Hyperinsulinemia induces hepatic iron overload by increasing liver TFR1 via the PI3K/IRP2 pathway

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Abstract
Dysmetabolic iron overload syndrome (DIOS) is frequently observed, but the underlying mechanism remains unclear. We propose the hypothesis that hyperinsulinemia, a common characteristic of DIOS, may stimulate liver transferrin receptor 1 (TFR1) expression via the PI3K/iron regulatory protein 2 (IRP2) pathway, leading to the occurrence of DIOS. The hepatic iron content, serum iron parameters, and expressions of TFRs and IRPs in the liver were determined in rats with temporary or long-lasting hyperinsulinemia induced by acute or chronic administration of insulin. The effect of insulin on TFR1 expression and its molecular mechanism were determined in HL-7702 cells in vitro. It was found that long-lasting hyperinsulinemia significantly increased TFR1 expression in the liver and induced mild-to-moderate hepatic iron overload, which was accompanied by a normal level of serum iron. Insulin markedly upregulated both protein and mRNA levels of TFR1 in HL-7702 cells. The stability of TFR1 mRNA stability, together with expression of IRPs expression, were both significantly increased by insulin treatment. Insulin-induced TFR1 expression was blocked by IRP2, but not by IRP1 interference, and disappeared when HL-7702 cells were pretreated with LY294002, triciribine hydrate, or rapamycin. In conclusion, the findings of this study indicate that hyperinsulnemia could induce hepatic iron overload by upregulating liver TFR1 via the PI3K/AKT/mTOR/IRP2 pathway, which may be one of the main reasons for the occurrence of DIOS.

Key Words
- Hyperinsulinemia
- Goto-Kakizaki rats
- Iron overload
- TFRs
- IRPs

Introduction
Dysmetabolic iron overload syndrome (DIOS) is now frequently found in patients with chronic metabolic diseases such as nonalcoholic fatty liver disease and metabolic syndrome (MS; Dongiovanni et al. 2011). DIOS has been confirmed to play an important role in the onset and development of chronic metabolic diseases by causing oxidative stress injury in the liver, increasing cellular lipid burden through enhancing liver cholesterol synthesis and participating in liver fibrosis (Sanyal 2005, Day 2006, O’Brien & Powell 2012). Iron reduction therapy, such as phlebotomy or an iron-restricted diet, could markedly decrease metabolic alterations and liver enzymes (Piperno et al. 2004, Dongiovanni et al. 2011, Valenti et al. 2011). However, the mechanism underlying the pathogenesis of DIOS remains unclear.

Hyperinsulinemia is a common characteristic of DIOS (Dongiovanni et al. 2011). Most recently, it has been
proposed that environmentally induced hyperinsulinemia is the causative factor rather than the consequence of insulin resistance in chronic metabolic diseases (Corkey 2012), indicating that patients with DIOS may experience a period with increased insulin activity and that the subsequent insulin resistance is an adaptive response to maintain the circulating levels of fat and glucose (Karpe et al. 2011). It has been known for a long time that insulin could promote cellular iron uptake in adipocytes by stimulating recycle of the transferring receptor (TFR) between the cell membrane and the cytoplasm (Davis et al. 1986, Tanner & Lienhard 1987). Results from more recent studies demonstrated (Ruivard et al. 2009, Tsuchiya et al. 2010) have indicated that iron absorption was decreased in DIOS patients without altering the level of serum iron, indicating that the hepatic iron overload may be associated with cellular iron uptake, which is mainly determined by the level of TFRs. It was also reported that TFR1 level was elevated obviously in patients with DIOS (Mitsuyoshi et al. 2009, Tsuchiya et al. 2010) and this elevation was significantly and positively correlated with the hepatic iron content (Mitsuyoshi et al. 2009). However, the effect of hyperinsulinemia on body iron stores and the level of liver TFRs remain elusive.

In this study, we observed, for the first time, to our knowledge, the effect of hyperinsulinemia on iron homeostasis and expression of hepatic TFRs in rats, and further studied the molecular mechanisms of insulin regulation of TFRs in a human liver cell line HL-7702. The results of our study help to elucidate the effect of insulin on body iron homeostasis and clarify whether and how hepatic TFRs are regulated by insulin, thus providing new insights into the etiology of DIOS and helping to improve the protection and treatment strategies.

Materials and methods

Animals

The animal experiments were approved by the Animal Ethics Committee of the Second Military Medical University (Shanghai, China). Adult female Sprague–Dawley (SD) rats (Shanghai-BK Co., Ltd., Shanghai, China) weighing 180–200 g were adapted to the environmentally controlled vivarium (23 ± 1 °C, 30–40% humidity) with a 12 h light:12 h darkness photocycle (lights on 0800 h). The rats were housed in cages on pine shaving bedding, with free access to food and water at all times.

Acute insulin administration

Twelve female SD rats were equally divided into two groups: the normal control (NC) group and the acute insulin administration (AI) group. The rats in AI group received a single i.p. injection of three units of insulin (Sigma), and rats in NC group received injections of saline alone. All rats were killed 4 h after injection.

Chronic insulin administration

Twelve female SD rats were equally divided into two groups: the NC group and the chronic insulin administration (CI) group. An Alzet minipump (Alzet, No 2001, Palo Alto, CA, USA) was implanted into the s.c. tissue on the back of each rat anesthetized with ketamine (250 mg/kg) to deliver insulin (Sigma) at 3 U/day in animals of the CI group, and saline in animals of the NC group. A group of sham-operated rats without pump implantation were used as a blank control group. These animals did not differ significantly from the animals in the NC group, and data obtained from this group of rats were grouped together with data from the NC rats. There were no deaths in all three groups and the rats were killed 5 days after surgery.

Iron status parameters

The iron level in the liver was quantitated using an atomic absorption spectrophotometer (Hitachi Z-8100) and normalized to the wet tissue weight for each sample. Serum iron concentrations and total iron-binding capacity (TIBC) were determined for non-hemolyzed serum samples using the colorimetric analysis kits (Nanjing Jian Cheng Biotechnology Institute, Nanjing, China). The transferrin saturation was calculated as plasmatic iron/TIBC and transferrin as TIBC/25.

Insulin and glucose analysis

Glucose was measured using fresh blood by cutting and pricking the tail (Glucometer Gluco Touch, Roche). The serum levels of insulin and corticosterone were measured using RIA Kits (North Biotechnology, Beijing, China).

Cell culture, treatment, and siRNA transfection

HL-7702 cells (Chinese Academy of Sciences, Shanghai, China) were grown in 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, and 50 μg/ml
streptomyacin sulfate (HyClone) at 37 °C in a humidified 5% CO₂ atmosphere, transferred to six-well plates, and then treated with 0.1, 1, 10, and 100 nM insulin for 12 h, or with 100 nM insulin for 4, 8, 12, and 24 h. To silence the expression of TFR1, IRP1, and IRP2, the cells were transfected with specific siRNA products (sc-37070, sc-40715, and sc-40713, Santa Cruz, USA) consisting of a pool of three target-specific 19–25 nt siRNAs, designed to knockdown gene expression, or negative control oligos (Santa Cruz Biotechnologies) in the presence of lipofectamine RNAiMAX, according to the manufacturer’s instructions (Invitrogen). The cells were maintained at 37 °C for 48 h with siRNA or negative control oligos and treated with 100 nM insulin for another 12 h. For tests of insulin pathway inhibitors, HL-7702 cells were pretreated with MAPK/Erk kinase (MEK) inhibitor U0126 (1 μM), Jun N-terminal kinase (JNK) inhibitor SP600125 (10 μM), PI3K inhibitor LY294002 (10 μM), AKT (also known as protein kinase B) inhibitor TH (10 μM), or mammalian target of rapamycin (mTOR) inhibitor rapamycin (Ra) (1 μM) for 30 min before exposure to 100 nM insulin or were treated only with GSK3 inhibitor TDZD8 (T8) (5 μM) for 12 h to mimic the inhibitory effect of insulin on GSK3.

**Real-time quantitative PCR analysis**

Total RNA isolated from HL-7702 cells was obtained using TRizol (Invitrogen) and reverse transcribed to cDNA with a RT regent kit (Primerscript, TAKARA Bio, Inc., Shiga, Japan). RT was carried out on 1 μg total RNA with a SYBR Green Kit (TAKARA) in a final volume of 20 μl, using Steponeplus (Life Technologies). The primer sequences for the human TFR1 are as follows: forward, TTCCA CCATC TCGGT CATCA and reverse, AGGTA TCCCT CTAGC CATTC AGT; for human β-actin: forward, ACACC TTCTA GGGTG CATCA and reverse, CATGA TGGAGTTGAAGG- TAG. The PCR conditions were as follows: 10 min at 95 °C followed by 40 cycles of 95 °C, 10 s; 60 °C, 20 s; and 72 °C, 25 s. The relative amount of each mRNA was normalized to RNA levels of the housekeeping gene β-actin, and the data were analyzed according to the 2^−ΔΔCT method.

**TFR1 mRNA stability**

Actinomycin D (ActD) (5 μg/ml) was added to the control and insulin-treated cells, and total RNA was isolated at 0, 2, and 4 h after ActD addition. RT-PCR was carried out with specific primers of human TFR1 and β-actin, which were also used for RT-PCR.

**Western blotting analysis**

Rat livers or HL-7702 cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer with 1 mM PMSF, complete protease inhibitor cocktail, and phosphatase inhibitor (Beyotime, Nanjing, China). Then the lysates were subsequently incubated at 4 °C for 10 min and centrifuged (13 000g) for 25 min at 4 °C. The supernatant was separated on a 10% Sodium dodecyl sulfate–pulsed agarose gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes (Merck) using a Bio-Rad Transblot. The blots were probed with anti-transferrin receptor-1 (Epitomics, USA; 1:1000 dilution), anti-transferrin receptor-2 (Abcam, UK; 1:500 dilution), anti-IRP1 (Epitomics, San Diego, CA, USA; 1:1000 dilution), anti-IRP2 (Abcam, Cambridge, UK; 1:500 dilution), anti-AKT (CST, Danvers, MA, USA; 1:1000 dilution), anti-phospho-AKT-SER473 (CST, USA; 1:1000 dilution), and anti-β-actin (BioWorld, Shanghai, China; 1:5000 dilution). Subsequently, the membranes were incubated with the peroxidase-conjugated goat anti-rabbit secondary antibodies (dilution, 1:8000). The signals quantified by densitometry were normalized to β-actin levels, or in the case of phosphoproteins, to the total levels of the same protein.

**Analysis of cellular iron uptake**

Intracellular iron was measured by Phen Green-FL (PG–FL, life) fluorescence signal quenching. The HL-7702 cells were divided into eight groups: control, insulin, apo-TF, apo-TF + insulin, holo-TF, holo-TF + insulin, TFR1 siRNA + holo-TF, and TFR1 siRNA + holo-TF + insulin. The cells were transfected with specific siRNA products for TFR1 or negative control siRNA for 48 h, and then incubated with 30 μM apo-transferrin (apo-TF, Merck Millipore) or 30 μM holo-transferrin (holo-TF, Merck Millipore) for 6 h with or without pre-treatment with 100 nM insulin for 6 h before detection of fluorescence. After incubation, the cells were washed with warm (37 °C) Dulbecco phosphate-buffered saline (DPBS) twice and then incubated with PG–FL diacetate (5 μM, Molecular Probes, Eugene, OR, USA) in DPBS for 20 min at 37 °C. The excess PG–FL was washed off twice with DPBS, and the PG–FL fluorescence intensity was recorded (excitation/emission: 490/520 nm) under a confocal laser scanning microscope (Olympus).

**Statistical analysis**

The values are represented as mean ± S.E.M. Statistical analysis was performed using the Statview software.
Insulin upregulates TFR1 in HL-7702 cells

The HL-7702 cells were treated with insulin for different times and at different concentrations. Compared with the blank control group, expression of TFR1 mRNA and protein were markedly increased after insulin treatment ($*P<0.05$, $**P<0.01$, and $***P<0.001$; Fig. 3A and B). In the time course, TFR1 expression increased to the maximum of 1.5-fold at 12 h ($P<0.001$; Fig. 3B), and in the concentration gradient, the maximal effect about 1.5-fold elevation was detected at 100 nM ($P<0.01$; Fig. 3B). No significant alteration in TFR2 expression was observed in HL-7702 cells treated with insulin depending on time or concentration gradient (Fig. 3B).

Insulin increases intracellular iron content through TFR1 in HL-7702 cells

The HL-7702 cells were transfected with negative control siRNA for 48 h and then incubated with 30 μM apotransferrin or 30 μM holo-transferrin for 6 h with or without insulin treatment (Fig. 3B). To gain insights into the effect of hyperinsulinemia on iron homeostasis, SD rats of the AI group received i.p. injection of insulin (three units) or saline. Protein levels of hepatic TFR1, TFR2, IRP1, and IRP2 were analyzed by western blotting. The values are expressed as mean ± S.E.M. for two groups compared with controls: *$P<0.01$.

Table 1 Effect of acute insulin injection on body weight, plasma glucose, plasma insulin, and corticosterone. Values are expressed as mean ± S.E.M.

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<th>Saline (n=6)</th>
<th>Insulin (n=6)</th>
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<tr>
<td>Body weight (g)</td>
<td>190.9±24.25</td>
<td>191.68±20.18</td>
</tr>
<tr>
<td>Plasma insulin (μU/ml)</td>
<td>39.53±12.97</td>
<td>302.88±15.71*</td>
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<tr>
<td>Plasma glucose (mmol/l)</td>
<td>6.83±0.76</td>
<td>3.98±1.29*</td>
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<tr>
<td>Corticosterone (ng/ml)</td>
<td>26.50±3.34</td>
<td>26.88±5.19</td>
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Compared with controls: *$P<0.01$.

(SAS Institute, Inc., Kerry, NC, USA). The statistical difference between two groups was assessed by the independent t-test. One-way ANOVA, followed by LSD test, was performed to analyze the difference between the three or more groups. The differences were considered significant at $P<0.05$.

Results

Effect of acute or chronic insulin on iron parameters and hepatic TFRs and IRPs expression in rats

To gain insights into the effect of hyperinsulinemia on iron homeostasis, SD rats of the AI group received insulin by a single injection those of the CI group received insulin through s.c. implantation of a mini-pump. It was found that plasma glucose was decreased and insulin level was increased in AI rats (Table 1) without significantly altering the expression of TFRs and iron regulatory proteins (IRPs) in the liver (Fig. 1) and the serum corticosterone (Table 1). The CI rats presented with significant hyperinsulinemia, represented by a fourfold increase in plasma insulin ($P<0.01$; Table 2) and a mild-to-moderate hepatic iron overload as compared with the control rats (240.25 ± 6.13 vs 198.50 ± 5.23 μg/g, $P<0.05$; Table 2), with no significant changes detected in blood iron parameters and corticosterone ($P>0.05$) (Table 2). The western blotting analysis results indicated that compared with the control group, TFR1, but not TFR2, was significantly elevated in CI rats (TFR1, CI vs NC, $P<0.05$; and TFR2, CI vs NC, $P>0.05$; Fig. 2A), accompanied with an approximately 1.5-fold increase in both IRP1 and IRP2 (IRP1, CI vs NC, $P<0.05$; and IRP2, CI vs NC, $P<0.05$) (Fig. 2A). The level of phospho-AKT and the phosphorylation ratio of AKT were both significantly increased in CI rats (pAKT, CI vs NC, $P<0.001$; and pAKT/AKT, CI vs NC, $P<0.001$; Fig. 2B), while the total AKT remained unchanged (Fig. 2B).

![Figure 1](image1.png)

Effect of acute insulin administration on expression of rat hepatic TFRs and IRPs. SD rats (180–200 g) received i.p. injection of insulin (three units) or saline. Protein levels of hepatic TFR1, TFR2, IRP1, and IRP2 were analyzed by western blotting. The values are expressed as mean ± S.E.M. for two groups (n=6/group). The significance of differences between the two groups was assessed by the independent-t test. Differences were considered significant at $P<0.05$.
Table 2 Effect of chronic insulin injection on body weight, plasma glucose, plasma insulin, iron parameters, and corticosterone

<table>
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<th>Saline (n=6)</th>
<th>Insulin (n=6)</th>
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<tr>
<td>Body weight (g)</td>
<td>235.3 ± 6.66</td>
<td>233.3 ± 7.79</td>
</tr>
<tr>
<td>Plasma insulin (μU/ml)</td>
<td>39.99 ± 11.12</td>
<td>165.43 ± 18.77</td>
</tr>
<tr>
<td>Liver iron (µg/g)</td>
<td>198.50 ± 5.23</td>
<td>240.25 ± 6.13*</td>
</tr>
<tr>
<td>Plasma iron (µmol/l)</td>
<td>72.00 ± 9.29</td>
<td>71.96 ± 8.99</td>
</tr>
<tr>
<td>TIBC (g/l)</td>
<td>107.05 ± 9.38</td>
<td>114.12 ± 8.89</td>
</tr>
<tr>
<td>Plasma transferrin (g/l)</td>
<td>4.28 ± 0.37</td>
<td>4.56 ± 0.35</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>66.17 ± 7.61</td>
<td>62.84 ± 6.99</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>27.00 ± 2.48</td>
<td>29.88 ± 3.97</td>
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TIBC, total iron binding capacity. Compared with controls: *P < 0.05 and **P < 0.01.

without pre-treatment with 100 nM insulin for 6 h before the detection of the fluorescence. We also observed the effect of insulin on the fluorescence activities of HL-7702 cells treated with holo-Tf (30 µM, for 6 h) under conditions in which the TFR1 expression was blocked by transfection of specific siRNA into HL-7702 cells (Fig. 4A). The fluorescence was significantly suppressed by holo-Tf (holo-Tf vs control, P < 0.01, holo-Tf+Ins vs control, and P < 0.05; Fig. 4B), and further decreased in HL-7702 cells pretreated with insulin (holo-Tf+Ins vs holo-Tf, P < 0.05; Fig. 4B). Moreover, we found that holo-Tf could no longer repress the fluorescence in HL-7702 cells lacking TFR1 (TFR1 siRNA+holo-Tf vs control, P > 0.05; Fig. 4B), and the fluorescence was still not changed by additional pretreatment with 100 nM insulin for 12 h (TFR1 siRNA+holo-Tf+insulin vs TFR1 siRNA+holo-Tf, P > 0.05; Fig. 4B).

Insulin upregulates IRPs and TFR1 mRNA stability in HL-7702 cells

IRPs are novel regulators of TFR1 and play positive roles in the maintenance of mRNA stability (Andrews & Schmidt 2007, Anderson et al. 2012). Our results indicated that insulin greatly stimulated IRP1 and IRP2 expression with the maximal effect present at 8 h (Fig. 5A). The most effective concentration of insulin on IRP1 and IRP2 expression was 100 nM (Fig. 5A). Analysis of the half-life of TFR1 mRNA in HL-7702 cells with or without insulin treatment showed that TFR1 mRNA stability was markedly increased in insulin-treated cells compared with that in untreated control cells (Fig. 5B).
Insulin stimulates TFR1 synthesis through IRP2, but not IRP1, specifically mediated by the PI3K/akt/mTOR pathway

To further determine the role of IRP1 and IRP2 in insulin-induced TFR1 expression, specific siRNAs for IRP1 or IRP2 was co- or singly transfected into HL-7702 cells (si-IRPs, for IRP1, P<0.001, for IRP2, P<0.01, Fig. 6A; si-IRP1, P<0.01, Fig. 6B; si-IRP2, P<0.001, Fig. 6C), followed by an additional 12-h treatment with 100 nM insulin. Results of western blotting analysis indicated that the insulin-induced expression of TFR1 was blocked in HL-7702 cells lacking both IRPs or only IRP2, but not influenced by IRP1 interference.

To examine the pathway mediating the regulation of insulin on TFR1 and IRP2, HL-7702 cells were pretreated with LY294002 (10 μM), U0126 (1 μM), or SP600125 (10 μM) for 30 min before exposure to 100 nM insulin for 12 h. Upregulation of TFR1 and IRP2 by insulin was entirely blocked by LY294002 (LY vs LY, P>0.05; Fig. 7A), but not suppressed in the presence of U0126 (U+1 vs U, P<0.01; Fig. 7A) or SP600125 (SP+1 vs SP, P<0.01; Fig. 7A). Also, the phospho-AKT and the phosphorylation ratio of AKT were both markedly increased by insulin treatment in HL-7702 cells (pAKT, I vs C, P<0.01 and pAKT/AKT, I vs C, P<0.001; Fig. 7B), which could be totally blocked in the presence of LY294002 (Fig. 7B).

To gain deeper insights of the downstream pathways of PI3K involved in the regulation of insulin on IRP2/TFR1, we also observed the protein level of IRP1 and IRP2 in HL-7702 cells with or without pretreatment with the AKT inhibitor TH (10 μM) or mTOR inhibitor rapamycin (Ra) (1 μM) for 30 min before exposure to 100 nM insulin for 12 h, and also in HL-7702 cells that were only treated with GSK3 inhibitor TDZD8 (T8) (5 μM) for 12 h to mimic the inhibitory effect of insulin on GSK3. Our results indicated that the upregulation of TFR1 and IRP2 by insulin could be totally blocked by TH (TH+1 vs T, P>0.05; Fig. 7C) or rapamycin (Ra+1 vs Ra, P>0.05; Fig. 7D), but this could not be replicated in the presence of TDZD8 (T8 vs C, P>0.05; Fig. 7E).

Discussion

In this study, we demonstrated that chronic administration of insulin leading to chronic hyperinsulinemia, a common characteristic of patients with DIOS, induced mild-to-moderate hepatic iron overload accompanied by overexpression of TFR1 in the liver. We also confirmed in human liver HL-7702 cells in vitro that insulin could
directly upregulate the protein level of TFR1 probably by enhancing its mRNA stability through IRP2, but not IRP1, which was mediated by the PI3K/AKT/mTOR pathway.

AI showed no effect on the blood iron parameters and liver iron content, indicating that acute hyperinsulinemia may not interfere with iron homeostasis. The duration of hyperinsulinemia was prolonged in rats with CI administered via a subcutaneously implanted mini-pump set to deliver insulin at a constant rate of about 3 U/day. Although serum iron levels remained unchanged in these rats with long-lasting hyperinsulinemia, the hepatic iron content was elevated mildly to moderately and statistically significantly, suggesting that hyperinsulinemia induced hepatic iron overload, probably through regulation of cellular iron homestasis rather systematic iron balance. It has been reported (Tsuchiya et al. 2010) that serum iron was normal in DIOS patients with hepatic iron overload, which is consistent with the iron status that we observed in the present animal experiment. Clairmont & Czech (1990) reported that the level of serum TFRs of rats was increased after 30-min AI, but returned to control values in 90 min, which may explain why liver TFR expression was unchanged in acute insulin-treated rats 4 h after injection in the present study. However, TFR1 was markedly increased in rats with chronic hyperinsulinemia without significant alteration in TFR2, indicating that long-lasting insulin infusion upregulated TFR1 in a sustainable way through a specific mechanism that was different from that for TFR2. It has been demonstrated in clinical studies that the liver TFR1 level is significantly upregulated (Mitsuyoshi et al. 2009, Tsuchiya et al. 2010) in patients with DIOS and even further increased as the stage of DIOS progressed, aggravating hepatic iron overload (Mitsuyoshi et al. 2009). It was also reported in clinical studies that accumulation of hepatic iron might impair the insulin effect by reducing hepatic insulin...
Figure 5
Insulin upregulates IRPs protein expression and enhances TFR1 mRNA stability. (A) HL-7702 cells were treated with 100 nM insulin for 4, 8, 12, and 24 h or for 8 h at concentrations of 0.1, 1, 10, and 100 nM. The protein levels of IRP1 and IRP2 were detected by western blotting analysis. *Significantly different from control, \( P < 0.05 \) (*** \( P < 0.001 \)). (B) Actinomycin D (5 \( \mu \)g/ml) was added to HL-7702 cells and incubated for 0, 2, and 4 h, in the presence or absence of 100 nM insulin pretreatment for 12 h. Total RNA was isolated and RT-PCR was carried out with specific primers for human TFR1 and \( \beta \)-actin. The values are expressed as mean \( \pm \) S.E.M., determined in three independent experiments. One-way ANOVA, followed by LSD \( t \) and SNK post hoc test, was performed to analyze differences between the groups. The differences were considered significant at \( P < 0.05 \).

extraction and metabolism and decreasing glucose uptake in muscle (Niederau et al. 1984, Merkel et al. 1988, Mascitelli et al. 2009), and thus causing hyperinsulinemia, which, together with our results, implied that the interaction between iron overload and hyperinsulinemia, and might partly explain the progressive increase in TFR1 following the aggravation of hepatic iron overload in patients with DIOS.

Regulation on TFR1 by IRPs/IRE was first discovered in the 1980s (Müllner & Kuhn 1988, M"ullner et al. 1989), which was confirmed as the most important mechanism underlying the response to intracellular iron load. Surprisingly, TFR2 had no IRE motif and could not be regulated by IRPs (Fleming et al. 2000). On the basis of these findings, we further detected the protein levels of IRPs in the liver. It was found that both IRP1 and IRP2 were dramatically increased in rats with long-lasting hyperinsulinemia, implying a great probability that the insulin-stimulated overexpression of hepatic TFR1 was secondary to the elevation of IRPs. There is also a possibility that hypoglycemia or the hormones involved in stress contribute to the alteration of the hepatic TFR1 expression. However, our results indicated that the classic stress-responsive hormone, corticosterone, was not changed in rats by acute or chronic insulin administration. Results from our previous experiments also indicated that hyperglycemia, but not hypoglycemia, increased the expression of TFR1 in HL-7702 cells (Qiao & Li 2010). We found that, for the same time of incubation, 12, 24, or 48 h, the TFR1 level in HL-7702 cells incubated in 25 mmol/l glucose was significantly higher than that in those incubated in a normal concentration of 5.5 mmol/l glucose.

To further investigate how insulin is involved in cellular iron regulation, we carried out an in vitro study in a human liver cell line HL-7702 and observed the effect of insulin on expression of TFRs by treating HL-7702 cells with human recombinant insulin for different times or at different concentrations. Our results indicated that insulin obviously upregulated TFR1 in a time- and dose-dependent manner but had no effect on TFR2, which is consistent with the results from our previous animal study, indicating that TFR1 synthesis can be directly stimulated by insulin, and that the increase in liver TFR1
Hyperinsulinemia induces hepatic iron overload

Insulin stimulates TFR1 expression through IRP2 but not IRP1. (A) HL-7702 cells were transfected with either control (si cont) or siRNA for both IRP1 and IRP2, and 48 h later were exposed to 100 nM insulin for another 12 h. Expression of IRP1, IRP2, and TFR1 was determined by western blotting analysis. **Significantly different from control, P<0.01 (**P<0.01 and ***P<0.001).

(A) and (C) The cells were transfected with invalid IRP1 (B) or IRP2 (C) siRNA, and 48 h later were exposed to 100 nM insulin for another 12 h. Expression of IRP1 or IRP2 and TFR1 s was determined by western blotting analysis. *Significantly different from control, P<0.05 (**P<0.01 and ***P<0.001).

Figure 6

content in CI rats was mainly due to long-lasting hyperinsulinemia. We subsequently studied the effect of insulin on cellular iron uptake and confirmed that cellular uptake of holo-Tf was markedly increased in the presence of insulin using a fluorescence indicator that is quenched by free iron ions. Our finding is consistent with results from several other studies (Davis et al. 1986, Tanner & Lienhard 1987, Clairmont & Czech 1990, Biswas et al. 2013) concerning insulin-stimulated iron uptake. What is more important, we also have shown that the stimulatory effect of insulin on cellular iron uptake was totally blocked by knocking down TFR1 via siRNA, implying that TFR1 plays a specific and critical role in the effect of insulin on cellular iron homeostasis.

On the basis of the results that we obtained from the animal experiments, we detected the protein levels of IRP1 and IRP2 in insulin-treated HL-7702 cells and found that they were also significantly elevated. Interestingly, compared with the stimulatory effect of insulin on TFR1, insulin-induced elevation of IRPs appeared 4 h earlier, because TFR1 acts as a transcription regulatory target of IRPs (Hentze et al. 2010) and therefore the elevation of the protein level of TFR1 involves a process of mRNA stabilization by IRPs, then an increase in transcription products, and finally the enhancement of translation, which makes TFR1 response to insulin later compared with that of IRP2. Knowing that IRPs increase TFR1 expression at the post-transcription level, we then measured the stability of TFR1 mRNA in HL-7702 cells in the presence or absence of insulin at different time points in the presence of ActD. The results indicated that the half-life of TFR1 was prolonged after insulin treatment, indicating that insulin promotes the expression of TFR1 probably by enhancing its mRNA stability. We also verified the role of IRP1 and IRP2 in the insulin-stimulated upregulation of TFR1 by knocking down IRP1, IRP2, or both in HL-7702 cells before administration of additional insulin. It was found that insulin-induced upregulation of TFR1 was totally inhibited by the interference with both IRPs or IRP2, but remained unchanged even though IRP1 was knocked down. These results indicate that IRP2 and IRP1 play completely different roles in the mediation of insulin regulation on TFR1, as only IRP2 was dominant and essential in this mechanism. Unlike IRP2, IRP1 is a bifunctional protein, serving either as cytosolic aconitase or a high-affinity...
IRE-binding protein, and most biofunctional IRP1 is in the form of cytosolic aconitase rather than an RNA-binding protein when iron is abundant (Haile et al. 1992, Stys et al. 2011, Anderson et al. 2012), implying the majority of the overexpressed IRP1 in the rats with persistent hyperinsulinemia in this study functioned as aconitase rather IRP. It has also been reported (Galy et al. 2005, Anderson et al. 2012) that IRP1-deficient animals could still maintain normal iron homeostasis, while IRP2-deficient mice displayed severe iron deficiency due to low expression of TFR1. These findings support the idea that IRP2 is the primary post-transcriptional regulator of TFR1 (Galy et al. 2005), as we observed in this study.

Figure 7
Effect of insulin on TFR1 and IRP2 expression is specifically mediated by PI3K pathway. (A) HL-7702 cells were pretreated with MEK inhibitor U0126 (1 μM), JNK inhibitor SP600125 (10 μM), or PI3K inhibitor LY294002 (10 μM) for 30 min and exposed to 100 nM insulin for 12 h. The protein levels of TFR1 and IRP2 were analyzed by western blotting. (B) HL-7702 cells were pretreated with the AKT inhibitor TH (10 μM) for 30 min and exposed to 100 nM insulin for 12 h. Protein levels of AKT and phospho-AKT were analyzed by western blotting. (C and D) HL-7702 cells were pretreated with the AKT inhibitor TH (10 μM), or the mTOR inhibitor rapamycin (Ra) (1 μM) for 30 min and exposed to 100 nM insulin for 12 h. Protein levels of IRP2 and TFR1 were analyzed by western blotting analysis. (E) HL-7702 cells were treated with insulin (100 nM) or GSK3 inhibitor TDZD8 (T8) (5 μM) for 12 h. The protein levels of IRP2 and TFR1 were analyzed by western blotting analysis. **Significantly different from control, P < 0.01 (***P < 0.001). The values are expressed as mean ± S.E.M., determined in three independent experiments. One-way ANOVA, followed by LSD post hoc test, was performed to analyze differences between the groups.

To further clarify how the downstream pathway of insulin is involved in the insulin-induced upregulation of TFR1 and to better understand the role of IRP2 in this mechanism, we used the inhibitors of PI3K and MEK and JNK pathways and found that LY294002, a novel inhibitor of PI3K, markedly blocked the stimulatory effect of insulin on TFR1 and IRP2 expression, but neither U0126 (an inhibitor of MEK) nor SP600125 (an inhibitor of JNK) showed any significant inhibitory effect on insulin-induced TFR1 and IRP2 synthesis, indicating that it was the PI3K pathway but not MEK or JNK that mediated the effect of insulin on the IRP2/TFR1 axis. We also found that AKT was markedly
activated both in rats under chronic mini-pump administration and in HL-7702 cells treated with insulin, further confirming the involvement of PI3K in the regulatory effects of insulin on TFR1. In addition, to better clarify the signal transduction downstream of the PI3K pathway, we observed the role of AKT, mTOR and GSK3 in insulin-induced elevation of IRP2/TFR1 by using specific inhibitors. We found that the upregulation of TFR1 by insulin disappeared when either AKT or mTOR was inhibited, and could not be replicated when the activation of GSK3 was suppressed by the inhibitor instead of insulin, demonstrating that the effect of insulin on IPR2/TFR1 was specially mediated, downstream of PI3K, by the AKT/mTOR pathway but not GSK3. It has been reported that the activation of IRP2 was regulated by proteasomal degradation, via the E3 ubiquitin ligase complex containing FBXL5 protein (Salahudeen et al. 2009, Vashisht et al. 2009), the stability of which depends on both iron and oxygen as the result of assembly of an iron–oxygen center. Therefore, the PI3K/AKT/mTOR pathway may stimulate IRP2 indirectly by interfering with its proteasomal degradation through FBXL5 or other components of the ubiquitin ligase complex, which needs to be verified in further studies.

Biswas et al. (2013) also reported that insulin upregulated TFR1 expression, however, by enhancing the transcription, but not the mRNA stability, via hypoxic-induced factor 1 alpha (HIF1α), in their most recent study in HepG2 cells. In this study, the results of the experiment with ActD confirmed that it was the post-transcriptional mechanism that mainly mediated the stimulatory effect of insulin on TFR1 in L-02 cells, because the half-life of TFR1 was still prolonged by insulin when the process of transcription was blocked. We then postulated that the choice of cell line may be partially responsible for the differences in the cellular mechanism observed in insulin-induced TFR1 expression, and this postulate was supported by the study of (Festa et al. 2000) who reported that IRP genes were markedly upregulated during differentiation of 3T3-L1 pre-adipocytes, which implied a relatively worse status of IRPs in carcinoma cells with poor differentiation. More importantly, compared with Sudipta Biswas’s study, we further confirmed the effect of insulin on TFR1 in vivo, and thus considered hyperinsulinemia to be an important risk factor for hepatic iron overload, which helped better to explain the occurrence of DIOS.

In summary, our study has demonstrated that long-lasting hyperinsulinemia could induce hepatic iron overload through its direct stimulatory effect on TFR1, mediated by the PI3K/IRP2 pathway, thus playing a critical role in the occurrence of DIOS. Food additives, which are considered to be the main contributors to diet-induced hyperinsulinemia, should be addressed in protection against DIOS. IRP2 or TFR1 antagonists may be potential drug targets to improve treatment.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution
M L developed the concept and design of the study and the critical revision of the manuscript and was responsible for obtaining the funding; X J and H W reviewed the concept and design of the study, provided technical or material support, participated in data acquisition, analysis and interpretation, drafted the manuscript, and performed the statistical analysis; W S, Z S, and H S participated in data acquisition and technical support. M L is the guarantor of this article and, as such, has full access to all the data in the study, and is responsible for the integrity of the data and the accuracy of the data analysis.

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