

## Regulatory effects of tumor necrosis factor-alpha and interleukin-6 on HAMP expression in iron loaded rat hepatocytes

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**Background/Aims:** To study the effect of iron and proinflammatory cytokines on the expression of HAMP and other iron regulatory genes in primary rat hepatocytes.

**Methods:** Primary hepatocytes from rats fed a control or iron-enriched diet were plated on extracellular matrix and incubated with inflammatory stimuli in the presence or absence of serum. Cells were also incubated with desferrioxamine or ferric ammonium citrate. mRNA levels were determined by Real-Time PCR.

**Results:** Hepatocytes from control rats increased their HAMP expression during culturing, whereas the opposite was seen in hepatocytes from carbonyl-iron loaded animals. In the presence of serum, tumor necrosis factor-alpha, lipopolysaccharide and interleukin-6 increased HAMP expression in hepatocytes from both control and iron-loaded rats. Under serum-free conditions only tumor necrosis factor-alpha increased HAMP mRNA levels. Desferrioxamine and ferric ammonium citrate decreased HAMP gene expression. Tumor necrosis factor-alpha significantly increased mRNA levels of TfR2 and decreased those of DMT1 and IREG1.

**Conclusions:** HAMP expression differs in cultured as compared with freshly isolated hepatocytes, and decreases in iron-loaded hepatocytes in serum free-media, suggesting that additional serum factors influence HAMP expression. Tumor necrosis factor-alpha regulates the mRNA levels of HAMP, IREG1, DMT1 and TfR2 in cultured hepatocytes from both iron-loaded and control animals.

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**Keywords:** Rat; Liver; Hepatocytes; Gene expression; Iron metabolism; Iron overload; Inflammation; Hepcidin; TNF-alpha

### 1. Introduction

Hepcidin is a cysteine-rich antimicrobial peptide [1,2], which act as an important regulator of body iron homeostasis by negatively controlling intestinal iron absorption and macrophage iron release [3,4]. The hepcidin protein is synthesized by hepatocytes and encoded by the HAMP gene [1,5], the transcription of which is stimulated by iron [6] and inflammatory stimuli, such as interleukin-6 (IL-6), lipopolysaccharide (LPS) and turpentine [6–8]. The mechanisms as to how iron and inflammation regulates HAMP gene expression are still poorly understood. Treatment with dietary or parenteral iron in vivo induces hepatic HAMP gene expression [6], whereas iron added to

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**Abbreviations:** BSA, bovine serum albumin; CoS, serum from rats fed the standard diet; DFO, desferrioxamine; DMT1, divalent metal transporter 1; EHS, Engelbreth-Holm-Swarm tumours; FAC, ferric ammonium citrate; FCS, fetal calf serum; FeS, serum from rats fed the carbonyl-iron supplemented diet; HAMP, hepcidin antimicrobial peptide; HFE, hemochromatosis gene; IL-6, interleukin 6; IREG1, iron regulated transporter 1; LPS, lipopolysaccharide; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; TNF-alpha, tumor necrosis factor-alpha.

human or murine hepatocytes in culture does not upregulate HAMP transcription [6,8]. HAMP mRNA is increased in human hepatocytes treated with IL-6 [8] but LPS still has a stimulatory effect on IL-6 knockout mice [9], suggesting that other cytokines may also act as stimulators of hepcidin transcription. Both IL-6 [10] and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [11,12] causes hypoferremia in humans and rodents. TNF- $\alpha$  is also known to induce ferritin-H [13–15].

Cellular uptake of transferrin-bound iron from the plasma occurs through receptor-mediated endocytosis via transferrin receptor-1 (TfR1) [16]. In addition, recent data suggests that iron also can be delivered to the hepatocyte as non-transferrin bound iron via the divalent metal ion transporter-1 (DMT1) [17]. TfR1 interacts with the HFE protein [18–20], the gene of which is mutated in the majority of patients with hereditary hemochromatosis [21]. TfR2 belongs to the transferrin receptor family and is highly expressed in hepatocytes [22]. Mutations in TfR2 have been associated with development of iron overload, suggesting a regulatory role in iron metabolism [23,24]. Inside the cell, DMT1 transports iron across the endosomal membrane to the cytosol [25,26], but its major role is the transport of ferrous iron through the apical membrane of enterocytes [27], thus being involved in the iron uptake from the gut. Iron export from cells is mediated via iron-regulated transporter 1 (IREG1) (also known as Ferroportin), which is highly expressed on the basolateral membrane of enterocytes and on the plasma membrane of reticuloendothelial cells [28–30].

Previous studies have indicated that hepatic HAMP gene expression is influenced by plating and culturing time [6] or whether or not serum is included in the medium [6,8]. Therefore, in the present paper we analyzed the HAMP gene expression in fresh hepatocytes and in hepatocytes cultured for different lengths of time in serum-containing and serum-free medium. The effects of cytokines on hepcidin mRNA expression are hitherto performed on hepatocytes without dietary iron overload. To explore the unknown combined effects of cytokines and dietary iron on hepcidin gene expression, we studied the effects of iron, TNF- $\alpha$ , LPS and IL-6 on HAMP mRNA levels in hepatocytes from control and iron-loaded liver, and the effects of TNF- $\alpha$  on other genes involved in iron uptake or release from cells.

## 2. Materials and methods

### 2.1. Isolation of rat hepatocytes

Male Sprague-Dawley rats fed a control rat diet or a diet supplemented with 2.5% (w/w) carbonyl iron (Sigma, St Louis, MO, USA) for 6–8 weeks were sacrificed according to the local institution's guidelines.

Hepatocytes were isolated after whole liver perfusion through the portal vein with collagenase as previously described [31,32] with some changes. The liver was perfused for 15 min with a Krebs-Ringer

bicarbonate buffer (Sigma, St Louis, USA) containing 0.13% Na-bicarbonate, 0.01% EGTA, 1 mM HEPES, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, followed by 15 min perfusion with 0.025% collagenase (Sigma, St Louis, USA) containing Nutmix F12 (Gibco BRL, UK) buffer with the same amount of HEPES and antibiotics as described above. Hepatocyte purity exceeded 99% as assessed by light microscopy, and cell viability was more than 95% as judged by the Trypan blue exclusion test. The iron concentration in control hepatocytes was 0.35  $\mu$ g Fe/mg protein and in iron-loaded hepatocytes 1.9  $\mu$ g Fe/mg protein [31].

### 2.2. Incubation of hepatocytes in 5% BSA for different lengths of time

To investigate the effect of culture plating on HAMP mRNA expression, hepatocytes from one control and one iron-loaded rat were cultured in 5% BSA for 12 different lengths of time. Freshly isolated hepatocytes ( $n=2.25 \times 10^6$ ) were plated on plastic cell culture dishes coated with an extracellular matrix (ECM) derived from Engelbreth-Holm-Swarm (EHS) tumours from mice. Cultures were incubated in 3 ml 199 Earles modified salts medium (Gibco BRL, UK) in the presence of 10% FCS (fetal calf serum) for 4 h. The medium was then changed to one containing 5% BSA (bovine serum albumin). Medium was refreshed after 17 and 41 h. Cells were harvested at the time points 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 41 and 65 h from the start of incubation with 5% BSA (Fig. 1(a)).

### 2.3. Incubation of hepatocytes in 5% BSA and three different sera

To investigate the effect of different sera on HAMP mRNA expression, hepatocytes from three control and three iron-loaded rats were plated and incubated for 41 h in 199 Earles modified salts medium containing either 5% BSA, 10% FCS, 10% serum from control or carbonyl-iron loaded rats, as demonstrated in Fig. 1(b). Media were refreshed after 4 and 17 h. Sera from control or carbonyl-iron loaded rats were obtained by extraction of blood into Vacutainer serum-tubes with gel (BD, Spain), followed by centrifugation for 10 min at 2400 rpm and collection of supernatants into new tubes.

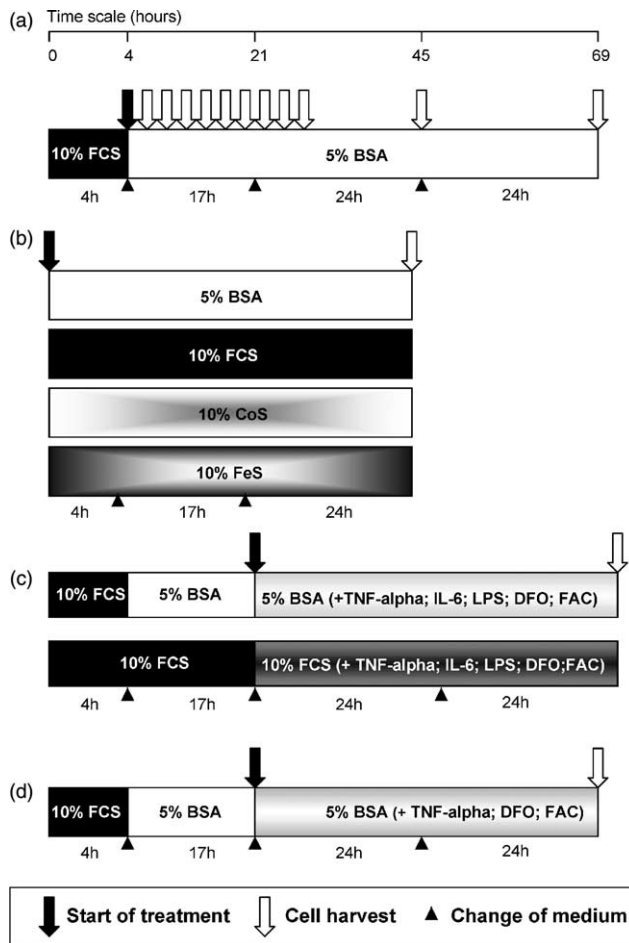
### 2.4. Incubation of hepatocytes in 5% BSA/10% FCS, with TNF- $\alpha$ , IL-6, LPS, desferrioxamine (DFO), or Ferric Ammonium Citrate (FAC)

To investigate the effects of various factors on HAMP mRNA expression, hepatocytes from three control and three iron-loaded rats were exposed to TNF- $\alpha$ , IL-6, LPS, DFO, or FAC in either 5% BSA or 10% FCS, as shown in Fig. 1(c). For analysis of the gene expression of TfR1, TfR2, DMT1, IREG1 and HFE, the cells were exposed to TNF- $\alpha$ , DFO, or FAC in 5% BSA, as shown in Fig. 1(d). Freshly isolated hepatocytes were plated and cultured in the 199 Earles modified salts medium containing 10% FCS for 4 h. Then the medium was changed to one containing 5% BSA, and cells were cultured over night (17 h). The cell cultures were then incubated for 48 h in 199 Earles modified salts medium containing 5% BSA with 10 ng/ml of TNF- $\alpha$ , 1 mM DFO, 100  $\mu$ M FAC, or left untreated [13]. Alternatively, the cells were cultured in the same medium containing either 5% BSA or 10% FCS with the addition of TNF- $\alpha$  (10 ng/ml), IL-6 (20 ng/ml), LPS (0.1  $\mu$ g/ml), DFO (1 mM), FAC (100  $\mu$ M) (all from Sigma, St Louis, MO, USA) for 48 h, or left untreated. Medium was refreshed after 24 h of incubation.

### 2.5. Preparation of total RNA and real time quantitative RT-PCR assays

Total RNA was extracted using the Ultraspec™-II RNA Isolation System (Biotex Laboratories, Inc., Houston, TX, USA) according to the manufacturer's instructions.

Two micrograms of quality-checked DNase-treated total RNA (RNase-free DNase Set, RNeasy Protect Mini Kit; Qiagen GmbH, Hilden,



**Fig. 1.** Experimental designs of the four different experiments, as described in Section 2. (a) Experiment no. 1. After incubation with 10% FCS for 4 h, cells from one control rat and one rat treated with carbonyl iron were incubated in 5% BSA for twelve different time periods (0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 41 and 65 h) for analysis of HAMP mRNA levels. (b) Experiment no. 2. Cells from three control and three iron-treated rats were incubated with either 5% BSA, 10% FCS, serum from control animals (CoS) (10%), or serum from carbonyl-iron treated animals (FeS) (10%) for 41 h, for determination of HAMP mRNA levels. (c) Experiment no. 3. After initial incubation with 10% FCS for 4 h, cells from three control and three iron-treated animals were incubated with either 5% BSA or 10% FCS with the addition of 10 ng/ml of TNF- $\alpha$ , 20 ng/ml IL-6, 0.1  $\mu$ g/ml LPS, 1 mM DFO or 100  $\mu$ M FAC for quantitation of HAMP mRNA levels. (d) Experiment no. 4. After incubation with 10% FCS for 4 h and 5% BSA for 17 h, cells from three control rats and three iron-treated animals were exposed to 10 ng/ml of TNF- $\alpha$ , 1 mM DFO or 100  $\mu$ M FAC in 5% BSA. After 48 h, cells were collected for measurement of TfR1, TfR2, DMT1, IREG1 and HFE mRNA levels. BSA, bovine serum albumin; FCS, fetal calf serum; CoS, serum from rats fed the standard diet; FeS, serum from rats fed the carbonyl-iron supplemented diet; TNF- $\alpha$ , Tumor necrosis factor alpha.

Germany) was reverse transcribed into cDNA with the SuperScript III RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen, USA). The relative amount of mRNA was quantified through singleplex Real Time RT-PCR analysis on an ABI PRISM<sup>®</sup> 7000 Sequence Detection System, using eukaryotic 18S ribosomal RNA as endogenous control (TaqMan Ribosomal RNA Control Reagents, Applied Biosystems). PCR amplification was performed in triplicates in accordance with the manufacturer's instructions (Table 1). Expression data were normalized to 18S rRNA and the relative mRNA

expression levels calculated according to the  $\Delta\Delta C_T$  (comparative  $C_T$ ) method (Applied Biosystems; User Bulletin #2: Relative Quantitation of Gene Expression). The identity of the PCR product for each gene under study was verified by direct sequence analysis in random samples.

## 2.6. Statistics

Differences in gene expression in different hepatocyte cultures were assessed by one-way analysis of ANOVA followed by Student-Newman-Keuls test. Values of  $P < 0.05$  were considered to indicate statistically significant differences.

## 3. Results

### 3.1. HAMP mRNA levels during incubation with 5% BSA for different lengths of time

HAMP mRNA levels were higher in hepatocytes obtained immediately after perfusion ('fresh hepatocytes') in the carbonyl iron-treated animal than in the animal fed standard chow diet. After plating, mRNA levels gradually dropped to steady-state levels after 10 h in hepatocytes from the normal rat and after approximately 30 h in hepatocytes from the iron-loaded animal (Fig. 2).

### 3.2. Effects of incubation with different sera on HAMP mRNA levels

HAMP mRNA expression decreased in iron-loaded hepatocytes after culturing for 45 h in 5% BSA, while it increased in cultured hepatocytes from control rats as compared levels detected in freshly isolated hepatocytes. The expression levels of HAMP mRNA was significantly higher in control hepatocytes cultured in the presence of 10% FCS or sera from control or carbonyl-iron treated animals than in those cultured in 5% BSA. However, the effect of FCS was more pronounced. Addition of 10% serum to the culture medium maintained the high expression level of HAMP in iron-loaded hepatocytes. There was no statistical significant difference in the effect on HAMP mRNA levels between FCS and sera from control or iron overloaded rats (Fig. 3).

### 3.3. Effects of treatments with TNF- $\alpha$ , IL-6, LPS, FAC and DFO, in medium with 5% BSA or 10% FCS on HAMP gene expression

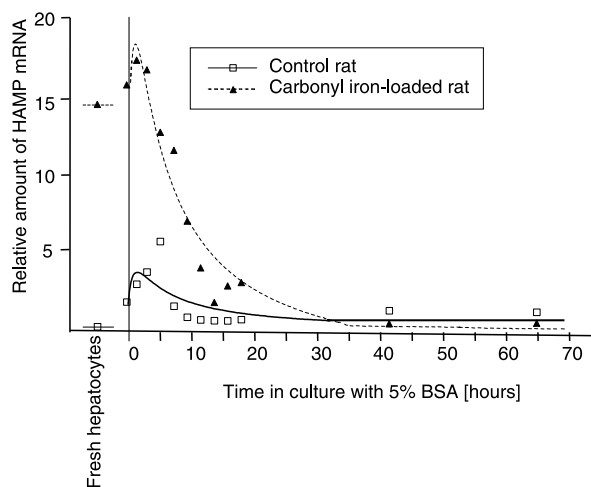
In hepatocytes from control animals cultured in the presence of 5% BSA, TNF- $\alpha$  treatment resulted in increased HAMP mRNA levels ( $P < 0.004$ ) while treatment with FAC and DFO resulted in decreased mRNA levels ( $P < 0.001$ ;  $P < 0.03$ ). In cultures with 10% FCS HAMP mRNA levels were increased in control hepatocytes exposed to TNF- $\alpha$ , IL-6 or LPS ( $P < 0.001$ ;  $P < 0.003$ ;  $P < 0.04$ ). Treatment with FAC and DFO lead to decreased levels of HAMP mRNA ( $P < 0.001$ ;  $P < 0.001$ ).

**Table 1****Primers and TaqMan probes for quantification of iron metabolism-related transcript expressed in rat hepatocytes, using real time quantitative RT-PCR**

Transcript	GenBank accession number	Oligonucleotides used in real time quantitative RT-PCR
HFE	AJ001517	Forward primer: 5'-TGGACCATCATGGGCAACTATA-3' Reverse primer: 5'-CAGAAGCCACTGG-TACTGTTGTCT-3' Probe: 5'-(6FAM) CCACAGTAAGGTCACGAA(MGB/NFQ)-3
TfR1	M58040	Forward primer: 5'-GAATACGTTCCCGTTGTTGA-3' Reverse primer: 5'-ATCCCCAGTTCCTAGAT-GAGCAT-3' Probe: 5'-(6FAM)CAGACCTTCAATTCTTT (MGB/NFQ)-3'
TfR 2	XM_222022	Forward primer: 5'-TGGGATGCTGAAGTGATCCA-3' Reverse primer: 5'-CCGAAAAGCTGTGAAG-GAA-3' Probe: 5'-(6FAM) TGCCCATGGACAGCAGCGCAT (MGB/NFQ)-3'
DMT1-IRE	AF008439	Forward primer: 5'-GCCTGTCGTTCTCTGGACTGT-3' Reverse primer: 5'-AGTATTGCCACCGCTGG-TATCT-3' Probe: 5'-(6FAM) CGGTAAGCATCTCTAAAGT (MGB/NFQ)-3
IREG1	AF394785	Forward primer: 5'-GCCTGGCTTCTCTATATGACG-3' Reverse primer: 5'-ACTCAGTCCCTGAGTG-TAAGCATATC-3' Probe: 5'-(6FAM) CTTCGACTGTATCACCACA (MGB/NFQ)-3'
HAMP	NM_053469	Forward primer: 5'-CGAGACACCAACTTCCCCATAT-3' Reverse primer: 5'-GCTCTGGCTCTCTATGTTATGCA-3' Probe: 5'-(6FAM) CTCTGCTGTAATGCTGT (MGB/NFQ)-3'

Gene-specific primers and TaqMan MGB probes were designed by using the Primer Express™ software v 2.0 (Applied Biosystems, Foster City, CA, USA) based on the GenBank rat cDNA sequence. MGB denotes Minor Groove Binder. NFQ denotes Non-Fluorescent Quencher.

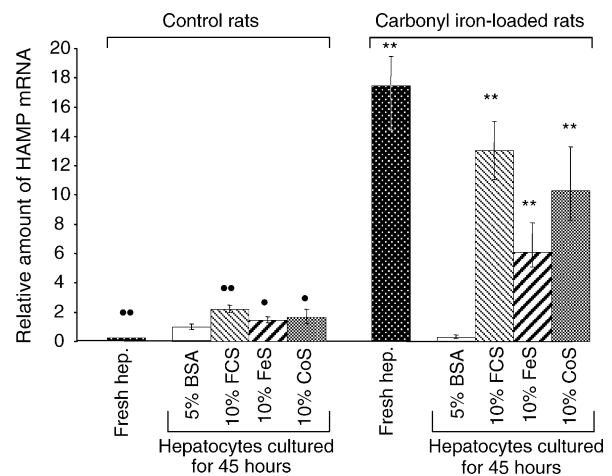
In hepatocytes from iron-loaded animals cultured in 5% BSA, HAMP mRNA levels were increased by treatment with TNF- $\alpha$  ( $P < 0.001$ ). Treatment with FAC resulted in decreased HAMP gene expression ( $P < 0.005$ ). In the presence of 10% FCS, HAMP mRNA levels were increased by TNF- $\alpha$ , IL-6 and LPS ( $P < 0.001$ ;  $P < 0.001$ ;  $P < 0.001$ ), whereas treatment with FAC lead to decreased levels ( $P < 0.001$ ) (Fig. 4).



**Fig. 2.** Graph showing time course experiment of HAMP expression in primary rat hepatocytes during culturing. In fresh hepatocytes obtained after collagenase perfusion, HAMP mRNA levels were increased in the carbonyl iron-treated liver as compared with that from one animal fed standard chow diet. After plating, there was a gradual drop in HAMP gene expression. This drop was most marked in cultured hepatocytes from the iron-treated rat, and after 41–65 h, these levels were lower than those found in hepatocytes from the control rat. The time course experiment was performed on hepatocytes isolated from one control rat and one iron-overloaded animal.

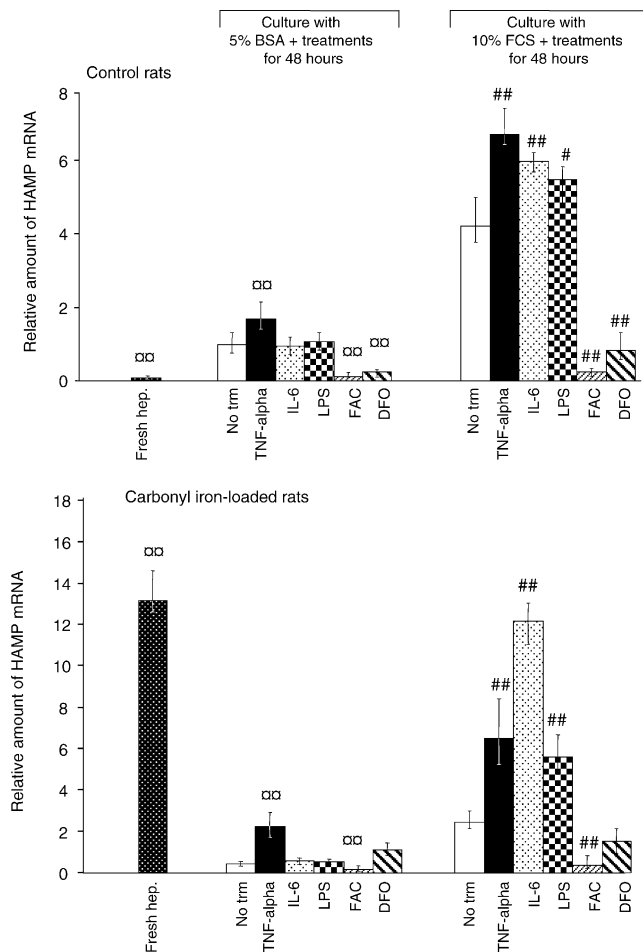
#### 3.4. Expression of iron regulatory genes in freshly isolated hepatocytes.

As seen in Figs. 3–4, gene expression of HAMP in freshly isolated hepatocytes was significantly higher in iron-treated animals as compared with animals receiving standard chow diet ( $P < 0.001$ ;  $P < 0.001$ ). Levels of TfR1 mRNA in freshly isolated hepatocytes were significantly lower in carbonyl



**Fig. 3.** Graphs showing HAMP expression in cells during incubation with different sera. Comparison of the effects of different sera in hepatocyte cultures on HAMP mRNA levels. In fresh hepatocytes, HAMP gene expression was increased by carbonyl iron loading, and decreased after culture for 45 h in 5% BSA. However, the addition of 10% serum to the culture medium normalized the HAMP response to iron loading of hepatocytes. No difference was seen between FCS, serum from control rats or from iron-loaded animals. FCS, fetal calf serum; CoS, serum from control rats; FeS, serum from carbonyl iron-treated animals. Statistical evaluation was performed by one-way analysis of ANOVA followed by Student-Newman-Keuls test ( $\cdot P < 0.05$ ,  $\cdot\cdot P < 0.01$  compared to control cells incubated in 5% BSA;  $\ast P < 0.05$ ,  $\ast\ast P < 0.01$  compared to carbonyl-iron loaded cells incubated in 5% BSA).



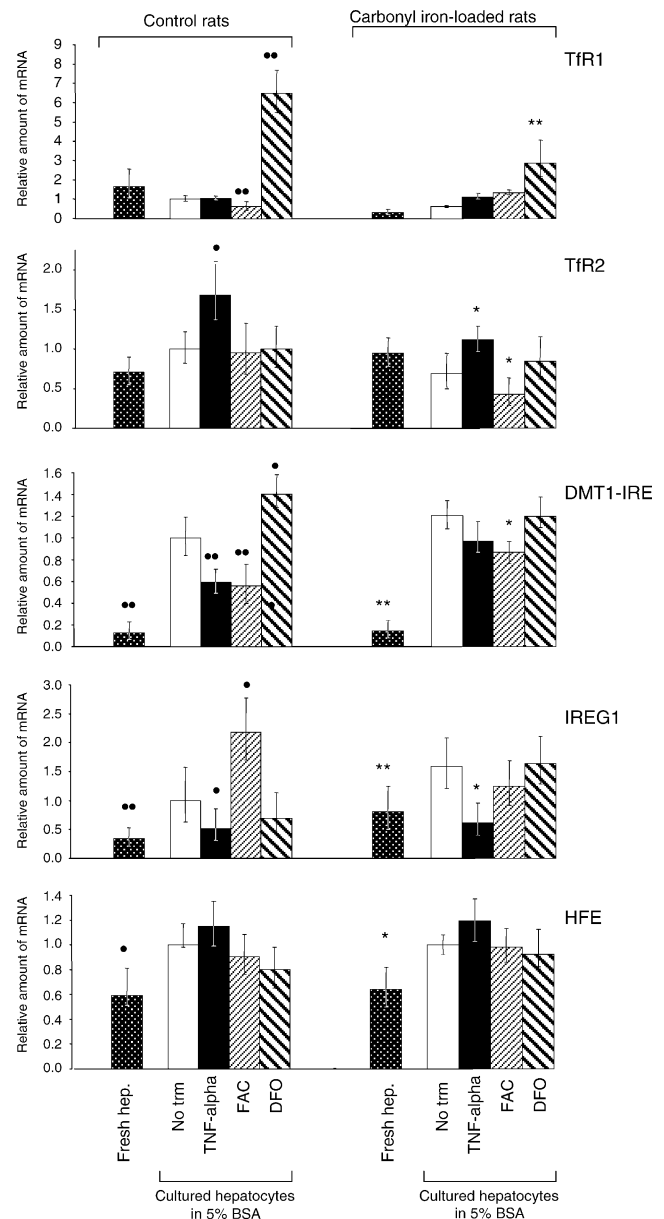


**Fig. 4.** Graphs showing expression of HAMP following cytokine incubation. In hepatocytes from control rats cultured in either 5% BSA or 10% FCS, treatment with TNF- $\alpha$  significantly increased, and DFO and FAC significantly decreased HAMP mRNA levels. In hepatocytes from iron-loaded rats cultured in 5% BSA, treatment with TNF- $\alpha$  significantly increased and FAC decreased HAMP mRNA levels, whereas the other treatments did not alter HAMP gene expression. The addition of 10% FCS to the medium led to an increased HAMP gene expression in response to LPS and IL-6, whereas the relative response to TNF- $\alpha$  was unaltered. Statistical evaluation was performed by one-way analysis of ANOVA followed by Student-Newman-Keuls test for control and carbonyl iron-loaded rats separately. (□  $P < 0.05$ , □□  $P < 0.01$  compared to cells with no in vitro treatment cultured in 5% BSA; #  $P < 0.05$ , ##  $P < 0.01$  compared to cells with no in vitro treatment cultured in 10% FCS). No trm denotes no treatment in vitro.

iron-treated cells as compared with those from control rats ( $P < 0.005$ ). Collagenase perfusion and isolation lead to a down-regulation of TfR2 transcripts in cells from both control and iron-loaded rats ( $P < 0.01$ ) (Fig. 5).

### 3.5. Effect of TNF- $\alpha$ , DFO and FAC on mRNA levels of TfR1, TfR2, DMT1, IREG1 and HFE

In hepatocytes from control rats, TNF- $\alpha$  increased mRNA expression of TfR2 ( $P < 0.02$ ) and decreased the mRNA levels of DMT1 and IREG1 ( $P < 0.003$ ;  $P < 0.04$ )



**Fig. 5.** Graphs showing gene expression of TfR1, TfR2, DMT1, IREG1 and HFE following in vitro incubation with TNF- $\alpha$ , FAC or DFO. Studies were performed on fresh hepatocytes and cultured hepatocytes from animals treated with the standard diet or the carbonyl-iron supplemented diet. Cultured hepatocytes were incubated with TNF- $\alpha$ , FAC, or DFO in 5% BSA for 48 h. mRNA levels were measured by quantitative real-time RT-PCR. For details see Section 2. TNF- $\alpha$  significantly increased TfR2 mRNA and decreased IREG1 mRNA in cultured hepatocytes from both control rats and animals fed a carbonyl-iron supplemented diet. Statistical evaluation was performed by one-way analysis of ANOVA followed by Student-Newman-Keuls test (\*  $P < 0.05$ , \*\*  $P < 0.01$  compared to control cells incubated in 5% BSA; \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to carbonyl-iron loaded cells incubated in 5% BSA). No trm denotes no treatment in vitro.

(Fig. 5). In hepatocytes from animals fed an iron-supplemented diet, TNF- $\alpha$  increased mRNA expression of TfR2 ( $P < 0.02$ ) and decreased the expression of IREG1 ( $P < 0.03$ ).

FAC increased mRNA expression of IREG1 ( $P < 0.03$ ) and decreased the expression of TfR1 and DMT1 levels ( $P < 0.004$ ;  $P < 0.004$ ) in cells from control rats. In cells from iron-loaded animals, FAC decreased mRNA levels of TfR2 and DMT1 ( $P < 0.04$ ;  $P < 0.04$ ).

DFO significantly increased TfR1 and DMT1 gene expression ( $P < 0.001$ ;  $P < 0.02$ ) in hepatocytes from control rats, and TfR1 expression in cells from iron-treated animals ( $P < 0.001$ ). HFE expression was unaltered by any of the treatments applied.

#### 4. Discussion

We found that HAMP expression in cultured hepatocytes depends on (a) the plating of cells, (b) the culturing time, (c) the intracellular iron content, and (d) the presence of serum in the culture medium. TNF-alpha, IL-6 and LPS all increased HAMP gene expression in serum-containing medium, whereas only TNF-alpha increased HAMP mRNA levels in serum-free medium. DFO and FAC both decreased HAMP gene expression. In serum-free medium, TNF-alpha also significantly increased mRNA levels of TfR2 and decreased those of DMT1 and IREG1 whereas levels of HFE mRNA were unaltered.

A previous study demonstrated a stronger HAMP gene expression in whole liver than in cultured hepatocytes [6]. We could not reproduce this finding when freshly isolated hepatocytes were compared with cultured cells from control animals. However, in the study by Pigeon et al., HAMP mRNA levels were higher in hepatocytes cultured in the presence of FCS than in the serum-free ones [6]. We saw an additional increase of HAMP mRNA levels when FCS was added to the culture. Hepatocytes from iron-loaded rats down-regulated their HAMP expression when cultured in serum-free but not in serum-containing medium. Thus, serum factors seem to be needed for an adequate HAMP response to iron loading in vivo.

HAMP gene expression was reduced in both control and carbonyl-iron loaded hepatocytes when adding iron to the medium as FAC, both in the presence and absence of serum. Thus, iron has opposite effects on HAMP gene expression in cultured as compared to freshly isolated hepatocytes [6,8]. It is well known that FAC is toxic to hepatocytes in culture [33]. However, in our model a general toxic effect of FAC can be ruled out as the only explanation for the decreased HAMP expression, since the expression of IREG1 increased as expected in response to FAC (Fig. 5). Thus, iron induces HAMP via indirect, yet undefined mechanisms.

The decreased HAMP expression in hepatocytes from iron-loaded rats grown in serum-free medium led us to speculate if a serum factor was needed. If so, such a factor may be elevated in serum from iron-loaded animals, indicating a systemic regulation of HAMP transcription by iron overload. To explore this, we compared HAMP mRNA levels in hepatocytes cultured in media containing FCS, serum from control rats, or serum from iron-loaded animals.

We found significantly increased HAMP mRNA levels in cells cultured in all these sera (Fig. 3), however, without any additional elevation in cells grown in serum from carbonyl-iron fed animals. This indicates there is a factor in serum needed for an adequate HAMP response to increased iron stores. However, this factor seems species-independent and not dependent on the iron status of the animal.

Previous studies by Nemeth et al. have showed that transcription of HAMP in primary human hepatocytes is induced by IL-6 but not significantly by TNF-alpha or IL-1 [8]. In hepatoma cells, TNF-alpha inhibited HAMP gene expression [10]. In murine hepatocytes, both IL-1 and IL-6 stimulated hepcidin gene expression [34]. In the present study, we have used extracellular matrix derived from Engelbreth-Holm-Swarm (EHS) tumours from mice for cell culturing. EHS have been shown to contain transforming growth factor-beta (TGF- $\beta$ ), but other cytokines are less frequently found [35]. It is well known that hepatocytes cultured on EHS matrix (Matrigel) display a more well preserved phenotype with regard to for example heme-containing proteins [36], and are less susceptible to oxidative stress [31]. To our knowledge, in all other studies on HAMP expression in hepatocytes, the cells have been cultured on collagen. This difference in culture conditions may be one explanation to the discrepancies in HAMP response to TNF-alpha. We also investigated whether TNF-alpha could mediate an effect on the HAMP gene expression in cultured rat hepatocytes subjected to different levels of iron loading. Our results showed that TNF-alpha increased HAMP mRNA both in control hepatocytes and cells from iron-loaded rats, both in absence and presence of fetal calf serum. IL-6 and LPS also increased HAMP mRNA in hepatocytes cultured in medium containing FCS, but not in the serum-free medium. Our findings may indicate that in vitro, TNF-alpha directly can stimulate HAMP gene expression. In vivo however, the cytokines influencing HAMP transcription possibly originate from reticuloendothelial cells, since supernatant from LPS-exposed monocytes increases HAMP mRNA levels [8]. However, whether this signaling is mediated only by IL-6, or involves many cytokines including TNF-alpha, possibly also ferritin remains to be elucidated. Since LPS induced HAMP transcription also in IL-6 knockout mice [9], the latter hypothesis seems more valid.

TNF-alpha may also participate in other aspects of the iron perturbations seen in inflammation, characterized by hypoferrremia and hyperferritinemia. It has been shown that TNF-alpha induces synthesis of ferritin H [13], and possibly also DMT1 in cultured human intestinal cells [37]. The present findings concerning the effects of TNF-alpha on DMT1 and IREG1 mRNA levels are well in line with the alterations occurring in inflammatory conditions, including iron retention in macrophages and decreased iron uptake in cells. Furthermore, our findings indicate that TNF-alpha, in addition to inducing transcription of ferritin and probably HAMP, also has a direct effect on the expression of several

genes involved in iron uptake and release. In particular, the induction of TfR2 by TNF- $\alpha$  is interesting as TfR2 has been suggested to act as a regulator of hepcidin synthesis [38] in parallel to the TfR1-HFE axis. A stimulating effect on the TfR2 gene expression could therefore influence HAMP mRNA levels. However, TfR2 does not seem to be indispensable for the HAMP response to other cytokines such as IL-6 [9], but enhanced levels of TfR2 induced by TNF- $\alpha$  may nevertheless influence the iron homeostasis.

Data is still controversial whether or not a functional HFE protein is necessary for the induction of HAMP expression by inflammation [9,39–42]. In the present study we found no effects on the HFE gene expression by TNF- $\alpha$ .

In conclusion, we found that IL-6 induced HAMP gene expression in rat hepatocytes cultured in the presence of serum, whereas TNF- $\alpha$  increased HAMP mRNA in both serum-containing and serum-free medium. TNF- $\alpha$  decreased gene expression of DMT1 and IREG1, involved in iron uptake and release from cells, and increased that of TfR2, with possible significance on iron homeostasis. Furthermore, we found an increased HAMP gene expression during culturing of control rat hepatocytes, whereas the opposite was seen in cultured hepatocytes from iron-loaded rats. The addition of serum normalizes the blunted HAMP response seen in cultured hepatocytes from iron-loaded animals, but we found no additional effect of serum from iron-loaded rats as compared to FCS. We suggest that TNF- $\alpha$  plays a role in iron homeostasis by regulating the mRNA levels of HAMP, DMT1, IREG1 and TfR2.

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