REGULATION OF ACTIVATING TRANSCRIPTION FACTOR 4 (ATF4) EXPRESSION BY FAT MASS AND OBESITY-ASSOCIATED (FTO) IN MOUSE HEPATOCYTE CELLS

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Abstract

Context. Abnormally increased hepatic glucose production contributes to hyperglycemia in diabetes. Interventions that suppress hepatic gluconeogenesis should be beneficial in improving glycemic control in patients with diabetes.

Objectives. It has been suggested that hepatic *FTO* is involved in glycemic control by regulating gluconeogenesis. Both *FTO* and activating transcription factor 4 (*ATF4*) positively regulate the expression of gluconeogenic genes in the liver, suggesting the possibility that *ATF4* mediates the stimulatory effect of *FTO* on hepatic gluconeogenesis. The present study aimed to determine the effect of altered expression or activity of *FTO* on *Atf4* and gluconeogenic gene expression in hepatocyte cells.

Methods. Mouse hepatocyte AML12 cells were treated with the *FTO* inhibitor rhein or transfected with an *FTO*-expressing plasmid. Levels of gluconeogenic glucose-6-phosphatase (*G6pc*) and *Atf4* mRNA and protein were measured.

Results. Rhein treatment significantly reduced *G6pc* mRNA levels as well as *Atf4* mRNA and protein levels. Conversely, enhanced *FTO* expression caused an increase in *G6pc* and *Atf4* mRNA levels.

Conclusions. These findings support the hypothesis that hepatic *FTO* participates in the regulation of hepatic gluconeogenic gene and *ATF4* expression. Reducing the activity of the hepatic *FTO-ATF4* pathway may be beneficial in reducing hepatic glucose production and ameliorating hyperglycemia in diabetes.

Keywords: liver, gluconeogenesis, demethylase, transcription factor, type 2 diabetes.

INTRODUCTION

Hepatic metabolism is critical for the maintenance of whole body glucose homeostasis. Gluconeogenesis is the major metabolic process that causes an increase in hepatic glucose production. Abnormally increased hepatic gluconeogenesis

contributes to an impaired glycemic control in diabetes. Thus, understanding the mechanism underlying both normal and abnormally increased hepatic glucose production may suggest new and better anti-diabetes drug candidates.

Common genetic variants of the fat mass and obesity-associated (*FTO*) gene are associated with obesity and type 2 diabetes (1,2). The association between *FTO* variants and risk of type 2 diabetes involves obesity-dependent and –independent components (3,4). *FTO* is ubiquitously expressed and previous studies have suggested that *FTO* participates in the regulation of metabolism in a tissue specific manner (5). Levels of *FTO* (the rodent homologue of human *FTO*) mRNA and protein are altered by nutrient and hormonal signals in mouse liver and hepatocyte cells (6, 7). Enhanced *FTO* expression leads to increased levels of gluconeogenic genes, while *FTO* knockdown causes an opposite effect (7-9). Moreover, liver-specific *FTO* overexpression results in an increased blood glucose level and impaired glucose tolerance in mice (8). Conversely, *FTO* inhibitor treatment or liver-specific *FTO* deficiency reduced blood glucose level and improved glucose tolerance in mice (9). These findings suggest that hepatic *FTO* participates in the regulation of whole body glucose metabolism through alterations in hepatic gluconeogenic gene expression by responding to nutrient and hormonal signals.

Activating transcription factor 4 (*ATF4*) is a transcription factor that belongs to the cAMP-responsive element-binding (CREB) protein family and plays a pivotal role in the regulation of glucose homeostasis. *Atf4* (the rodent homologue of human *ATF4*) deficiency causes a reduction in gluconeogenic gene expression in the liver and improves glucose homeostasis in mice (10-13). A recent study showed that levels of *ATF4* protein are elevated in the liver of transgenic mice with enhanced liver specific *FTO* expression (14). These findings led to the hypothesis that *ATF4* mediates the

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stimulatory effect of *FTO* on hepatic gluconeogenesis by up-regulating gluconeogenic gene expression. Conversely, it is hypothesized that reduced *FTO* activity in liver causes a reduction of gluconeogenic gene expression by suppressing *ATF4* expression. To address this hypothesis, we investigated the effect of altered *FTO* expression and activity on *Atf4* mRNA and gluconeogenic gene expression in mouse hepatocyte cell line.

MATERIALS AND METHODS

Cell culture

The immortalized mouse hepatocyte cell line AML12 (ATCC®, CRL-2254™) was maintained in DMEM/Ham's F-12 (1:1) medium supplemented with insulin (5 ng/mL), dexamethasone (40 ng/mL), sodium selenite (5 ng/mL), transferrin (5 µg/mL), fetal bovine serum (10%) and antibiotics at 37°C in humidified 5% CO₂ atmosphere.

To determine the effect of *FTO* inhibition on the expression of gluconeogenic genes and *Atf4* mRNA and protein, subcloned cells were grown to 60-70% confluency and treated with the *FTO* inhibitor rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid, Cayman Chemical, Ann Arbor, MI) at various concentrations (10 fM, 100 pM, 10 nM, 1 µM, 20 µM or 50 µM) for 24 h. Rhein is one of the major components of *Rheum palmatum* L and has the ability to inhibit demethylase activity of *FTO* (15,16). Cells were harvested at the end of the 24-h treatment. To determine the time course, cells were treated with rhein at 1 µM and harvested 0, 2, 4, 6, 12 and 24 h later. Rhein was initially dissolved in dimethyl sulfoxide (DMSO) and further diluted in 0.2-µm filter sterilized water to prepare stock solutions. Working solutions of rhein were freshly prepared by further diluting the stock solution in maintenance media immediately before use. DMSO solution (0.1%) was used as control vehicle.

To determine the effect of increased *FTO* level on the expression of gluconeogenic genes and *Atf4* mRNA, cells were subcultured and grown overnight in the regular DMEM/F-12 (1:1) to 60-70% confluency and transfected with an *FTO*-expression vector (pAdTrack-CMV-*FTO*, 1 µg) using TurboFect (Thermo Fisher Scientific, Ottawa, ON). Mock-transfected cells were used as control. Cells were harvested 24 h after transfection.

RNA analysis

Total RNA was extracted from cells using

TRIzol reagent (T9424, Sigma-Aldrich, St Louis, MO) and converted to cDNA using iScript™ Reverse Transcription Supermix (170-8840, Bio-Rad Laboratories, Hercules, CA). The mRNA expression levels were measured by real-time PCR using the ABI 7500 Fast thermal cycler (Applied Biosystems, Foster City, CA) as described previously (17). All primer pairs were designed using Primer Express software (Version 3.0, Applied Biosystems). Primer sequences used in this study were as follows: *FTO* (Accession number: NM_011936) forward: 5'-GACATCGAGACACCAGGATTAACA-3' (exon 4), *FTO* reverse: 5'-GTGAGCCAGCCAAAACACAGT-3' (exon5), *G6pc* (Accession number: NM_008061) forward: 5'-TCTTTCCCATCTGGTTCCATCT-3' (exon 1), *G6pc* reverse: 5'-AATACGGGCGTTGTCCAAAC-3' (exon 2), phosphoenolpyruvate carboxykinase 1 (*Pck1*, Accession number: NM_011044) forward: 5'-GCTGGCCCCGGGAGTCACC-3' (exon 7), *Pck1* reverse: 5'-TGCCGAAGTTGTAGCCGAAGAAGG-3' (exon 10), *Atf4* (Accession number: X61507) forward: 5'-CAGCGTTGGCCTTTGCA-3' (exon 1), *Atf4* reverse: 5'-CGCCAACACTTCGCTGTTTC-3' (exon 2), cyclophilin (Accession number: X52803) forward: 5'-AAGCATACAGGTCCTGGCATCT-3' (exon 4), and cyclophilin reverse: 5'-TGCCATCCAGCCATTTCAGT-3' (exon 4/5). Levels of mRNA were normalized to cyclophilin mRNA levels, and are expressed as means (% of the control group) ± standard error of the mean (S.E.M.). All experiments were performed in triplicates and the coefficient of variation was less than 5% for each triplicate.

Assessment of cytotoxicity

Cytotoxicity of rhein was evaluated by the amount of lactate dehydrogenase (LDH) released from the cytoplasm. In brief, AML12 cells (1.7 x 10⁵ cells in 100 µL medium) were plated in a 96-well culture plate and grown overnight under normal culture condition. Cells were treated with 0.1% DMSO as a vehicle control or rhein (10 nM or 1 µM) for 24 h. The culture plate was centrifuged at 514 x g for 4 min and 50 µL cell-free medium (culture supernatant) was used for LDH assay. Medium LDH levels were measured with the Pierce™ LDH Cytotoxicity Assay Kit (cat no. 88953, Thermo Scientific, Waltham, MA) and absorbance was measured at 490 nm with the background correction at 655 nm using a microplate reader (iMark Absorbance Microplate Reader, Bio-Rad Laboratories). Cytotoxicity(%) was calculated as follows, percent cytotoxicity(%) = [(Experimental

$\text{OD}^{490} - \text{Spontaneous OD}^{490} / (\text{Maximum OD}^{490} - \text{Spontaneous OD}^{490}) \times 100$.

Western blot analysis

Cells were lysed in protein lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol and 1% Triton X-100) supplemented with EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Total protein concentrations were determined by Bradford assay using serial diluted bovine serum albumin as standard. Proteins (12 μg) were separated on 10% stain-free gels (Bio-Rad Laboratories) at 250V and transferred to pre-wet low fluorescent polyvinylidene difluoride membranes (Bio-Rad Laboratories) at 100V for 1.5 h. The blots were rinsed with Tris-buffered

saline (TBS) and images of stain-free blots were acquired using the ChemiDoc MP imager (Bio-Rad Laboratories) to check protein transfer efficiency and quality. After blocking with 5% non-fat milk in 1x TBS with 0.1% Tween 20 (TBST) for 1 h at room temperature, membranes were probed with primary antibodies for *ATF4* (sc-390063, Santa Cruz Biotechnology, Dallas, TX, 1:1,000, mouse monoclonal) or pan-actin (no. 4968, Cell Signaling Technology, Danvers, MA, 1:1,000, rabbit polyclonal) at 4°C overnight. After three washes in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (anti mouse or rabbit) at 1:10,000 dilution in blocking buffer for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence and

Figure 1. Dose-dependent and time-dependent effect of rhein treatment on gluconeogenesis gene expression in AML12 cells. A and B: Cells were treated with rhein (10 fM, 100 pM, 1 μM , 20 μM or 50 μM) for 24 h. DMSO (0.1%)-treated cells were used as control. Levels of G6pc and Pck1 mRNA were measured by real-time PCR. Values in control group were set to 100%. C: Cells were treated with rhein (10 nM or 1 μM) or DMSO for 24 h. Cytotoxicity (%) was assessed by LDH release into the culture medium. D: Cells were treated with rhein (1 μM) or DMSO for 2-24 h. Levels of G6pc mRNA were measured by real-time PCR. Values in control group were set to 100% at each time point. Data are means $\pm$ S.E.M. ($n = 7-12/\text{group}$, 24/group and 10-17/group in A and B, C and D, respectively). *: $P < 0.05$, #: $P < 0.01$, †: $P < 0.005$, ‡: $P < 0.0001$ by one-way ANOVA followed by Dunnett's test (vs. control group, A and B), Student's t-test or Wilcoxon test (D).

Figure 2. Reduced ATF4 and G6pc mRNA and protein levels in rhein-treated AML12 cells. Cells were treated with rhein (100 pM, 10 nM, 1 μ M, 20 μ M or 50 μ M) for 24 h. DMSO (0.1%)-treated cells were used as control. A: Levels of ATF4 and G6pc mRNA were measured by real-time PCR. Values in control group were set to 100%. Data are means $\pm$ S.E.M. (n = 7-14/group). *: P < 0.05, †: P < 0.005, §: P < 0.001, &: P < 0.0005, ¶: P < 0.0001 by one-way ANOVA followed by Dunnett's test. B: Representative images of ATF4 Western blot and stain-free protein blot. C: Quantification data of ATF4 protein normalized to total protein in stain-free blot. Values in control group were set to 100%. Data are means $\pm$ S.E.M. (n = 8-9/group). Groups that do not share the same letter are significantly different (P < 0.05 by Kruskal–Wallis test followed by Wilcoxon test with a Bonferroni correction).

imaged using the ChemiDoc MP imager. The intensity of the bands was quantified by densitometry using the ImageLab software ver. 6 (Bio-Rad Laboratories) and normalized to total protein from stain-free blot for each sample. Blots were stripped after incubation with ATF4 antibody using low pH glycine buffer and reprobed with pan-actin antibody.

Statistical analysis

Data are presented as means $\pm$ S.E.M. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's test (vs. control group). When data were not normally distributed, data were analyzed by Kruskal–Wallis test followed by Wilcoxon test with a Bonferroni correction (vs. control group). Comparisons between two groups were done using Student's t-test (for parametric data) or Wilcoxon test (for nonparametric data). In all cases, differences were taken to be significant if p-values were below 0.05. Outliers were identified by Discordance test and omitted from the analysis.

RESULTS

Dose-dependent and time-dependent effect of rhein treatment on gluconeogenesis gene expression in AML12 cells

Rhein treatment at 100 pM, 1 μ M, 20 μ M and 50 μ M for 24 h significantly reduced levels of G6pc mRNA by 67.2% (P < 0.05), 65.2% (P < 0.0001), 64.6% (P < 0.005) and 84.3% (P < 0.0001), respectively, compared to the control DMSO treatment (Dunnett's test, Fig. 1A). Level of G6pc mRNA was not significantly altered by rhein treatment at a lower concentration (10 fM, P = 0.65). Similarly, rhein treatment at 10 fM (P = 1.00) and 100 pM (P = 0.70) did not cause significant changes in Pck1 mRNA level, while higher concentrations of rhein (1, 20 and 50 μ M) significantly reduced Pck1 mRNA level by 67.6% (P < 0.0001), 46.5% (P < 0.01) and 79.4% (P < 0.001), respectively, compared to the control DMSO treatment (Dunnett's test, Fig. 1B). Rhein treatment at 10 nM and 1 μ M did not significantly increase cytotoxicity compared to the control DMSO treatment as assessed by LDH release into the culture medium in AML12 cells (F [2, 69] = 2.1875, P = 0.1199 by one-way ANOVA, Fig. 1C). Since rhein treatment at 1 μ M produced a marked reduction in G6pc mRNA level without causing a significant cytotoxicity, this dose was used in a time course study. Rhein treatment (1 μ M) caused significant changes in G6pc mRNA level in AML12 cells in a time-dependent manner (H(5) =

Figure 3. Increased Atf4 and G6pc mRNA levels in Fto overexpressing AML12 cells. Cells were transfected with an Fto-expressing plasmid (1 µg) for 24 h (+Fto). Mock transfected cells were used as control. Levels of Fto (A), Atf4 (B) and G6pc (C) mRNA were measured by real-time PCR. Values in control group were set to 100%. Data are means ± S.E.M. (n = 11-15/group). *: P < 0.05, ¶: P < 0.0001 by Student's t-test or Wilcoxon test.

11.68, $P < 0.05$ by Kruskal–Wallis test) with significant reductions after 2-h (52.1% reduction, $P < 0.05$), 12-h (48.1% reduction, $P < 0.01$) and 24-h (59.5% reduction, $P < 0.005$) treatment (Fig. 1D). Rhein treatment for 4 and 6 h at the same concentration did not cause significant changes in *G6pc* mRNA level (4 h: $P = 0.34$, 6 h: $P = 0.51$, Student's t-test, Fig. 1D).

Effect of rhein treatment on Atf4 mRNA and protein expression in AML12 cells

To further determine the effect of *FTO* inhibition on *Atf4* gene and protein expression, AML12 cells were treated with rhein in a separate study. Rhein treatment at 100 pM, 10 nM and 1 µM significantly lowered levels of *Atf4* mRNA as well as *G6pc* mRNA in AML12 cells compared to the control treatment (Fig. 2A). There was a significant positive correlation between *Atf4* mRNA and *G6pc* mRNA levels ($r = 0.3487$, $P < 0.05$). Western blot analysis showed that *ATF4* protein levels were significantly reduced by rhein treatment (20 µM, Fig. 2B and C). Since rhein treatment significantly altered levels of pan-actin ($F[3, 8] = 5.2260$, $P < 0.05$ by one-way ANOVA), total protein for each sample from the stain-free blot images was used to normalize the intensity of the *ATF4* protein bands (Fig. 2B).

Effect of enhanced FTO expression on Atf4 mRNA expression in AML12 cells

To increase *FTO* level, AML12 cells were transfected with an *FTO*-expression vector for 24 h. *FTO* mRNA levels were significantly increased 24 h after transfection of the *FTO*-expressing vector compared to the control mock transfection ($P < 0.0001$ by Wilcoxon test, Fig. 3A). We have shown that this protocol produced a significant 143.9% increase of *FTO* protein level in AML12 cells in the earlier study (7). Levels of *Atf4* and *G6pc* mRNA were significantly increased by 58.3% and 119.3%, respectively, in *FTO*-overexpressing cells compared to the control cells ($P < 0.05$, Student's t-test, Fig. 3B and C).

DISCUSSION

There is increasing evidence to support the role for *FTO* in the regulation of gluconeogenesis. However, the mechanism by which *FTO* regulates gluconeogenesis is not fully understood. It has been suggested that the regulation of gene expression by *FTO* is mediated by transcription factors such as forkhead box protein O1 (FOXO1), a major transcription factor

controlling gluconeogenic gene expression (8,9,18). The transcription factor *ATF4* belongs to the family of basic zipper-containing proteins and is a positive regulator of gluconeogenic gene expression. Levels of gluconeogenic genes and proteins were increased by enhanced *Atf4* expression in primary mouse hepatocytes, while they were reduced by *Atf4* knockdown (13). Complete absence of *Atf4* resulted in improved glucose and pyruvate tolerance and reduced *G6pc* and *Pck1* mRNA levels and fasting blood glucose in mice (10,19). Both hepatic *Atf4* and extra-hepatic (i.e. osteoblastic) *Atf4* contribute to these beneficial effects (11,13). Intriguingly, levels of *ATF4* protein are elevated in the liver of transgenic mice in which *FTO* expression is selectively increased in the liver (14). In the present study, we demonstrated that *FTO* inhibition caused a reduction of *Atf4* mRNA and protein levels in hepatocyte cells, while enhanced *FTO* expression led to an increase in *Atf4* mRNA expression. These changes were paralleled by changes in gluconeogenic *G6pc* mRNA expression. These data support the hypothesis that hepatic *ATF4* mediates the effect of *FTO* on glucose metabolism by modulating gluconeogenesis.

The mechanism by which *ATF4* mediates *FTO*-induced gluconeogenic gene expression is currently unknown. Probable mechanisms involve the function of *FTO* as a nucleic acid demethylase. *FTO* is a Fe(II)- and 2-oxoglutarate-dependent dioxygenase that catalyzes demethylation of 3-methylthymine (m³T) in single-stranded DNA and 3-methyluracil (m³U) and 6-methyladenosine (m⁶A) in single-stranded RNA. To support the role of *FTO* in regulating *Atf4* expression, there are 18 putative m⁶A sites with the consensus sequence RRACH in *Atf4* mRNA (Genbank accession no: X61507). Consequently, *FTO* may promote demethylation at m⁶A sites on *Atf4* mRNA, leading to an increased expression of *Atf4* and its target genes such as gluconeogenic genes.

Secondly, a cooperative interaction between *ATF4* and FOXO1 affects glucose metabolism. FOXO1 co-localizes with *ATF4*, physically interacts with *ATF4*, and promotes transcriptional activation of *ATF4* (20). Although heterozygous null mutation in the *FoxO1* gene alone or the *Atf4* gene alone did not affect blood glucose level, double heterozygous resulted in reduced blood glucose level in mice (20). Thus, it is probable that *FoxO1* and *ATF4* regulates glucose metabolism in a synergistic manner by mediating *FTO*-induced gluconeogenesis.

Thirdly, ubiquitin-specific peptidase 14 (USP14) may be involved in the regulation of hepatic

gluconeogenic gene expression by *FTO* and *ATF4*. USP14 is the deubiquitinating enzyme and stabilizes CREB to potentiate glucagon action, thereby promoting gluconeogenesis. Increased *Usp14* expression in the liver led to increases in gluconeogenic gene expression and blood glucose in mice. Overexpression of hepatic *Usp14* also caused impairments in glucose and pyruvate tolerance in mice, while hepatic *Usp14* knockdown produced an opposite effect in high fat diet-induced obese mice (21). Enhanced *Atf4* expression increases *Usp14* promoter activity and levels of *Usp14* mRNA and protein in mouse primary hepatocytes as well as mouse liver (21). Moreover, enhanced *FTO* expression promotes phosphorylation of CREB (18). Collectively, these findings suggest the possibility that USP14 mediates stimulatory effects of the *FTO-ATF4* pathway on CREB-mediated gluconeogenic gene expression.

In conclusion, present findings suggest that hepatic *FTO* promotes gluconeogenesis by suppressing *ATF4* expression. Abnormally increased activity of the *FTO-ATF4* pathway may contribute to the increased hepatic glucose production, while the inhibition of this pathway (i.e. *FTO* inhibitor treatment) may be beneficial in suppressing hepatic gluconeogenesis and ameliorating hyperglycemia in diabetes. The present study opens a new avenue of research to determine whether hepatic *FTO-ATF4* pathway is a viable target for diabetes treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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