Hepatic FTO expression is increased in NASH and its silencing attenuates palmitic acid-induced lipotoxicity


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1. Introduction

Genome-wide association studies (GWAS) have identified variants of the FTO gene that appear to be correlated with obesity in humans [1]. In animal models, transgenic overexpression of FTO leads to obesity [2] whereas loss of function mutations of FTO decreased body weight [3]. Furthermore, FTO is increased in muscle of type 2 diabetes patients, and its overexpression in myotubes alters insulin signaling, and induces mitochondrial dysfunction [4], suggesting that FTO may have an etiological role in metabolic disorders. FTO belongs to the AlkB family of enzymes and is an iron and 2-oxoglutarate-dependent dioxygenase that specifically demethylates N6-methyladenosine (m6A) in mRNA [5–7]. Recently, selective FTO inhibitors have been developed that block its enzymatic activity [8], and thus serve as potential treatments for metabolic disorders that have dysregulation of FTO.

Non-alcoholic fatty liver disease (NAFLD) is a common co-morbidity associated with obesity, and is represented by a clinical spectrum that includes hepatosteatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis. The severity of NASH is characterized by histological criteria such as hepatocyte ballooning, inflammation, focal fibrosis, and steatosis [9]. Advanced NASH is a serious liver condition since the hepatocyte apoptosis and ensuing fibrosis usually are not reversible. The prevalence of NASH varies depending on the population and geographical location, but it is estimated to occur in more than 15% of obese individuals with other features of the metabolic syndrome [9]. Currently, there is no FDA-approved drug for NASH.

Lipotoxicity, from saturated free fatty acids such as palmitic acid (PA), is a major pathological feature of NASH. It is caused by the accumulation of toxic lipid intermediates in the liver that leads to cellular dysfunction and death [9]. At a cellular level, PA induces reactive oxygen species (ROS) that cause lipid peroxidation and stress in mitochondria and endoplasmic reticulum (ER). PA also
serves as a precursor for the generation of ceramides that can alter the membrane compositions of organelles to induce intracellular stress pathways [10], leading to mitochondrial dysfunction, activation of the caspase cascade, and cell death [11].

In this study, we have demonstrated that the hepatic FTO levels are significantly increased in both human NASH patients and a diet-induced rodent model of NASH. Furthermore, we demonstrated that depletion of FTO decreased PA-induced lipotoxicity in hepatic cells by restoring mitochondrial function and reducing ER stress.

2. Materials and methods

2.1. Microarray analysis

For analysis of FTO expression in human NASH and steatosis, a publically available database was used [12]. The data set is accessible at the ArrayExpress public repository for microarray data under the accession number E-MEXP-3291 (http://www.webcitation.org/5zyojNu7T). The distribution of FTO gene expression in Control, NASH, and steatotic samples was ascertained via boxplots, and the statistical significance of expression differences across the three groups was determined via ANOVA. All calculations were performed in the statistical package, R 3.2.3.

2.2. Immunohistochemistry

FTO protein expression in the liver specimens was examined by immunohistochemistry (IHC). Samples used in the IHC were archived tissues. All patients gave written informed consent, and all tissue samples were collected with approvals from respective ethics committees. Standard IHC techniques were used to stain the FFPE histological samples using the Leica Bond III Automated Stainers. Staining for FTO was noted to be nuclear and results were scored using 0–3+ intensity with 3+ being strongest and 1+ the weakest, a percentage of cells for each sample was also given with a range from 0 to 100%.

2.3. Animal model

C57BL/6J mice (6–8 weeks old) were fed on either normal chow or methionine/choline-deficient (MCD) diet for 6 weeks. The study was carried out in strict accordance to the standards as described in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animal and approved by National University of Singapore Institutional Animal Care and Use Committee (NUS IACUC) at Duke-NUS Medical School.

2.4. Cell culture

Human hepatocellular carcinoma cell line, HepG2 (HB-8065) were cultured at 37 °C in Dulbecco’s modified Eagle’s medium, DMEM (Life Technologies) containing 4.5 g/L D-glucose, L-glutamine and 10% fetal bovine serum (FBS) (Sigma-Aldrich), in a humidified incubator containing 5% CO2 environment.

siRNA transfection was performed using Lipofectamine RNAiMAX (Life Technologies) according to manufacturer’s instructions. Forty-eight hours after transfection, cells were treated overnight with or without BSA-conjugated PA (0.75 mM).

2.5. Cell viability assay

Cell number was evaluated by the crystal violet staining as
described previously [13]. Briefly, cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde and stained with 0.1% crystal violet in 2% methanol. Dye was extracted with 10% acetic acid (Merck Millipore) and absorbance was measured at 595 nm with a microplate reader (Tecan).

2.6. Western blotting

Western blotting was done as described previously [14]. Cells or tissue samples were lysed using mammalian lysis buffer (Sigma-Aldrich), and immunoblotting was performed as per the manufacturer’s guidelines (Bio-Rad, Hercules, CA). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD).

2.7. Cellular oxygen consumption rate (OCR) analysis

Oxygen consumption was measured at 37°C using an XF24 extracellular analyzer (Seahorse Bioscience Inc., North Billerica, MA). HepG2 cells (40,000) were seeded in 24-well plates and transfected with either negative or FTO siRNA. Forty-eight hours after transfection, palmitic acid (0.75 mM) was added overnight. Mitochondrial metabolic parameters were assessed using Mitoset test kit (Seahorse Biosciences, Cat. 103015-100) according to manufacturer’s instructions. Every point represents an average from five different wells.

2.8. Protein oxidation detection

Protein carbonyl formation was measured using an Oxyblot system (Millipore, S7150) according to the manufacturer’s instructions [15]. In brief, the protein lysate was denatured with 12% SDS, and the carbonyl groups in the protein side chains were subsequently derivatized to 2,4-dinitrophenylhydrazine (DNPH) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNPH-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting using an anti-DNP primary antibody provided in the kit.

2.9. Measurement of cellular ceramide

To quantify levels of ceramide, cells were washed with PBS and trypsinized. The cell pellet was collected and re-suspended homogeneously in 100 μL cold PBS. 10 μL was used for protein measurement, and lipids were extracted using ice-cold chloroform/methanol (1:2). The organic phase was separated with chloroform and Milli-Q water. The organic phase was collected into a fresh tube, dried with a gentle nitrogen stream, and stored at −80°C freezer for liquid chromatography/mass spectroscopy analysis as described previously [14].

Fig. 2. FTO protein level is significantly increased in the liver of rodent model of NASH. (A) Immunoblots and (B) densitometric analysis of FTO, cleaved caspase 3 and CHOP in mouse livers harvested from mice on normal chow or methionine/choline-deficient (MCD) diets for 6 weeks.

Fig. 3. Knockdown of FTO protects against PA-induced ER-stress and apoptosis in HepG2 cells. HepG2 cells were transfected with negative control or FTO siRNA. 48 h after transfection, cells were treated with or without BSA-conjugated PA (0.75 mM) overnight. (A) Knockdown of FTO decreased PA-induced apoptosis. Cell viability was measured by crystal violet staining. (B) Knockdown of FTO decreased PA-induced cleaved caspase 3, and CHOP.
2.10. Statistical analysis

Cell cultures were performed in triplicates. All results were expressed in terms of mean ± standard deviation (SD) values, unless otherwise indicated. Differences between groups were compared using ANOVA analysis; \( p < 0.05 \) was considered statistically significant.

3. Results and discussion

3.1. FTO expression is significantly increased in livers from NASH patients and a rodent model of NASH

Hepatic FTO expression was analyzed in a cohort of Caucasian patients with hepatosteatosis or NASH that were compared to

![Graphs and images showing the effects of FTO knockdown on oxidative stress and mitochondrial function.](image)

Fig. 4. Knockdown of FTO protects against PA-induced mitochondrial dysfunction. HepG2 cells were transfected with negative control or FTO siRNA. 48 h after transfection, cells were treated with or without BSA-conjugated PA (0.75 mM) overnight. (A) Oxyblot analysis showing that knockdown of FTO decreased PA-induced oxidative stress. (B) Knockdown of FTO partially restored mitochondrial metabolic parameters upon PA treatment. Cellular oxygen consumption rate (OCR) was measured using Mitostress test kit. (C) Metabolomics analysis showing that knockdown of FTO decreased C-16 ceramide upon PA treatment.
healthy subjects. Hepatic FTO mRNA expression was increased in patients with hepatosteatosis and NASH compared to healthy subjects. However, a significant increase in FTO mRNA was observed in NASH patients when compared to healthy subjects (Fig. 1A). In order to validate FTO expression in NASH at the protein level, we performed IHC staining using liver biopsies obtained from healthy subjects and NASH patients from Singapore General Hospital. As shown in Fig. 1B and Supplementary Table 1, hepatic nuclear FTO staining was increased in NASH patients and associated with increased fat accumulation and NAFLD Activity Score (NAS) score [16]. The latter is based upon current histopathology guidelines [17] that characterize and grade hallmarks of NASH such as inflammation, ballooning and degree of steatosis, and glycogenated nuclear staining.

We then examined FTO protein expression in an experimental rodent NASH model in which mice were fed a methionine/choline-deficient (MCD) diet. Unlike fatty liver models of benign steatosis that are induced by high fat diet (HFD), this animal model exhibits several phenotypic characteristics of human NASH, including large areas of mixed inflammatory cell infiltration, hepatocyte necrosis, and discrete perivenular and pericellular fibrosis [18]. We found that MCD diet induced liver damage, as evidenced by increased expression of the apoptotic marker-cleaved caspase 3, and ER stress marker-CHOP, respectively (Fig. 2A). Interestingly, FTO protein level and nuclear FTO staining were significantly increased in mice fed MCD diet, suggesting that hepatic FTO may have a significant role in NASH progression. Several previous studies have suggested that hepatic expression of FTO in rodents is sensitive to nutritional status since FTO mRNA level is reduced by fasting [6] and increased by HFD [19,20]. However, unlike the dietary models of fatty liver disease such as HFD feeding, the MCD mice did not gain weight and/or increase dietary fat intake. The development of fatty liver and steatosis in this model is due to the inability of the liver to secrete VLDL which leads to triglyceride accumulation and inflammation. Of note, the histological characteristics of liver damage resemble human NASH. Based on these results, it is possible that the increase in FTO expression in NASH may not be driven by fatty acid accumulation in hepatocytes per se but may be induced by inflammation and/or its sequelae. This notion is further supported by our in vitro experiments in which FTO expression was not up-regulated by PA treatment in hepatic cell culture (Fig. 3). These results notwithstanding, the precise cause of FTO up-regulation in humans and the rodent model of NASH still needs to be determined.

3.2. Genetic ablation of FTO protects against hepatic cell apoptosis by PA

To understand the biological effect of FTO activity in influencing hepatocyte survival in NASH, we next examined a cell culture-based model of hepatic lipotoxicity. To induce lipotoxicity in hepatocytes, PA was added to cultured HepG2 human hepatoma cells. As shown in Fig. 3, FTO knockdown cells had higher viability than control cells after PA treatment (Fig. 3A). PA induced the expression of the apoptotic marker-cleaved caspase 3 and ER stress marker-CHOP, respectively (Fig. 2A). Interestingly, FTO protein level also was significantly increased in mice fed MCD diet, suggesting that hepatic FTO may have a significant role in NASH progression. Several previous studies have suggested that hepatic expression of FTO in rodents is sensitive to nutritional status since FTO mRNA level is reduced by fasting [6] and increased by HFD [19,20]. However, unlike the dietary models of fatty liver disease such as HFD feeding, the MCD mice did not gain weight and/or increase dietary fat intake. The development of fatty liver and steatosis in this model is due to the inability of the liver to secrete VLDL which leads to triglyceride accumulation and inflammation. Of note, the histological characteristics of liver damage resemble human NASH. Based on these results, it is possible that the increase in FTO expression in NASH may not be driven by fatty acid accumulation in hepatocytes per se but may be induced by inflammation and/or its sequelae. This notion is further supported by our in vitro experiments in which FTO expression was not up-regulated by PA treatment in hepatic cell culture (Fig. 3). These results notwithstanding, the precise cause of FTO up-regulation in humans and the rodent model of NASH still needs to be determined.

We next measured reactive oxygen species (ROS) production and mitochondrial function to better understand the protective mechanism(s) mediated by FTO knockdown. Using immunoblot-based detection of carbonyl groups introduced into proteins by oxidative reactions, we found that PA treatment significantly increased intracellular ROS, and knockdown of FTO decreased PA-induced oxidative stress (Fig. 4A). We next tested mitochondrial function by measuring cellular oxygen consumption rate to further explore the metabolic consequences of FTO signaling in a lipotoxic environment. Basal oxygen consumption, ATP turnover, maximal respiration and spare respiratory capacity all were impaired by PA treatment in control hepatic cells, but they were restored in FTO knockdown cells treated with PA (Fig. 4B). Thus, FTO knockdown protected against ROS production and mitochondrial dysfunction caused by PA. Our results indicate that the increased FTO expression is likely to be accounted for the increased ROS and mitochondrial dysfunction that is commonly found in NASH.

Generation of lipotoxic lipid species such as ceramides from PA has been associated with mitochondrial dysfunction [21,24–26] and decreased mitochondrial oxygen consumption in hepatocytes [26]. Therefore, we next measured cellular ceramide content by metabolomics. C-16 ceramide, the most abundant and toxic ceramide, was significantly increased after PA treatment, whereas this induction was attenuated after FTO knockdown (Fig. 4C). Altered ceramide content in the mitochondrial membrane perturbs the membrane structure and inhibits Complex I and III of the electron transport chain [27,28]. Thus, attenuation of PA-induced C-16 ceramide formation may be a novel mechanism by which decreased FTO led to mitochondrial protection from PA-induced lipotoxicity.

In conclusion, our study shows that hepatic FTO mRNA and protein levels are increased in rodent and human NASH, and its silencing reduces PA-induced lipotoxicity in human hepatic cells.

Conflict of interest

The authors declare no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.09.086.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.09.086.

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