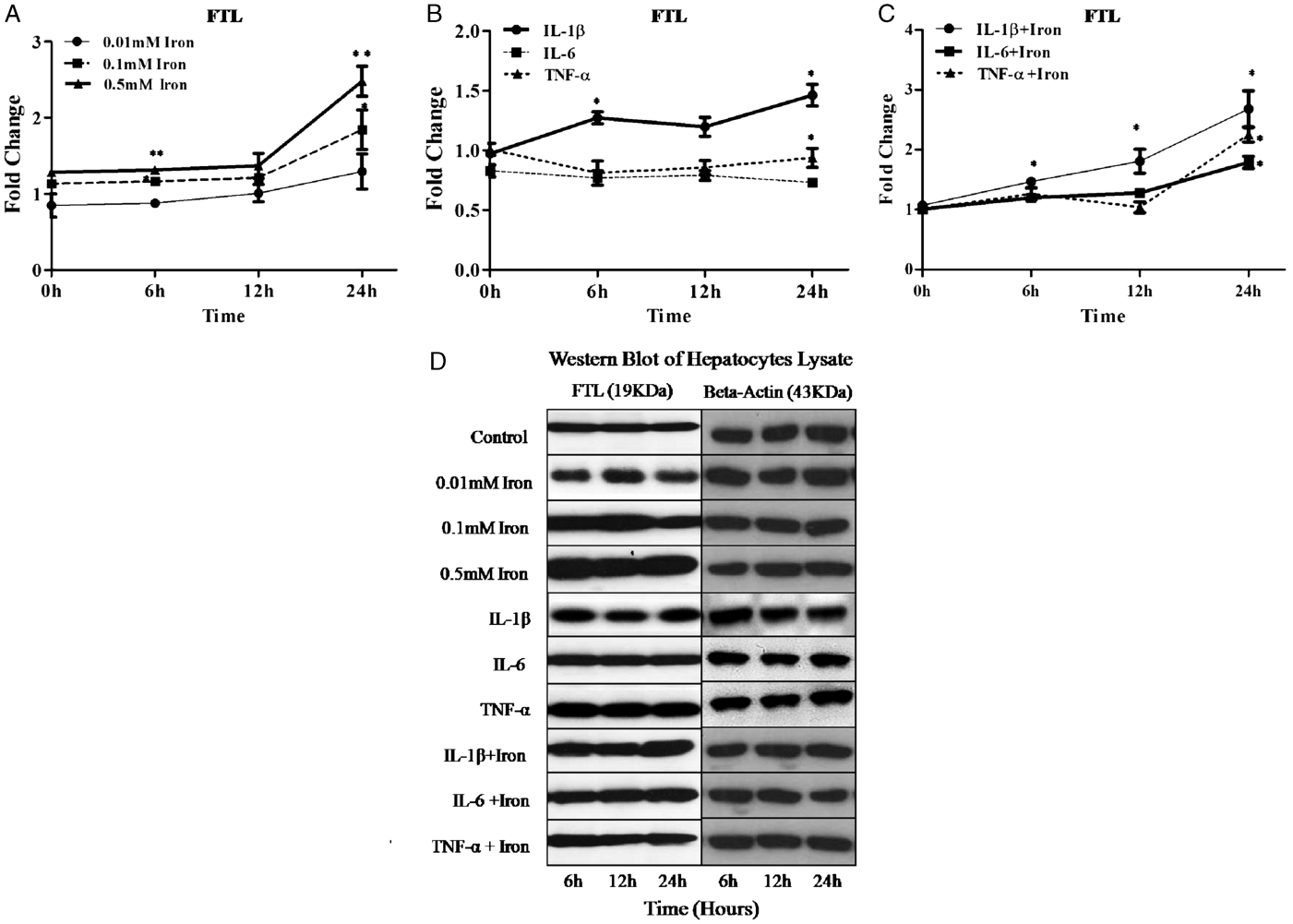

FIG. 5. **Quantitative RT-PCR analysis of total mRNA from rat hepatocytes**. Fold change in mRNA expression of FTL gene expression after iron treatments (A), cytokine treatment (B), cytokines + iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value ± SEM. $^{*}P < 0.05$, $^{**}P < 0.001$ 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 control level throughout the course of 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 iron. 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 expression caused by iron was further enhanced in the presence of AP cytokines, mainly by IL-6.

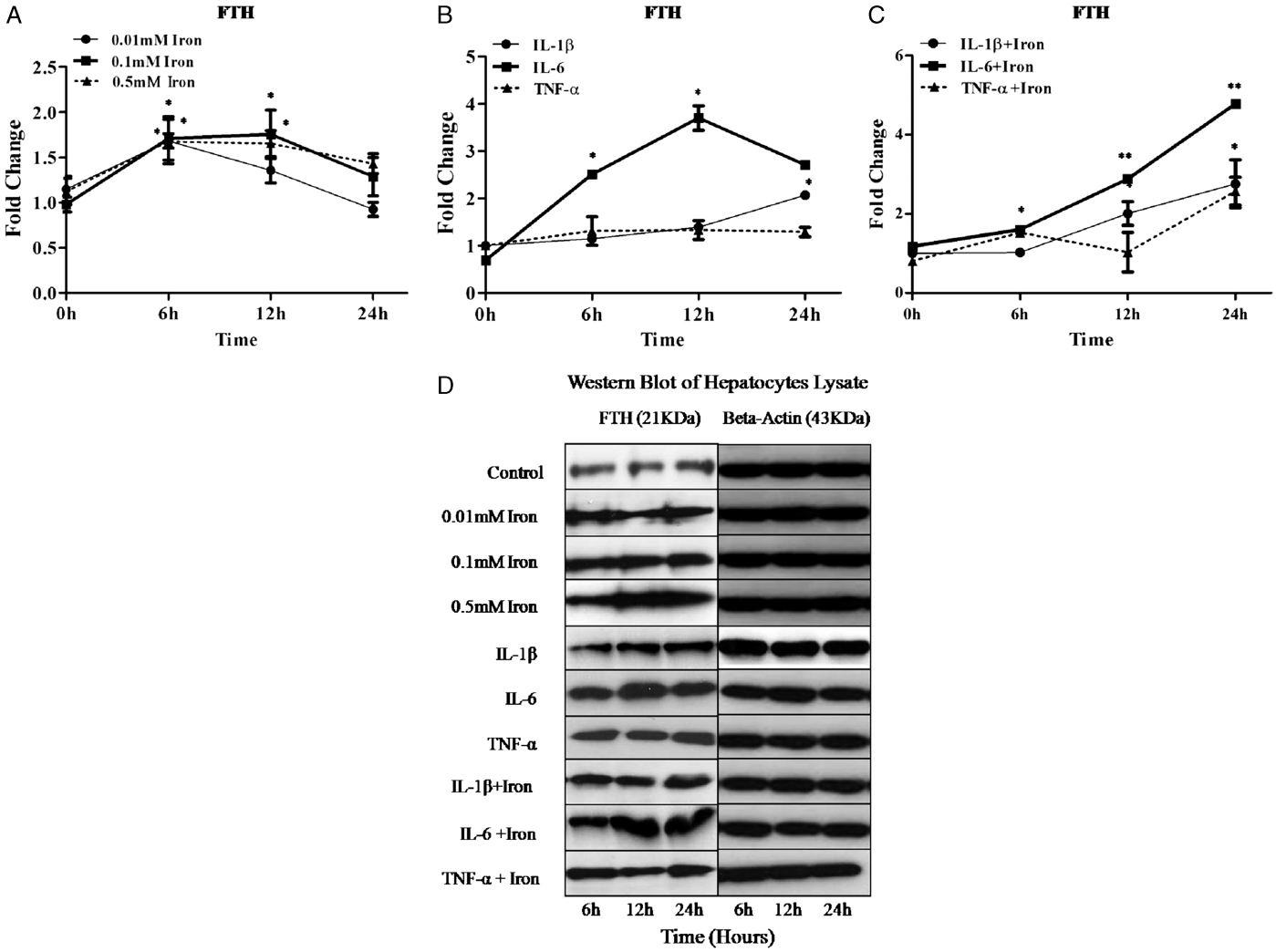

FIG. 6. **Quantitative RT-PCR analysis of total RNA from rat hepatocytes**. Fold change in mRNA expression of FTH gene expression after iron treatments (A), cytokine treatment (B), cytokines + iron treatment (C) at different time points related to nontreated controls for each time point. Quantitative RT-PCR was normalized by using housekeeping gene β-actin. Results represent mean value ± SEM. $^{*}P < 0.05$, $^{**}P < 0.001$ analyzed by *t* test (n = 4). Western blot analysis of FTH (21 kd) from total protein of rat hepatocytes. β-Actin (43 kd) 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. Transferrin receptor 2 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 activated 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 -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 cytokines 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 stimulated 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. Lipocalin 2 release into the

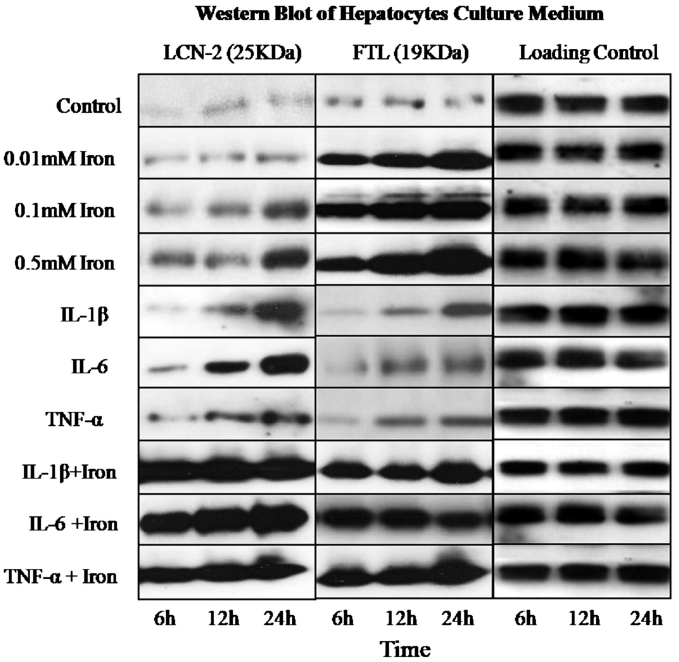

FIG. 7. **Western blot analysis of LCN-2 (25 kd) and FTL (19 kd) from total protein of rat hepatocytes supernatant**. Ponceau S staining was used for equal loading.

supernatants 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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