ATF4 Protein Deficiency Protects against High Fructose-induced Hypertriglyceridemia in Mice*

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Background: Hypertriglyceridemia is the most common lipid disorder with incompletely understood mechanisms.

Results: ATF4 deficiency attenuates lipogenesis in the liver and protects against high fructose-induced hypertriglyceridemia in mice.

Conclusion: ATF4 plays a pivotal role in regulating hepatic lipid metabolism.

Significance: ATF4 is a contributing factor for the pathogenesis of hypertriglyceridemia.

Hypertriglyceridemia is the most common lipid disorder in obesity and type 2 diabetes. It results from increased production and/or decreased clearance of triglyceride-rich lipoproteins. To better understand the pathophysiology of hypertriglyceridemia, we studied hepatic regulation of triglyceride metabolism by the activating transcription factor 4 (ATF4), a member of the basic leucine zipper-containing protein subfamily. We determined the effect of ATF4 on hepatic lipid metabolism in Atf4−/− mice fed regular chow or provided with free access to fructose drinking water. ATF4 depletion preferentially attenuated hepatic lipogenesis without affecting hepatic triglyceride production and fatty acid oxidation. This effect prevented excessive fat accumulation in the liver of Atf4−/− mice, when compared with wild-type littermates. To gain insight into the underlying mechanism, we showed that ATF4 depletion resulted in a significant reduction in hepatic expression of periloxime proliferator-ac- tivated receptor-γ, a nuclear receptor that acts to promote lipogenesis in the liver. This effect was accompanied by a significant reduction in hepatic expression of sterol regulatory element-binding protein 1c (SREBP-1c), acetyl-CoA carboxylase, and fatty-acid synthase, three key functions in the lipogenic pathway in Atf4−/− mice. Of particular significance, we found that Atf4−/− mice, as opposed to wild-type littermates, were protected against the development of steatosis and hypertriglyceridermia in response to high fructose feeding. These data demonstrate that ATF4 plays a critical role in regulating hepatic lipid metabolism in response to nutritional cues.

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4 The abbreviations used are: TG, triglyceride; ATF4, activating transcription factor 4; MTP, microsomal triglyceride transfer protein; apoB, apolipoprotein B; PPPR, periloxime proliferator-activated receptor; SREBP-1c, sterol regulatory element-binding protein 1c; FAS, fatty-acid synthase; ACC, acetyl-CoA carboxylase; CPT1, carnitine palmitoyltransferase 1; ACOX1, acyl-coenzyme A oxidase 1; qRT, quantitative RT; HBSS, Hanks’ buffered saline solution; C/EBP, CCAAT/enhancer-binding protein; CARE, C/EBP-ATF4-response element.
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**Experimental Procedures**

**Animal Studies**—Atf4+/− heterozygous mice were bred for generating homozygous Atf4 null (Atf4−/−) mice and wild-type (WT) littermates (Atf4+/+) in Black Swiss background, as described (18). Mice were fed regular rodent chow and water ad libitum in sterile cages with a 12-h light/dark cycle. For fructose feeding, mice were fed regular chow with free access to drinking water containing 30% (w/v) fructose. For blood chemistry, mice were fasted for 16 h and tail vein blood samples were collected into capillary tubes pre-coated with potassium/EDTA (Sarstedt, Numbrecht, Germany). The study was performed in male Atf4−/− and sex/age-matched wild-type littermates. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh.

**VLDL-TG Production Assay**—Mice were fasted for 16 h, followed by intravenous injection of tyloxapol (0.5 g/kg, Sigma) to inhibit systemic TG clearance. Aliquots of tail vein blood were taken at different times for determining plasma TG levels. VLDL-TG production rates were defined as the amount of hepatic TG produced per unit time, as described (35).

**Fat Tolerance Test**—Mice were fasted for 16 h, followed by an oral bolus of olive oil (10 μl/g). Aliquots of blood (25 μl) were taken from the tail vein at different times for determining plasma TG and cholesterol levels, as described (35, 36).

**Glucose Tolerance Test**—Mice were fasted for 5 h, followed by intraperitoneal injection of glucose (2 g/kg). Blood glucose levels were measured prior to and at different times after glucose injection.

**Insulin Tolerance Test**—Mice were injected intraperitoneally with recombinant regular human insulin (Lilly) at the dose of 0.75 units/kg body weight, followed by the determination of blood glucose levels at different times.

**RNA Isolation and Real Time qRT-PCR**—RNA isolation from liver (20 mg) was performed using the RNeasy mini kit (Qiagen, Valencia, CA). Real time qRT-PCR was used for quantifying mRNA concentrations using the LightCycler-RNA amplification kit (Roche Diagnostics), as described (35). The primers used are as follows: Acox1 forward 5′-TCCCGATCTTCGCAAGGAGC-3′ and reverse 5′-CTGGTGAAGCAGGTGGGACA-3′; Ppar-α forward 5′-CCTGAACTCGAGTGGAGATT-3′ and reverse 5′-GGTCTTCTCTGCAATCTCGAC-3′; Sreb-1c forward 5′-GGAGCCATGGATGAGCG-3′ and reverse 5′-GCTTCCAGAGAGGAGGCA-3′; Cpt1 forward 5′-GACCTGCAGCTGCAATAC-3′ and reverse 5′-CTCACAGACGTAACCCTCTGAG-3′; Acc forward 5′-TGCAGACGTACCGAGAA-3′ and reverse 5′-GGGAGGCCCCAGCAACA-3′; Fas forward 5′-GCTGCCGAAAACCTCAGGAAT-3′ and reverse 5′-AGAGACGTGTACCTCCTCGGAC-3′; ApoB forward 5′-TCGGCAGGCCCTTACTGCTG-3′ and reverse 5′-CAGATTGGGGGACCCTCAG-3′; Mttp forward 5′-TCCAGGGTGTGGTCTAGCTAT-3′ and reverse 5′-CCTTGGCTTGCATCACTG-3′; Ppar-γ forward 5′-GGCATCGCTGTGAGCAGC-3′ and reverse 5′-GACCGCTTGTGAAGGACG-3′; and β-actin forward 5′-GGGAATGTGACGACAGCT-3′ and reverse 5′-GTCACTGTGCCTGAAATC-3′. All primers were obtained commercially from Integrated DNA Technologies (Coralville, IA). β-Actin mRNA was used as control for normalizing hepatic mRNA levels.

**Hepatic mRNA Superarray**—Aliquots of total RNA (5 μg) isolated from liver tissues were subjected to Superarray analysis, using the ABI 7900 HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA). This assay allows simultaneous determination of 96 mRNA species in a given sample. We used the mouse insulin signaling and carbohydrate metabolism pathway (PAMM-030, BABioscience, Valencia, CA) according to the manufacturer’s instructions for quantifying mRNA levels of genes in insulin action and glucose and lipid metabolism in the liver.

**FPLC Fractionation of Lipoproteins**—Aliquots (400 μl) of plasma pooled from Atf4−/− mice and WT littermates were applied to two head-to-tail linked Tricorn high performance Superose S-6 10/300GL columns using an FPLC system (GE Healthcare), followed by elution with PBS at a constant flow rate of 0.25 ml/min. Fractions (500 μl) were eluted for determining TG and cholesterol levels, as described (35, 36).

**Hepatic Lipid Content**—Aliquots of liver tissue (20 mg) were homogenized in 400-μl HPLC-grade acetone. After incubation with agitation at room temperature overnight, aliquots (50 μl)
of acetone/extract lipid suspension were used for the determination of triglyceride concentrations using the Infinity triglyceride reagent (Thermo Electron). Hepatic lipid content was defined as milligrams of triglyceride/g of total liver proteins, as described (35, 37).

Cell Culture—HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Walkersville, MD) and were transduced with Adv-ATF4 or control Adv-Empty vector as described (38). All adenoviral vectors were produced in HEK293 cells, as described (36).

Mouse Primary Hepatocytes—Mouse primary hepatocytes were isolated from C57BL/6 mice using the protocol, as described (38). The liver was infused in situ through the inferior vena cava first with 10 ml of HBSS supplemented with EGTA (1 mM), then with 10 ml of EGTA-free HBSS, and finally with 10 ml of HBSS supplemented with collagenase, type V (1.95 mg/ml, Sigma). The liver was harvested, minced, and incubated at 37 °C for 10 min. The cells were dispersed in HBSS, filtered through a 70-μm nylon mesh, and washed three times with HBSS. Hepatocytes were plated in collagen-coated 12-well plates at 2 × 10^5 cells/well and cultured in hepatocyte maintenance medium (Lonza) supplemented with dexamethasone, insulin, and GA-1000 according to the manufacturer’s instructions (Lonza), as described (38). For adenovirus transduction, primary hepatocytes were incubated with Adv-ATF4 or control Adv-Empty vector at a predefined dose (200 pfu/cell). After 24 h of incubation, primary hepatocytes and conditioned medium were collected for analysis.

Immunoblot Assay—Primary hepatocytes (~1 × 10^5 cells) were lysed in 100 μl of M-PER (Pierce) containing 2 μl of Halt Protease Inhibitor Mixture (Pierce). Protein extracts were obtained by centrifugation at 13,000 rpm for 10 min. To obtain protein extracts from liver tissue, 20 mg of liver tissue were homogenized in 400 μl of M-PER supplemented with 4 μl of Halt Protease Inhibitor Mixture (Pierce), followed by centrifugation at 13,000 rpm for 10 min. Aliquots of proteins (20 μg) were resolved on 4–20% SDS-polyacrylamide gels and subjected to immunoblot analysis. Proteins were blotted onto Immun-Blot PVDF membrane (Bio-Rad) and subjected to Western blot assay. Antibodies used in immunoblot analysis were anti-SREBP-1c (sc-8984, Santa Cruz Biotechnology), anti-PPAR-γ (sc-7196, Santa Cruz Biotechnology), anti-FAS (ab128870, Abcam), anti-ACC (ab63531, Abcam), anti-PPAR-α (sc-9000, Santa Cruz Biotechnology), anti-CPT1 (ab87498, Abcam), and anti-ACOX1 (ab59964, Abcam). Control anti-actin IgG has been described (38).

Statistics—Statistical analyses of data were performed by analysis of variance using StatView software (Abacus Concepts). Analysis of variance post hoc tests were performed to study the significance between different conditions. Data were expressed as the mean ± S.E. p values <0.05 were considered statistically significant.

RESULTS

ATF4 Deficiency Improves Plasma Lipid Metabolism in Mice—To characterize the role of ATF4 in lipid metabolism, we bred Atf4+/− heterozygous mice for generating knock-out mice (Atf4−/−). Atf4−/− mice, as opposed to age/sex-matched wild-type littermates (WT), exhibited significantly lower plasma TG

### Table 1

**Plasma lipid profiles in Atf4−/− and WT mice**

|                | Regular chow | High fructose |
|----------------|--------------|---------------|
| **WT**         |              |               |
| TG (mg/dl)     | 130 ± 10     | 359 ± 25      |
| Cholesterol (mg/dl) | 296 ± 5 | 211 ± 9        |
| NEFA (mEq/liter) | 1.37 ± 0.06  | 0.55 ± 0.06   |
| **Atf4−/−**    |              |               |
| TG (mg/dl)     | 60 ± 6a      | 162 ± 11b     |
| Cholesterol (mg/dl) | 233 ± 6 | 165 ± 4b      |
| NEFA (mEq/liter) | 1.39 ± 0.16  | 0.37 ± 0.05a  |

*p < 0.01.  **p < 0.001 versus WT control.

**FIGURE 1. Growth curve and plasma lipoprotein profiles. A, body weight. B, plasma TG levels. C, plasma cholesterol levels. Aliquots of plasma (500 μl) pooled from individual mice from both groups (n = 7 per group, 15 months old) were subjected to FPLC-aided gel filtration chromatography for the fractionation of VLDL, LDL, and HDL particles, followed by the determination of plasma TG and cholesterol concentrations in different fractions. ***, p < 0.001 versus WT control.
and cholesterol levels, without significant alterations in plasma nonesterified fatty acid levels (Table 1). We monitored the growth of Atf4−/− mice and age/sex-matched WT littermates (n = 7 per group) from birth to 15 months. Atf4−/− mice, as opposed to WT littermates, exhibited growth retardation (Fig. 1A). This improvement in TG metabolism was reproduced in Atf4−/− mice at 12 and 15 months of age. To corroborate these findings, we determined plasma lipoprotein profiles, demonstrating that Atf4−/− mice had lower plasma VLDL-TG (Fig. 1B) and LDL-cholesterol levels (Fig. 1C). In contrast, HDL-cholesterol levels remained unchanged in Atf4−/− mice versus WT littermates (Fig. 1C).

A significant reduction in plasma TG levels could arise from increased systemic TG clearance and/or decreased VLDL-TG secretion from the liver. To distinguish these possibilities, we treated mice with tyloxapol, an antagonist of lipoprotein lipase, to block systemic TG hydrolysis, followed by the determination of hepatic VLDL-TG output. As expected, plasma TG levels increased with time following tyloxapol administration in Atf4−/− and WT control mice (Fig. 2A). However, the rate of VLDL-TG production, defined as plasma TG increment per unit time, remained unchanged in Atf4−/− versus WT groups (Fig. 2B).

To account for the underlying physiology of improved TG metabolism in Atf4−/− mice, we performed a fat tolerance test.
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Mice were orally gavaged with a bolus of olive oil, followed by the determination of plasma TG levels. In contrast to WT littermates, Atf4−/− mice exhibited significantly improved plasma TG profiles during the fat tolerance test (Fig. 2C). Furthermore, Atf4−/− mice had significantly reduced epididymal fat mass (Fig. 2D). This effect persisted even after normalizing to body weight (Fig. 2E and F). Atf4−/− mice, as opposed to WT littermates, had relatively higher food intake after normalizing to body weight (Fig. 2G).

As control, we determined the impact of ATF4 on glucose metabolism. ATF4 depletion resulted in a significant reduction in blood glucose levels under both fed (Fig. 2H) and fasting (Fig. 2I) conditions, accompanied by enhanced glucose tolerance (Fig. 2J) and whole-body insulin sensitivity (Fig. 2K) in Atf4−/− mice versus WT controls.

**TABLE 2**

Hepatic genes whose expression is differentially regulated in Atf4−/− mice

| Gene symbol | Up- or down-regulation | Fold regulation | p value |
|-------------|------------------------|----------------|--------|
| Cbl         | ††                      | 1.5179         | 0.0382 |
| G6pc        | ††                      | 2.4914         | 0.0131 |
| Igf1r       | ††                      | 1.8771         | 0.0319 |
| Igfbp1      | ††                      | 7.9187         | 0.0048 |
| Ins2        | ††                      | 2.1361         | 0.0167 |
| Prkcz       | ††                      | 1.6961         | 0.0173 |
| Gusb        | ††                      | 1.5024         | 0.0039 |
| Ppary       | ††                      | −2.0634        | 0.0081 |
| Cfd         | ††                      | −6.8764        | 0.0358 |
| Cebpa       | ††                      | −1.8303        | 0.0002 |
| Dusp14      | ††                      | −3.6699        | 0.0031 |
| Gpd1        | ††                      | −2.4333        | 0.0016 |

We then determined the effect of ATF4 depletion on hepatic expression of MTP and apolipoprotein B (apoB), two key functions in hepatic VLDL-TG production. ApoB is the core component of VLDL particles. MTP catalyzes the rate-limiting step in transferring lipid to nascent apoB polypeptides for VLDL-TG assembly (12, 39–41). We did not detect significant differences in hepatic mRNA levels of Mttp (Fig. 3D) and ApoB (Fig. 3E), consistent with the lack of alterations in hepatic VLDL-TG production rates in Atf4−/− mice versus WT littermates (Fig. 2, A and B).

Furthermore, we determined the impact of ATF4 depletion on hepatic expression of key enzymes in fatty acid oxidation, including peroxisome Ppar-α, and its two downstream targets Cpt1 and Acox1. We detected about 30% reduction in hepatic Ppar-α (Fig. 3F) and Acox1 levels (Fig. 3G) in Atf4−/− mice, although this reduction did not reach a significant level, when compared with WT control. In contrast, hepatic Cpt1 mRNA levels remained unchanged in Atf4−/− versus control mice (Fig. 3H). These data indicate that ATF4 depletion impacts hepatic lipid metabolism preferentially by attenuating lipogenesis in the liver.

![FIGURE 3. Effect of ATF4 depletion on hepatic gene expression and lipid content.](image-url)
To support this interpretation, we determined hepatic TG content, demonstrating that Atf4/H11002 mice had significantly lower fat content in the liver (Fig. 3). Furthermore, we quantified hepatic expression levels of Ppar-γ, a nuclear receptor whose activation is linked to enhanced lipid synthesis and increased fat storage in the liver (42–44). We detected a significant reduction in hepatic Ppar-γ expression (Fig. 3), in keeping with attenuated lipogenesis and decreased lipid content in the liver of Atf4/H11002 mice.

To provide further mechanistic underpinning for altered hepatic lipid metabolism in Atf4/H11002 mice, we subjected liver tissues to mRNA Superarray analysis for determining hepatic expression of genes in insulin signaling and carbohydrate metabolism. This assay revealed a 2-fold reduction in hepatic Ppar-γ mRNA abundance in the liver of Atf4/H11002 mice (Table 2), consistent with the reduction of hepatic fat content in Atf4/H11002 mice versus WT littermates (Fig. 3).

Atf4/H11002 Mice Are Protected against Fructose-induced Hypertriglyceridemia—Excessive fructose consumption is known to induce lipid disorder, culminating in the development of hyperlipidemia and hepatic steatosis in both humans and rodents (29–34). Based on the above findings, we hypothesized that ATF4 deficiency would protect mice from developing hypertriglyceridemia in response to fructose feeding. To test this hypothesis, we provided Atf4/H11002 mice and WT littermates with free access to drinking water containing 30% fructose. Fructose drinking for 8 weeks resulted in hypertriglyceridemia, accompanied by elevated plasma cholesterol levels in WT mice (Table 1). In contrast, Atf4/H11002 mice maintained significantly lower plasma levels of TG and cholesterol after 8 weeks of fructose drinking (Table 1). To corroborate these findings, we subjected plasma of Atf4/H11002 and WT mice to FPLC-mediated gel filtration chromatography for the fractionation of lipoproteins. We showed that Atf4/H11002 mice exhibited markedly lower VLDL-TG levels (Fig. 4A) with concomitant reduction in LDL cholesterol and HDL cholesterol levels (Fig. 4B). Atf4/H11002 mice also displayed significantly lower nonesterified fatty acid levels, consistent with their improved lipid profiles in response to fructose consumption (Table 1).

Atf4/H11002 Mice Stave Off Fructose-induced Steatosis—To determine the potential beneficial effect of ATF4 depletion on hepatic steatosis, we quantified hepatic lipid content following 8 weeks of fructose drinking. Atf4/H11002 mice, as opposed to WT littermates, had significantly reduced hepatic TG content (Fig. 4C). Furthermore, Atf4/H11002 mice had significantly reduced fat mass (Fig. 4D), accompanied by less weight gain (6.6 ± 0.3 g) relative to WT littermates (10.5 ± 0.5 g, p < 0.01) during the 8-week high fructose feeding. This effect on fat mass was maintained after normalizing to body weight (Fig. 4, E and F).
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To support these findings, we determined the effect of ATF4 depletion on hepatic expression of genes in lipogenesis **versus** fatty acid oxidation in response to fructose drinking. **Atf4**/−/− mice were associated with attenuated hepatic lipogenesis, culminating in a significant reduction in hepatic **Ppar-γ** expression (Fig. 5A). Hepatic expression of **Srebp-1c** (Fig. 5B), **Acc** (Fig. 5C), and **Fas** (Fig. 5D) mRNA levels was also reduced in **Atf4**/−/− mice, but the degree of reduction did not reach significant levels, when compared with WT littermates. Instead, we detected a significant reduction in hepatic mRNA levels of **Ppar-α** (Fig. 5E), **Cpt1** (Fig. 5F), and **Acox1** (Fig. 5G), three key functions in fatty acid oxidation, correlating with the reduction of hepatic fat content (Fig. 4C) and plasma nonesterified fatty acid levels (Table 1) in **Atf4**/−/− mice. Hepatic expression of **MTP** (Fig. 5H) and apoB (Fig. 5I), two enzymes catalyzing the rate-limiting step of VLDL-TG production in the liver, remained unchanged in **Atf4**/−/− mice **versus** WT littermates.

To provide further evidence that hepatic ATF4 depletion attenuated lipogenesis and protected against high fructose-induced steatosis, we determined hepatic abundance of key proteins in lipogenesis **versus** fatty acid oxidation pathways. We showed that hepatic levels of lipogenic proteins **Ppar-γ** (Fig. 6A), **Fas** (Fig. 6B), and **Acc** (Fig. 6C) were significantly reduced, consistent with reduced fat accumulation in the livers of **Atf4**/−/− mice (Fig. 4C). In contrast, **Srebp-1c** nuclear protein levels remained unchanged (Fig. 6D). Hepatic **Ppar-α** protein levels were also reduced in **Atf4**/−/− mice (Fig. 6E), although the reduction did not reach a significant level in comparison with WT controls. We did not detect significant differences in hepatic **Acox1** (Fig. 6F) and **Cpt-1** (Fig. 6G) protein levels in **Atf4**/−/− mice **versus** WT littermates.

**Effect of ATF4 Gain-of-Function on Hepatic Lipid Metabolism in Mouse Primary Hepatocytes**—To provide further physiological underpinning for the hypothesis that ATF4 is pivotal for regulating hepatic lipid metabolism, we determined the effect of ATF4 gain-of-function on hepatic lipogenesis. We postulated that ATF4 would stimulate lipogenesis and instigate hepatic steatosis. To test this hypothesis, we prepared primary mouse hepatocytes, which were subsequently transduced with Adv-ATF4 at a moderate dose for elevating hepatic ATF4 expression levels by 2–3-fold, when compared with Adv-empty control vector-treated hepatocytes. We detected a significant elevation of TG in both cells (Fig. 7A) and conditioned medium (Fig. 7B) of primary hepatocytes that were pretreated with Adv-ATF4 vector. This effect correlated with the induction of hepatic lipogenesis, as evidenced by significantly increased **Srebp-1c**, **Acc**, and **Fas** mRNA levels in primary hepatocytes with elevated ATF4 expression (Fig. 7C). In contrast, hepatic mRNA levels of **Ppar-α**, **Cpt-1**, and **Acox1** in fatty acid oxidation remained unchanged (Fig. 7C). Likewise, hepatic ATF4 production did not result in significant changes in **Ppar-γ** mRNA expression in primary hepatocytes (Fig. 7C).

To corroborate these findings, we subjected ATF4 and control vector-treated primary hepatocytes to immunoblot analysis. We confirmed that adenovirus-mediated ATF4 production resulted in a significant induction in **Srebp-1c** (Fig. 7D), **Fas** (Fig.
In this study, we characterized the role of ATF4 in hepatic lipid metabolism in Atf4−/− mice. We showed that Atf4−/− mice, as opposed to wild-type littermates, exhibited significantly reduced fasting plasma TG and VLDL-TG levels. Atf4−/− mice also had significantly improved postprandial TG profiles during fat tolerance. In response to high fructose feeding, Atf4−/− mice were protected against the development of hypertriglyceridemia. To gain insight into the underlying mechanism, we showed that ATF4 depletion resulted in a significant reduction in hepatic expression of lipogenic genes, without significantly affecting the expression of hepatic genes that are involved in fatty acid oxidation or VLDL-TG production in the liver. This effect contributed to the attenuation of hepatic lipogenesis and prevention of excessive fat infiltration in the liver of Atf4−/− mice. We recapitulated this finding in Atf4−/− mice under both regular chow and high fructose feeding conditions. In keeping with our finding, two independent
studies demonstrate that ATF4 deficiency protects mice from developing steatosis in response to high fat feeding (24, 25). Furthermore, we showed that ATF4 gain-of-function was associated with increased TG synthesis and secretion secondary to augmented lipogenesis in primary hepatocytes of C57BL/6J mice. Together these data characterize ATF4 as a significant factor for regulating hepatic lipid metabolism in response to nutritional cues.

The underlying mechanism by which ATF4 loss-of-function curbs hepatic lipogenesis and improves lipid metabolism is presently unknown. It is plausible that this observed improvement in lipid metabolism is secondary to enhanced glucose catabolism in Atf4−/− mice. Implicit in this assertion is that ATF4 exerts a direct impact on glucose metabolism via osteocalcin, a bone hormone that is secreted specifically from osteoblasts (22). ATF4 acts in cooperation with the forkhead transcription factor FoxO1 to stimulate osteoblast expression of embryonic stem cell phosphatase (Esp), an enzyme that catalyzes osteocalcin carboxylation, resulting in inactivation of osteocalcin (22, 45). As a result, ATF4 loss-of-function is asso-

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**FIGURE 7. Hepatic protein expression in primary hepatocytes.** Primary hepatocytes of C57BL/6J mice were transduced in culture with Adv-ATF4 (ATF4) and control Adv-Empty (Control) vectors at a predefined dose (200 pfu/cell). Each condition was run in triplicate. After a 24-h incubation, both hepatocytes and conditioned medium were harvested for determining TG concentration in cells (A) and medium (B). In addition, aliquots of ATF4 and control hepatocytes were subjected to real-time qRT-PCR assay for determining hepatic mRNA expression. Hepatic mRNA levels, after normalizing to β-actin mRNA levels, were compared between control and ATF4 groups (C). Other aliquots of cells were subjected to Western blot analysis for determining hepatic protein levels of Srebp-1c (D), Fas (E), Acc (F), and Ppar-γ (G), using actin protein as control. *, *p < 0.05 and **, *p < 0.001 versus control.
associated with enhanced insulin secretion from \(\beta\)-cells, contributing to a significant improvement in both fasting and postprandial glucose profiles in \(\text{Atf4}^{-/-}\) mice (22). We recapitulated these findings, demonstrating that ATF4-deficient mice exhibited significantly lower fasting blood glucose levels and enhanced glucose tolerance. Despite having relatively higher food intake, \(\text{Atf4}^{-/-}\) mice, as opposed to WT littermates, are lean, in accordance with enhanced energy expenditure (22, 24). ATF4 depletion seems to enhance insulin sensitivity in the liver (26). The improvement in glucose homeostasis along with enhanced hepatic insulin sensitivity contributes to improved lipid metabolism in \(\text{Atf4}^{-/-}\) mice (23, 25).

As an alternative mechanism, ATF4 exerts a direct effect on hepatic lipid metabolism. In support of this mechanism, we showed that ATF4 depletion curbs hepatic expression of \(\text{Ppar-\gamma}\), a nuclear receptor that functions in complex with retinoid X receptor to promote lipogenesis in the liver (42, 46–48). Hepatic \(\text{Ppar-\gamma}\) expression is up-regulated in mice with hepatic steatosis (44, 49) or dietary obesity (50–52). This observation is recapitulated in humans, as obese patients with hepatosteatosis exhibited markedly increased \(\text{Ppar-\gamma}\) activity in the liver (53, 54). Conversely, mice with liver-specific \(\text{Ppar-\gamma}\) deletion are refractory to fat-induced steatosis, due to the reduction of lipogenesis in the liver (42, 47). Thus, ATF4 may act through \(\text{Ppar-\gamma}\) to modulate hepatic lipid metabolism in ATF4-deficient mice. Consistent with this notion, we showed that hepatic \(\text{Ppar-\gamma}\)-expression was significantly decreased, coinciding with the attenuation of hepatic lipogenesis and reduction of hepatic fat infiltration in \(\text{Atf4}^{-/-}\) mice under both regular chow and high fructose feeding conditions.

Nonetheless, ATF4 gain-of-function did not seem to impact \(\text{Ppar-\gamma}\)-expression in mouse primary hepatocytes, despite the effect that ATF4 stimulated lipogenesis, contributing to increased TG synthesis and secretion. This suggests that ATF4 alone is not sufficient to promote hepatic \(\text{Ppar-\gamma}\)-expression, or alternatively, ATF4-mediated effect on \(\text{Ppar-\gamma}\)-expression is through an indirect mechanism. It is noteworthy that ATF4 does not bind to DNA alone. Instead, ATF4 binds together with \(\text{CCAT/enhancer-binding protein (C/EBP)}\) to the \(\text{C/EBP-ATF4-response element (CARE)}\) in target promoters (13). ATF4 binds to one-half of the CARE motif, and the other half of the CARE is bound by C/EBP. Although ATF4 acts in concert with C/EBP to enhance the transcription of target genes (13, 55), it is unknown how ATF4 cooperates with C/EBP for binding at the cognate site and for trans-activating promoter activity, nor is it clear whether C/EBP binding facilitates the recruitment of ATF4 at the CARE site or whether the occupancy of the CARE by both ATF4 and C/EBP is necessary for enhancing promoter activity. A recent study shows that ATF4 interacts with the forkhead transcription factor FoxO1 in regulating osteocalcin expression in osteoblasts (45). Further studies are needed to determine the molecular interplay of ATF4 with C/EBP or FoxO1 in regulating hepatic lipid metabolism.

In conclusion, we showed that ATF4 depletion diminished hepatic lipogenesis with little impact on hepatic VLDL-TG production or fatty acid oxidation. This effect prevented excessive fat accumulation in the liver and protected against the development of hypertriglyceridemia in fructose-fed ATF4-deficient mice. Our data characterize ATF4 as a contributing factor for hypertriglyceridemia. Further investigation is warranted to know whether ATF4 is a potential therapeutic target for treating hypertriglyceridemia in type 2 diabetes.

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