Biometals (2012) 25:749–759
DOI 10.1007/s10534-012-9543-9

# Hepatic and adipocyte cells respond differentially to iron overload, hypoxic and inflammatory challenge

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Received: 12 December 2011 / Accepted: 19 March 2012 / Published online: 5 April 2012


**Abstract** Adipose tissue secretes numerous pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α that can lead to insulin resistance (IR). In the liver, both IL-6 and TNF-α induce IR by inhibiting phosphorylation or ubiquitination of IRS1. In IR development, Fe is a risk factor in type-2 diabetes development. We studied the expression of genes related to inflammation, hypoxia, and mitochondrial function in hepatic (HepG2) and adipose (3T3-L1) cells. HepG2 and 3T3-L1 cells were incubated with 20 μM Fe, 40 μM Fe, or 40 μM Fe/20 mM glucose for 7 days and then challenged with 20 ng/ml IL-6 and/or 100 μM $CoCl_2$ for 20 h. We measured intracellular Fe levels and the relative expression of hepcidin, NF-κB, IL-6, TNF-α, hypoxia inducible factor 1α (HIF-1α), and mitofusin 2 (Mfn-2) mRNA using qRT-PCR. The intracellular Fe concentration in HepG2 cells did not change with 20 or 40 μM Fe. However, levels were decreased with Fe/glucose and IL-6 and/or $CoCl_2$. 3T3-L1 cells showed an increase in intracellular Fe with high Fe plus either IL-6 or $CoCl_2$. HepG2 cells incubated with 40 μM Fe alone or Fe/glucose and challenged with IL-6 and/or $CoCl_2$ showed increased IL-6, NF-κB, and TNF-α mRNA expression and decreased mRNA expression of Mfn-2 in all experimental conditions. 3T3-L1 cells incubated with 40 μM Fe alone or Fe/glucose and challenged with IL-6 showed increased NF-κB mRNA expression and decreased Mfn-2 expression in all experimental conditions. Thus, high Fe, inflammation, and hypoxia trigger the expression of genes related to inflammation and Fe metabolism in HepG2 cells, in 3T3-L1 cells the same stimuli increased NF-kB and hepcidin expression.

**Keywords** Inflammation · Hypoxia · Hepcidin · Adipocytes · Hepatocytes

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## Introduction

Obesity is a critical health issue in developed and developing countries. Obesity is associated with a number of health problems that involve the development of insulin resistance (IR), type-2 diabetes (T2D), cardiovascular disease, and fatty liver disease. Adipose tissue, especially visceral adipose tissue, also plays a pivotal role in the development of these diseases.

Adipose tissue is an important metabolic and endocrine organ that secretes numerous biologically active proteins called adipokines, including leptin, adiponectin, many chemokines, and cytokines (Rasouli and Kern 2008). A number of these adipokines are linked to immunity and the inflammatory response, and their production is generally increased in obesity (Hotamisligil 2006; Wellen and Hotamisligil 2005).

Obesity itself is characterized by a chronic state of low-grade inflammation in which circulating levels of several inflammatory markers such as tumor necrosis factor (TNF)α, interleukin (IL)6, plasminogen activator inhibitor (PAI)1, and monocyte chemotactic protein (MCP)1 are present at low levels but levels higher than in non-obese (Hotamisligil 2006; Wellen and Hotamisligil 2005; Galic et al. 2010).

There are at least two prevailing hypotheses regarding the development of adipose tissue inflammation. One hypothesis states that adipocyte enlargement results in failure of microvasculature expansion, with subsequent hypoxia, increased expression of hypoxia inducible factor 1α (HIF-1α), adipocyte necrosis, adipokine secretion, and fibrosis (Trayhurn and Wood. 2004; Pasarica et al. 2009; Rutkowski et al. 2009). Studies on murine adipocytes (both primary cultures and 3T3-L1 cells) indicate that the expression of several genes, including GLUT1, matrix metalloproteinase 9, PAI-1, TNF-α, and vascular endothelial growth factor (VEGF), is stimulated by hypoxia (Ye et al. 2007; Hosogai et al. 2007). The second plausible hypothesis in the development of adipose tissue inflammation is related to the demonstration of resident macrophages in adipose tissue (Xu et al. 2003; Weisberg et al. 2003). The infiltration of macrophages into adipose tissue may be linked to the hypoxia observed in this tissue. Recent studies have suggested that macrophages infiltrate adipose tissue as part of a scavenger function, in response to adipocyte necrosis (Cinti et al. 2005). The adipose tissue of obese rodents and humans contains increased numbers of macrophages, and once they are activated, these macrophages secrete cytokines such as TNF-α, IL-6, and IL1 (Wellen and Hotamisligil 2003).

The low-grade inflammation in obesity that originates in adipose tissue infiltrated with macrophages can inhibit signaling downstream of the insulin receptor as a primary mechanism through which inflammation leads to IR. Exposure of cells to TNF-α or elevated levels of free fatty acids stimulate inhibitory phosphorylation of serine residues in the insulin receptor substrate 1 (IRS1) (Aguirre et al. 2000, 2002). This phosphorylation reduces both tyrosine phosphorylation of IRS1 in response to insulin and the ability of IRS1 to associate with the insulin receptor, thereby inhibiting downstream signaling and insulin action (Aguirre et al. 2002). Furthermore, several serine/threonine kinases are activated by inflammatory or stressful stimuli and contribute to inhibition of insulin signaling, including JNK, inhibitor of NF-κB kinase, and protein kinase C θ (Zick 2003).

Cytokines produced by adipocytes and macrophages, especially IL-6 and TNF-α, target the liver. IL-6 upregulates suppressor of cytokine signaling 3 (SOCS3) in mouse liver (Klover et al. 2003). SOCS proteins constitute a family of intracellular proteins that play a major role in immune cell proliferation, differentiation, migration, and modulation of immune responses (Yoshimura et al. 2007). SOCS3 and SOCS1 in particular have been implicated in the development of hepatic IR. SOCS3 leads to phosphorylation of a serine in IRS-1, leading to inhibition of the downstream insulin signal (Lebrun and Obberghen 2008; Senn et al. 2003). SOCS1 can be activated through IL-6 and TNF-α signaling in different tissues such as liver, pancreas, and adipocytes. SOCS1 promotes ubiquitination and degradation of IRS1 and IRS2 both in cultured cells and in mouse liver, contributing to IR (Rui et al. 2002).

Besides the relationship between inflammation and development of T2D, several studies have shown a relationship between Fe and diabetes etiopathogenesis (Fernández-Real et al. 2002; Jiang et al. 2004; Valenti et al. 2007). Fe is a transitional metal and a strong prooxidant that catalyzes several cellular reactions that result in the production of reactive oxygen species (ROS) through the Fenton/Haber–Weiss reaction (Tajima et al. 2012). Species such as hydroxyl radicals are considered to be main factors in Fe-associated diseases such as diabetes, atherosclerosis, and cardiovascular disease (Rajpathak et al. 2009; Tajima et al. 2012). Hepatocytes play a central role in systemic Fe homeostasis through the Fe-regulatory hormone, hepcidin. Hepcidin is a 25-amino acid peptide hormone that negatively regulates the main Fe flow that enters the plasma compartment: the absorption of dietary Fe in the duodenum, the release of recycled Fe from macrophages, and the release of stored Fe from hepatocytes. Fe, which increases hepcidin synthesis, may include di-ferric plasma transferrin and Fe stored in hepatocytes (Ganz and Nemeth 2011). Hepcidin is mainly synthesized in hepatocytes, but it is also produced in intestinal cells, pancreatic cells, adipocytes, and monocytes (Nemeth and Ganz 2006; Vokurka et al. 2010; Zhang and Rovin 2010).

Besides Fe levels in plasma, inflammation also stimulates hepcidin expression, especially through IL-6, which induces signaling and activation of

STAT3 via binding to the hepcidin promoter to activate its transcription (Wrighting and Andrews 2006; Truksa et al. 2007). Therefore, hepcidin is considered an acute phase protein because it is linked to inflammatory states and leads to Fe overload in some tissues such as liver, pancreas, and macrophages.

Considering that adipose tissue, hepatocytes, Fe, and inflammation interact in the development of T2D, we challenged HepG2 cells and 3T3-L1 cells differentiated to adipocytes with high Fe and/or glucose concentrations in the presence of an acute inflammatory and/or hypoxic stimulus (using IL-6 and/or $CoCl_2$, respectively). Then, we measured the mRNA expression of genes related to inflammation including hepcidin, *NF-κB*, *IL-6*, and *TNF-α*, a gene related to hypoxia, *HIF-1α*, and a gene related to mitochondrial function, mitofusin 2 (*Mfn-2*).

## Methods and materials

### Cell culture

HepG2 cells were maintained in α Minimal Essential Medium (MEM), 10 % fetal bovine serum (FBS), and 10 IU/ml penicillin and streptomycin. 3T3L1 cells were cultivated in DMEM, 10 % FBS, and 10 UI/ml antibiotics. Differentiation of 3T3L1 cells was induced 2 days post-confluence with differentiation medium containing 10 % FBS, 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine for 72 h. Cells were maintained in post-differentiation medium containing 10 % FBS and insulin (10 μg/ml). Both cell types were maintained at standard temperature and $CO_2$. HepG2 cells and differentiated 3T3L1 cells were challenged with 20 or 40 μM Fe or 20 mM glucose plus 40 μM Fe for 7 days. The cells cultivated with 20 μM of Fe were considered the control group. The sixth day, cells were exposed to 20 ng/ml IL-6 as an inflammatory stimulus or 100 μM $CoCl_2$ as a chemical hypoxic stimulus or the combination of both stimuli (20 ng/ml IL-6/ 100 μM $CoCl_2$). The cells were seeded in 6-well plates, and media were changed every 2 days.

### Cellular Fe content

For total metal quantification, HepG2 and 3T3L1 cells were lysed with nitric acid (65 %) (Supra Pure, Merck, Chemical Co., Darmstadt, Germany) overnight at 60 °C. Fe determination was made using atomic absorption spectrophotometry with graphite furnace (Perkin Elmer, SIMAA 6100, Perkin-Elmer Corporation, Norwalk, CT, USA). Total metal content was expressed in μg metal/μg protein per sample.

### Real-time PCR

RNA from HepG2 and differentiated 3T3L1 cells was extracted using Trizol Reagent according to the manufacturer's protocol (Invitrogen. Carlsbad, California, USA). RNA (1 μg) was reverse-transcribed using an Affinity-Scrip cDNA Synthesis kit (Stratagene. Santa Clara, California, USA). Real-time PCR was performed using Brilliant II SYBR™ Green QPCR Master Mix (Stratagene) on a Max Proo™ system 3000. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-2-microglobulin (B2 M) were used as housekeeping genes for normalization. Relative expression levels were calculated as described in Pfaffl (2001), using the following formula:

$$\text{Ratio} = \left(E_{\text{target}}\right)^{\Delta Ct_{\text{Target}}}(\text{Control} - \text{Sample})/ \left(E_{\text{ref}}\right)^{\Delta Ct_{\text{ref}}}(\text{Control} - \text{Sample})$$

Primer sequences used for qRT-PCR in HepG2 cells were: GAPDH, Forward (F): CCAGCAAGA-GCAC AAGAGGA and Reverse (R): TCAAGGGGTCTAC ATGGCAA; B2M, F: GATGCCGCATTTG-GATTG GA and R: TGGAGCAACCTGCTCAGATA; Hepcidin, F: GACACCAGAGCAAGCTCAA and R: GAAA ACAGAGCCACTGGTCA; NF-κB, F: TGCATCCAA AGGTG-CTCAGA and R: GCAGCTGGCAA-AGCT TAGTA; Mfn-2, F: AACTGTCTGGGACCTT-TGCT CA and R: ACTGTCCAACCAACCGGCT-TTA; IL-6, F: ATGTCTGAGGCTCATTCTGC and R: GCGGC TACATCTTTGGAATC; TNF-α, F: GTTCCTCAGCC TCTTCTCCT and R: ACAACATGGGCTACAGGC TT; HIF-1α, F: GAAAGCGCAAG-TCCTCAA-3′ and R: GGTAGGAGATGGAGATGCAA.

Primer sequences used for qRT-PCR in 3T3-L1 cells were: GAPDH, F: AGGCCGGTGCTGAG-TATGTC and R: TGCCTGCTTCACCACCTTCT; B2M, F: TTC CACCCGCCTC-ACATTCAAATC and R: GGCCAT ACTGGCATGCTTAACTCT; Hepcidin, F: TATGAC GGG-CAAGATCACCTGGAA and R: TGTTTGGCA CGGATCTTGTGCT; NF-κB, F: ACAACTAT-GCG GGAGATCCTTCGT and R: GAATCT-GGCTCAGG

AATCTTGGGT; Mfn-2, F: AACTG-TCTGGGACCT TTGCTCA and R: ACTGTCCAACC-CAACCGGCT TTA; IL-6, F: AGAG-ACTTCCATCCAGTTGCCT and R: TCTGTTGGGAGTGGTATCC-TCTGT; TNF-α, F: ATTC-AGTGACAAGCCTGTAGC and R: ACCTG GGAGTAGACAAGGTACAAC; and HIF-1α, F: TCAA GAAACGACCACTGCTAAGGC and R: CAAAGTG GCAGACAGGTTAAGGCT.

### Statistical analysis

Statistical analysis was performed with Graph-Pad Prism 5.0 software (CA, USA). The results are expressed as means ± SEM. Differences were evaluated using one-way ANOVA. Statistical significance was considered when $p < 0.05$.

## Results

The intracellular Fe concentration in HepG2 cells did not change when challenged with inflammatory and/or hypoxic stimuli in the presence of physiologic (20 μM) or high (40 μM) Fe concentration in the medium (Figs. 1a, b). However, when the cells were cultivated with high Fe/glucose, the metal concentration decreased in the presence of these stimuli (Fig. 1c). In 3T3-L1 cells, the intracellular Fe concentration did not change when the cells were cultured in physiologic Fe in the presence of IL-6 alone or $CoCl_2$ alone, but did decrease in the presence of both stimuli (Fig. 2a). The intracellular Fe content in cells challenged with high Fe and IL-6 increased compared with high Fe alone. Fe levels also increased with $CoCl_2$ in the presence of high Fe (Fig. 2b). In 3T3-L1 cells challenged with high Fe/glucose, the intracellular Fe concentration increased in the presence of IL-6 alone or $CoCl_2$ alone and IL-6/$CoCl_2$ (Fig. 2c).

The expression of HIF-1α mRNA, which responds to hypoxia, increased when HepG2 cells were challenged with $CoCl_2$ and basal Fe; with $CoCl_2$ and high Fe; with $CoCl_2$, high Fe, and glucose; and also with $CoCl_2$, high Fe, IL-6, and glucose (one-way ANOVA: $p < 0.001$ for all; Tables 1, 2, and 3). HIF-1α mRNA expression increased in 3T3-L1 cells exposed to IL-6 in the presence of basal Fe concentration (one-way ANOVA: $p < 0.001$; Table 4) and also in those exposed to high Fe and CoCl2, IL-6, and high Fe (One-way ANOVA: $p < 0.05$; Table 5) and in cells exposed to high Fe and glucose (One-way ANOVA: $p < 0.05$; Table 6).

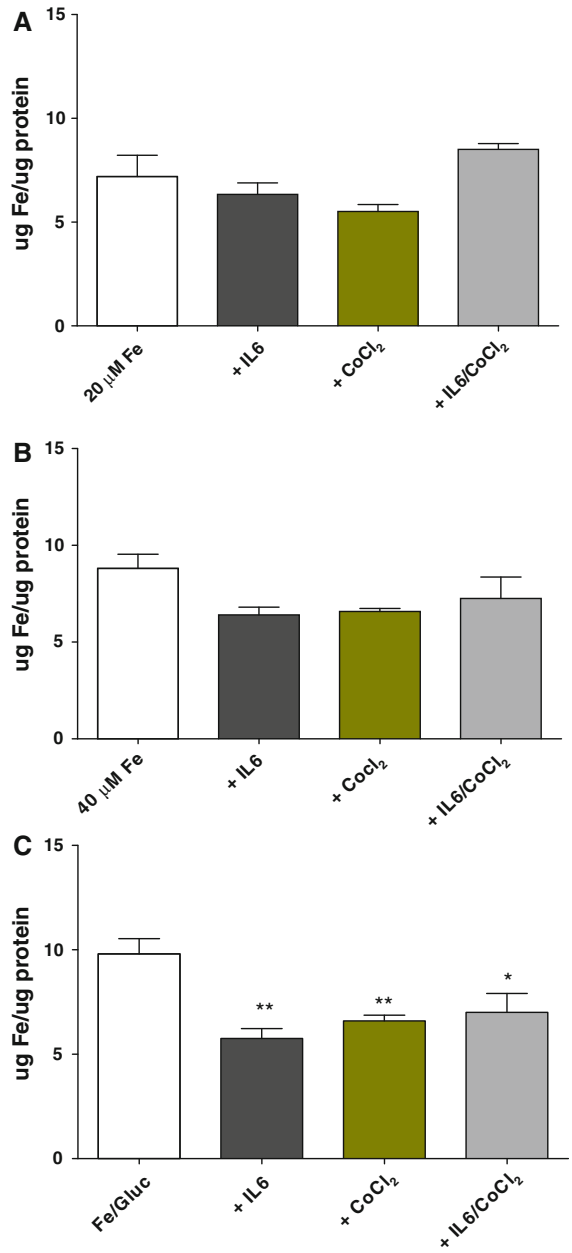


**Fig. 1** Intracellular Fe concentration in HepG2 cells. **a** Cells challenged with 20 μM Fe for 7 days. **b** Cells challenged with 40 μM Fe for 7 days. **c** Cells challenged with 40 μM Fe plus 20 mM glucose (Gluc) for 7 days. On the sixth day cells were challenged with 20 ng/ml IL6 and/or 100 μM $CoCl_2$ for 20 h. Results are expressed as the means ± SEM. One-way ANOVA: * $p < 0.05$; ** $p < 0.01$

NF-κB mRNA expression was increased in HepG2 cells exposed to basal Fe levels and $CoCl_2$ and to basal

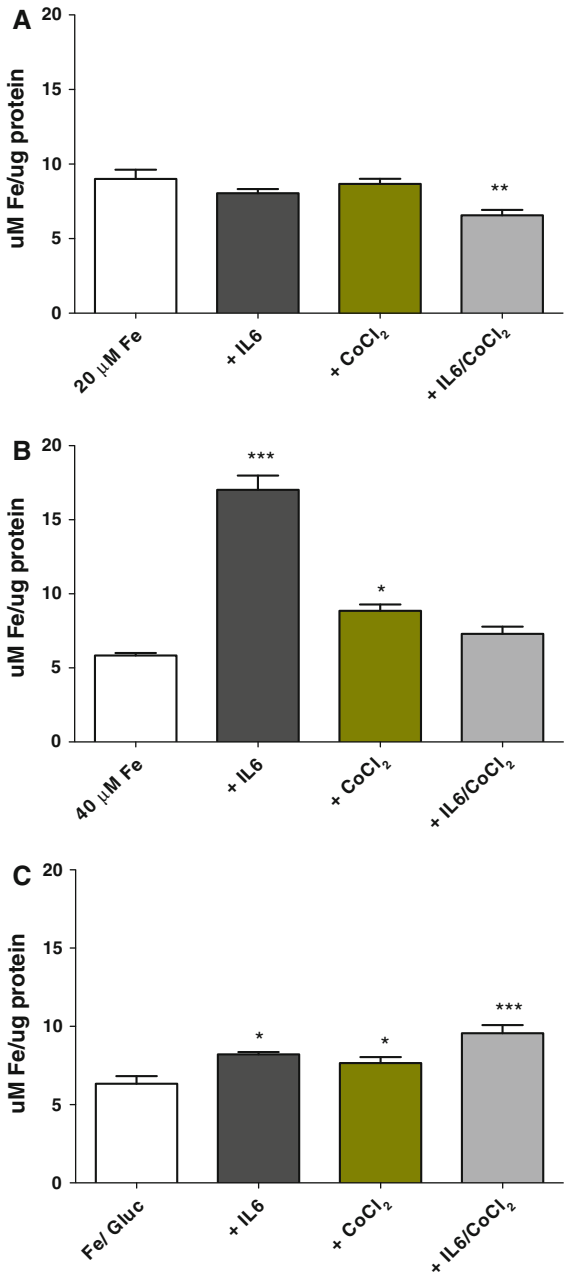


**Fig. 2** Intracellular Fe concentration in 3T3-L1 cells. **a** Cells challenged with 20 μM Fe for 7 days. **b** Cells challenged with 40 μM Fe for 7 days. **c** Cells challenged with 40 μM Fe plus 20 mM glucose for 7 days. On the sixth day cells were challenged with 20 ng/ml IL6 and/or 100 μM $CoCl_2$ for 20 h. Results are expressed as the means ± SEM. One-way ANOVA: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

Fe and the combination of inflammatory and hypoxic stimuli (one-way ANOVA: $p < 0.05$; $p < 0.01$, respectively; Table 1). However, when these cells were cultivated in high Fe, the expression of NF-κB was remarkably higher, especially in high glucose (One-way ANOVA: $p < 0.001$ for all; Tables 2, 3). In HepG2 cells exposed to the basal Fe concentration, IL-6 and TNF-α mRNAs decreased with inflammatory and/or hypoxic stimuli (One-way ANOVA: $p < 0.001$ for all; Table 1). However, IL-6 and TNF-α mRNA expression increased in HepG2 cells cultivated in high Fe with or without glucose (Tables 2, 3). IL-6 mRNA expression decreased in 3T3-L1 cells exposed to the basal Fe concentration with IL-6 and/or $CoCl_2$ (one-way ANOVA: $p < 0.001$; Table 4), and TNF-α mRNA increased only in cells exposed to 20 μM Fe and IL-6 (one-way ANOVA: $p < 0.001$; Table 4).

NF-κB mRNA decreased in 3T3-L1 cells cultivated in basal Fe concentration and inflammatory and/or hypoxic stimuli, but when the cells were exposed to high Fe and glucose and IL-6, NF-κB mRNA increased. In 3T3-L1 cells cultivated in high Fe and/or high glucose, both IL-6 and TNF-α mRNAs increased (one-way ANOVA: $p < 0.01$; $p < 0.05$, respectively), but not when the cells were challenged with inflammatory and/or hypoxic stimuli (Tables 5, 6).

HepG2 cells exposed to the basal Fe concentration and IL-6 showed increased expression of hepcidin mRNA (one-way ANOVA: $p < 0.001$; Table 1). The same tendency was seen in cells cultivated with 40 μM Fe (one-way ANOVA: $p < 0.001$ for all; Table 2). On the other hand, in 3T3-L1 cells, hepcidin mRNA was increased only in basal Fe conditions in the presence of both $CoCl_2$ and IL-6 (one-way ANOVA: $p < 0.001$; Table 4). In cells exposed to high Fe concentration alone, hepcidin mRNA increased, and in high glucose, hepcidin expression was downregulated in the presence of IL-6 or $CoCl_2$ (one-way ANOVA: $p < 0.01$; $p < 0.001$, respectively; Tables 5, 6).

Mfn-2 mRNA was significantly decreased in HepG2 cells in all conditions, except when cells were cultivated in high Fe/glucose and $CoCl_2$ (one-way ANOVA: $p < 0.001$ for all; Tables 1–3). In 3T3-L1 cells, Mfn-2 mRNA expression decreased in the basal Fe concentration (one-way ANOVA: $p < 0.001$; Table 4), and also with high Fe and/or glucose in the presence of IL-6 and/or $CoCl_2$ (one-way ANOVA: $p < 0.001$; $p < 0.01$, respectively; Tables 5, 6). However, Mfn-2 mRNA increased in 3T3-L1 cells cultivated with high Fe and glucose without other stimuli (one-way ANOVA: $p < 0.01$; Table 4).

**Table 1** mRNA expression in HepG2 cells cultivated with 20 μM Fe for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | HepG2 | | | |
|---|---|---|---|---|
| | 20 μM Fe | IL-6/20 μM Fe | $CoCl_2$/20 μM Fe | $CoCl_2$/IL-6/20 μM Fe |
| *HIF-1α* | 1 | 0.4 ± 0.1 | 25.9 ± 9.3*** | 2.8 ± 0.5 |
| *Hepc* | 1 | 61.0 ± 14.9*** | 4.3 ± 0.2 | 1.4 ± 0.4 |
| *NF-κB* | 1 | 1.8 ± 0.4 | 2.7 ± 0.9* | 3.8 ± 1.4** |
| *IL-6* | 1 | 0.2 ± 0.2*** | 0.4 ± 0.1*** | 0.1 ± 0.04*** |
| *TNF-α* | 1 | 0.4 ± 0.1*** | 0.3 ± 0.06*** | 0.2 ± 0.07*** |
| *Mfn-2* | 1 | 0.1 ± 0.05*** | 0.3 ± 0.06 | 0.2 ± 0.07*** |

*Hepc* Hepcidin; *Mfn-2* Mitofusin-2

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

**Table 2** mRNA expression in HepG2 cells challenged with high Fe for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | HepG2 | | | | |
|---|---|---|---|---|---|
| | 20 μM Fe | 40 μM Fe | IL-6/40 μM Fe | $CoCl_2$/40 μM Fe | $CoCl_2$/IL-6/40 μM Fe |
| *HIF-1α* | 1 | 7.8 ± 1.5 | 13.4 ± 3.8 | 109.2 ± 46.4*** | 5.1 ± 2.1 |
| *Hepc* | 1 | 0.4 ± 0.1 | 55.5 ± 18.9*** | 2.8 ± 0.7 | 0.9 ± 0.2 |
| *NF-κB* | 1 | 11.2 ± 1.9 | 189 ± 54.8*** | 182 ± 53.0*** | 13.1 ± 2.1 |
| *IL-6* | 1 | 0.2 ± 0.06 | 44.4 ± 7.0* | 83.9 ± 37.7*** | 23.2 ± 8.7 |
| *TNF-α* | 1 | 2.3 ± 0.4 | 82.8 ± 18.6*** | 84.9 ± 21.4*** | 157 ± 32.1*** |
| *Mfn-2* | 1 | 0.2 ± 0.06*** | 0.2 ± 0.1*** | 0.4 ± 0.1*** | 0.01 ± 0.0*** |

*Hepc* Hepcidin; *Mfn-2* Mitofusin-2

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: * $p < 0.05$, *** $p < 0.0001$

## Discussion

Adipose tissue in obese persons is considerably more hypoxic than that in lean persons. This difference in $O_2$ tension results in marked differences in gene expression, including expression of the inflammatory cytokines TNF-α and IL-6 (Trayhurn and Wood 2004). Increased cytokine expression has also been demonstrated in 3T3-L1 cells cultured in hypoxic conditions (1 % $O_2$) (Ye et al. 2007). We challenged 3T3-L1 cells with chemical hypoxia ($CoCl_2$) plus physiologic or high Fe and high glucose concentrations. Under these conditions, we could not demonstrate an increase in either cytokines or NF-κB mRNA expression. Normally, in the presence of oxygen, cells synthesize and degrade HIF-1α, the main regulator of $O_2$ homeostasis (Hosogai et al. 2007). In our study, Fe (basal and high concentration) was a strong inhibitor of HIF-1α expression in 3T3-L1 cells in chemical hypoxia. However, the inflammatory stimulus IL-6 increased HIF-1α mRNA expression in the same Fe concentrations.

In HepG2 cells cultured with $CoCl_2$, Fe, and high glucose, HIF-1α mRNA expression increased, but this did not occur with the high Fe concentration in the absence of $CoCl_2$, demonstrating that these cells were effectively in hypoxic conditions. Hypoxia in the liver switches the metabolism from aerobic to anaerobic, inducing a decrease in mitochondrial activity and an increase in ROS production (Everett et al. 2010). Hypoxia may also induce inflammation by increasing

**Table 3** mRNA expression in HepG2 cells challenged with high Fe/glucose for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | HepG2 | | | | |
|---|---|---|---|---|---|
| | 20 μM Fe | 40/20[a] | IL-6/40/20[a] | $CoCl_2$/40/20[a] | $CoCl_2$/IL-6/40/20[a] |
| HIF-1α | 1 | 6.7 ± 2.4 | 1.8 ± 0.2 | 58.4 ± 11.5*** | 93.4 ± 21.1*** |
| Hepc | 1 | 0.5 ± 0.1 | 1.5 ± 0.4 | 2.8 ± 0.8*** | 0.7 ± 0.2 |
| NF-κB | 1 | 7.2 ± 1.0 | 362 ± 83.9*** | 460 ± 82.1*** | 206 ± 60.8*** |
| IL-6 | 1 | 1.8 ± 0.6 | 513 ± 84.4*** | 4.4 ± 1.3 | 0.4 ± 0.1 |
| TNF-α | 1 | 3.9 ± 0.3 | 260 ± 62.6*** | 83.9 ± 21.5 | 388 ± 119.4*** |
| Mfn-2 | 1 | 0.8 ± 0.2 | 0.2 ± 0.04*** | 0.7 ± 0.1 | 0.3 ± 0.04*** |

*Hepc* Hepcidin; *Mfn-2* Mitofusin-2

[a] 40/20: 40 μM Fe/20 mM Glucose

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: *** $p < 0.0001$

**Table 4** mRNA expression in 3T3-L1 cells challenged with 20 μM Fe for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | 3T3-L1 | | | |
|---|---|---|---|---|
| | 20 μM Fe | IL-6/20 μM Fe | $CoCl_2$/20 μM Fe | $CoCl_2$/IL-6/20 μM Fe |
| *HIF-1α* | 1 | 2.6 ± 0.8*** | 0.6 ± 0.03 | 1.1 ± 0.9 |
| *Hepc* | 1 | 0.6 ± 0.1 | 0.2 ± 0.02 | 46.5 ± 0.6*** |
| *NF-kB* | 1 | 0.4 ± 0.05*** | 0.7 ± 0.1* | 0.6 ± 0.1** |
| *IL-6* | 1 | 0.3 ± 0.08*** | 0.6 ± 0.08*** | 0.6 ± 0.05*** |
| *TNF-α* | 1 | 1.8 ± 0.4*** | 0.3 ± 0.07** | 0.7 ± 0.1 |
| *Mfn-2* | 1 | 0.3 ± 0.08*** | 0.6 ± 0.08*** | 0.6 ± 0.05*** |

*Hepc* Hepcidin; *Mfn-2* Mitofusin-2

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

serum IL-6, IL-6 receptors, and C-reactive protein (CRP) in healthy volunteers who spent three nights at a higher altitude (3,400 m) (Hartmann et al. 2000). We observed that hypoxia increased cytokine expression only in hepatic cells. We also tested whether combined inflammation and hypoxia had an additive effect on the expression of HIF-1α and genes related to inflammation. In 3T3-L1 cells cultured in high Fe and both stimuli (inflammatory and hypoxic), we observed a synergistic increase in NF-κB (Table 5). Also, in HepG2 cells, TNF-α was similarly increased in high glucose and both stimuli.

Cytokines and other chemokines synthesized in adipose tissue can induce IR in obese persons by inhibiting signaling downstream of the insulin receptor, increasing mitochondrial ROS, or activating several serine/threonine kinases (Lin et al. 2005; Furukawa et al. 2004; Aguirre et al. 2000; Zick 2003). We challenged 3T3-L1 and HepG2 cells with the inflammatory stimulus IL-6. 3T3-L1 cells cultured in the basal Fe concentration with IL-6 showed decreased expression of IL-6 and NF-κB mRNA, but increase in TNF-α mRNA expression; cells incubated in high Fe and/or glucose plus IL-6 showed increase NF-κB expression. On the other hand, HepG2 cells exposed to high Fe and/or glucose and IL-6 showed increased expression of genes related to inflammation (IL-6 and TNF-α).

**Table 5** mRNA expression in 3T3-L1 cells challenged with high Fe for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | 3T3-L1 | | | | |
|---|---|---|---|---|---|
| | 20 μM Fe | 40 μM Fe | IL-6/40 μM Fe | $CoCl_2$/40 μM Fe | $CoCl_2$/IL-6/40 μM Fe |
| *HIF-1α* | 1 | 3.2 ± 1.1*** | 1.6 ± 0.6 | 0.9 ± 0.06 | 2.6 ± 0.6* |
| *Hepc* | 1 | 1.7 ± 0.6** | 0.9 ± 0.07 | 0.3 ± 0.05* | 0.9 ± 0.2 |
| *NF-κB* | 1 | 9.1 ± 2.1 | 64.8 ± 9.4*** | 5.9 ± 0.2 | 144 ± 36.1*** |
| *IL-6* | 1 | 2.3 ± 0.9** | 0.9 ± 0.1 | 0.7 ± 0.2 | 0.8 ± 0.3 |
| *TNF-α* | 1 | 2.3 ± 0.7** | 1.5 ± 0.4 | 0.3 ± 0.03 | ND |
| *Mfn-2* | 1 | 1.2 ± 0.4 | 0.06 ± 0.01*** | 0.4 ± 0.1** | 0.3 ± 0.1** |

*ND* not determined; *Hepc* Hepcidin; *Mfn-2* Mitofusin-2

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

**Table 6** mRNA expression in 3T3-L1 cells challenged with high Fe/glucose for 7 days. On the sixth day, cells were challenged with 20 ng/ml IL-6 or 100 μM $CoCl_2$ or 20 ng/ml IL-6/100 μM $CoCl_2$ for 20 h

| Gene | 3T3-L1 | | | | |
|---|---|---|---|---|---|
| | 20 μM Fe | 40/20[a] | IL-6/40/20[a] | $CoCl_2$/40/20[a] | $CoCl_2$/IL-6/40/20[a] |
| *HIF-1α* | 1 | 1.3 ± 0.1* | 0.7 ± 0.1 | 0.7 ± 0.1* | 2.5 ± 0.4* |
| *Hepc* | 1 | 1.1 ± 0.2 | 0.4 ± 0.1*** | 0.1 ± 0.08*** | 0.8 ± 0.2 |
| *NF-κB* | 1 | 4.4 ± 1.1 | 89.7 ± 17.3*** | 0.6 ± 0.08 | 0.7 ± 0.2 |
| *IL-6* | 1 | 1.5 ± 0.4* | 0.8 ± 0.2 | 0.6 ± 0.08 | 0.6 ± 0.1 |
| *TNF-α* | 1 | 1.8 ± 0.6* | 1.5 ± 0.2 | 0.7 ± 0.2 | ND |
| *Mfn-2* | 1 | 1.5 ± 0.2** | 0.09 ± 0.02*** | 0.5 ± 0.2** | 0.4 ± 0.1** |

*ND* not determined; *Hepc* Hepcidin; *Mfn-2* Mitofusin-2

[a] 40/20: 40 μM Fe/20 mM glucose

Results are relative to basal conditions (20 μM Fe) and are expressed as the means ± SEM

Asterisks show differences from results with 20 μM Fe

One-way ANOVA: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

Adipose tissue is a main source of IL-6 secretion, accounting for 15–35 % of the circulating levels (Eder et al. 2009; Bastard et al. 2006). However, in adipose tissue, a high proportion of IL-6 is not produced by mature adipocytes but rather by cells from the vascular stromal fraction including pre-adipocytes, endothelial cells, and macrophages (Fain et al. 2004). We could not demonstrate induction of IL-6 or TNF-α mRNA in adipocytes in the presence of inflammatory and hypoxic stimuli, perhaps because our model does not include other stimuli such as insulin, saturated fatty acids, or macrophages. However, in HepG2 cells, we demonstrated that high Fe and/or glucose concentrations together with an inflammatory stimulus increased the expression of TNF-α, IL-6, and NF-κB. A possible consequence is that TNF-α and IL-6 induce hepatic IR. In addition, IL-6 upregulates the expression of SOCS3, a potent inhibitor of insulin signal transduction (Senn et al. 2003). On the other hand, TNF-α inhibits insulin signaling in the liver via mechanisms that include the activation of serine kinases such as JNK-1 and the induction of SOCS3 and SOCS1; SOCS1 induces ubiquitination of IRS1 and IRS2 in the liver (Tarantino 2011; Popa et al. 2007).

Fe is linked to the development of IR and T2D (Huang et al. 2011). However, the molecular mechanisms by which Fe is associated with diabetes development are not well understood. We demonstrated that

HepG2 cells exposed to high Fe and/or glucose concentrations in inflammatory or hypoxic conditions showed increased expression of cytokines such as IL-6 and TNF-α, but not when exposed to the physiologic Fe concentration and the same stimuli. Therefore, Fe induces the expression of these inflammatory genes.

Fe metabolism is tightly regulated by the hormone hepcidin. This protein is secreted not only by hepatocytes, but also by adipocytes and intestinal and pancreatic cells (Nemeth and Ganz 2006; Vokurka et al. 2010; Zhang and Rovin 2010). Inflammation and Fe upregulate the hepcidin synthesis (Nemeth et al. 2004; Nemeth and Ganz 2006). We observed that hepcidin expression increased in 3T3-L1 cells only when cells were exposed to basal Fe concentration and IL-6 and $CoCl_2$, and high Fe concentration. Hepcidin mRNA increased in HepG2 cells incubated with physiologic or high Fe concentrations combined with IL-6, but not with the combination of high Fe and glucose. In vitro stimulation of fresh human hepatocytes with IL-6 induces strong expression of hepcidin mRNA, and hepcidin synthesis by hepatocytes is transcriptionally regulated by IL-6 (Nemeth et al. 2004; Nemeth et al. 2003). The binding of IL-6 to its receptor results in phosphorylation of the intracellular signaling molecule STAT3. Phospho-STAT3 dimerizes and then is translocated to the nucleus, where interacts with a characterized IL-6 response element in the hepcidin promoter (Fleming et al. 2011). Increased hepcidin levels in response to inflammation result in decreased Fe export and macrophage Fe retention. Hepcidin binds to ferroportin, which is the only known cellular Fe exporter in vertebrates, leading to Jak-2-mediated downregulation of ferroportin and decreased Fe export to the plasma (Domenico et al. 2010). We did not observe an increase in hepcidin expression with high Fe concentration in HepG2 cells. Expression and synthesis of hepatic hepcidin requires molecules such as transferrin receptor TfR1 and TfR2 that function as intracellular or extracellular Fe sensors coupled to one or more transduction pathways. TfR1 and TfR2, together with the membrane human hemochromatosis protein (HFE) that interacts with both receptors, may serve as sensors of diferric transferrin (holotransferrin) (Ganz and Nemeth 2011). HFE binding to TfR1 is modulated by holotransferrin. Increasing concentrations of holotransferrin displace HFE from its association with TfR1. In addition, free HFE interacts with TfR2, which is stabilized by holotransferrin binding. The Fe-Tf/HFE/TfR2 complex stimulates hepcidin expression through a pathway that is not completely understood (Schmidt et al. 2008; Gao et al. 2009). Therefore, a possible explanation for the absence of hepcidin expression in HepG2 cells cultivated in high Fe may be decreased diferric transferrin in the medium with the consequent lack of signaling in the cells.

We also studied the expression of Mfn-2, which participates in the maintenance of mitochondrial tubules or networks in mammalian cells (Bach et al. 2003; Santel and Fuller 2001). Mfn-2 regulates mitochondrial metabolism and is mainly synthesized in muscle. Repression of Mfn-2 reduces glucose oxidation, mitochondrial membrane potential, and cell respiration (Pich et al. 2005). Mfn-2 can be downregulated by cytokines such as TNF-α and IL-6, as well as by ROS and high glucose (Bach et al. 2005; Pich et al. 2005; Yu et al. 2006). We observed that Mfn-2 expression decreased in 3T3-L1 and HepG2 cells in the presence of high Fe and/or glucose concentrations and inflammatory and/or hypoxic stimulus. Therefore, mitochondrial activity in both cell types may be decreased, and together with high cytokine expression and Fe and/or glucose, oxidative stress may be triggered with a consequent alteration in glucose metabolism and IR.

In summary, we showed that inflammation, hypoxia, high Fe, and high glucose concentrations interact in HepG2 cells, triggering and amplifying the inflammatory response through cytokines such as TNF-α and IL-6. Several lines of evidence show that Fe is related to T2D development, especially through oxidative stress. However, we demonstrated that excess Fe is related to the inflammatory status. Unleashing an inflammatory response can induce IR in the liver, because we observed that Fe does not necessarily enter the cell. On the other hand, we did not observe an increase in the inflammatory response when 3T3-L1 cells were treated with the different stimuli.

**Acknowledgments** This research was supported by the Diabetes and Endocrinology Society of Chile (SOCHED) and FONDECYT No. 1085173 and 1110080.

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