Research Article

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Activating transcription factor 3 is a target molecule linking hepatic steatosis to impaired glucose homeostasis

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Background & Aims: Non-alcoholic fatty liver disease (NAFLD) contributes to impaired glucose tolerance, leading to type 2 diabetes (T2D); however, the precise mechanisms and target molecules that are involved remain unclear. Activating transcription factor 3 (ATF3) is associated with β-cell dysfunction that is induced by severe stress signals in T2D. We aimed to explore the exact functional role of ATF3 as a mechanistic link between hepatic steatosis and T2D development.

Methods: Zucker diabetic fatty (ZDF) rats were utilized for animal experiments. An *in vivo*-jetPEI siRNA delivery system against ATF3 was used for loss-of-function experiments. We analyzed the baseline cross-sectional data derived from the biopsy-proven NAFLD registry (n = 322). Human sera and liver tissues were obtained from 43 patients with biopsy-proven NAFLD and from seven healthy participants.

Results: ATF3 was highly expressed in the livers of ZDF rats and in human participants with NAFLD and/or T2D. Insulin resistance and hepatic steatosis were associated with increased ATF3 expression and decreased fatty acid oxidation via mitochondrial dysfunction and were attenuated by *in vivo ATF3* silencing. Knockdown of *ATF3* also ameliorated glucose intolerance, impaired insulin action, and inflammatory responses in ZDF rats. In patients with NAFLD and/or T2D, a significant positive correlation was observed between hepatic ATF3 expression and surrogate markers of T2D, mitochondrial dysfunction, and macrophage infiltration.

Conclusions: Increased hepatic ATF3 expression is closely associated with hepatic steatosis and incident T2D; therefore, ATF3 may serve as a potential therapeutic target for NAFLD and hepatic steatosis-induced T2D.

Lay summary: Hepatic activating transcription factor 3 (ATF3) may play an important role in oxidative stress-mediated hepatic steatosis and the development of type 2 diabetes (T2D) in a Zucker diabetic fatty (ZDF) rat model and in human patients with non-alcoholic fatty liver disease (NAFLD). Therefore, ATF3 may be a useful biomarker for predicting the progression of NAFLD and the development of T2D. Furthermore, given the significant association between hepatic ATF3 expression and both hepatic steatosis and impaired glucose homeostasis, *in vivo ATF3* silencing may be a potential central strategy for preventing and managing NAFLD and T2D.

Keywords: Non-alcoholic fatty liver disease; Oxidative stress; Zucker diabetic fatty rat; Human patients; *In vivo* silencing; Activation transcription factor 3.
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Introduction

Obesity is a major underlying risk factor for type 2 diabetes (T2D).[1] A common pathogenic event in both animals and humans with obesity and T2D is hepatic lipid accumulation, which is the earliest phenotype of non-alcoholic fatty liver disease (NAFLD).[2] NAFLD has reached epidemic levels worldwide because its incidence has gradually increased in the non-obese Asian population as well as the obese populations of the United States and Europe.[3] Non-alcoholic steatohepatitis (NASH) is the histologic form of NAFLD that is associated with increased morbidity and mortality, and it is closely linked to hepatic insulin resistance and abnormal

glucose metabolism.[4] However, the exact mechanism by which NAFLD induces T2D is poorly understood, and effective pharmacotherapies for NAFLD-induced T2D are unsatisfactory.

Hepatic oxidative stress, including endoplasmic reticulum (ER) and mitochondrial stress, is closely associated with the progression from simple steatosis to NASH and with the development of insulin resistance and T2D.[5] In the liver and adipose tissue of both diet-induced and *ob*/*ob* mice, lipid-induced ER and oxidative stress exacerbated hepatic steatosis via impaired hepatic lipid, glucose, and insulin metabolism.[6,7] Hepatic steatosis also contributes to the development of metabolic dysfunction associated with obesity, such as macrophage infiltration in adipose tissues, lipid accumulation and insulin resistance in skeletal muscle and liver, hyperglycemia, and hyperinsulinemia.[8,9] Moreover, several liver-derived endocrine factors that affect the peripheral metabolism have been identified[10–12] as key hepatokines associated with insulin resistance and NAFLD. However, the specific target molecule underlying metabolic dysfunction and linking hepatic steatosis to T2D remains obscure.

Stress-inducible activating transcription factor 3 (ATF3) can function as either a transcriptional activator or repressor.[13] Although several studies using *ATF3* transgenic or knockout (KO) mice have been performed,[14–16] it is still unclear whether the physiological role of ATF3 is beneficial or detrimental in the development of metabolic dysfunction. ATF3 is induced by various stresses and promotes a compensatory or adaptive homeostatic response, alleviating cellular stress.[17] However, recent studies have shown that ATF3 is also associated with β-cell dysfunction induced by severe stress signals related to T2D.[18,19] Therefore, persistent ATF3 expression induced by excessive reactive oxygen species (ROS) or ER stress likely has detrimental effects, overwhelming its initial compensatory role. Nonetheless, the exact functional role of ATF3 as a target molecule responsible for oxidative stress-mediated hepatic steatosis and impaired glucose metabolism is largely unknown. Hence, the aim of this study was to investigate whether ATF3 has a beneficial or detrimental effect on hepatic steatosis and incident T2D in Zucker diabetic fatty (ZDF) rats and human participants with NAFLD.

Patients and methods

Human participants

We prospectively enrolled this cross-sectional cohort derived from the ongoing NAFLD registry (NCT02206841; n = 322) of the Seoul Metropolitan Government Seoul National University Boramae Medical Center. The eligibility criteria and liver biopsy indications are presented in the Supplementary information and described elsewhere.[20] Informed consent was obtained from all participants under a protocol approved by the institutional review board (#20130320/16-2013-45/041). The study was conducted in accordance with the Declaration of Helsinki.

Statistical analysis

In human studies, data are expressed as a percentage, median (interquartile range) or mean ± standard deviation. Data are reported as the mean ± standard error of the mean in animal studies and *in vitro* experiments. Group comparisons were performed using an unpaired *t* test and one- or two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* tests, where $p < 0.05$ indicates statistical significance. In the human studies, continuous variables are described as the mean (95% confidence intervals [CI]). Categorical variables are shown as counts and percentages. Comparisons between groups were performed using Student's *t* test or the Mann-Whitney *U* test and Kruskal-Wallis test when appropriate. Differences between categorical variables were assessed with the chi-squared test or Fisher's exact test. Correlation analyses were performed using Spearman's analysis. The SPSS statistical package (SPSS Inc., version 18.0, Chicago, IL, USA) was used for all analyses.

Additional procedures and detailed methods are described in the Supplementary material and CTAT form.

Results

Hepatic steatosis is correlated with impaired insulin action and glucose intolerance

To clarify the relationship between NAFLD and T2D, we used ZDF rats, which phenotypically mimic human participants with obesity and T2D. Impaired glucose tolerance (IGT) and insulin resistance observed during the progression of T2D in ZDF rats significantly exacerbated hepatic steatosis, as shown by the serum alanine aminotransferase (ALT), hepatic triglyceride (TG), and cholesterol levels as well as the liver/body weight ratios (Fig. S1A, B). In parallel, a reduction in the insulin action due to hepatic insulin resistance was also observed in the livers of ZDF rats (Fig. S1C, D). Insulin receptor substrate 1 (IRS-1) phosphorylation at inhibitory Ser-307 was increased in the livers of 6-week-old ZDF rats compared to those of Zucker lean rats and was enhanced in 19-week-old rats with hyperglycemia, hypoinsulinemia, and pancreatic β-cell dysfunction. In contrast, active IRS-1 (Tyr-941) phosphorylation was increased in 6-week-old ZDF rats and was significantly decreased in 19-week-old rats. This trend was consistent with the changes in phosphorylation of Akt, a downstream signaling molecule of IRS-1. Phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, the rate-limiting enzymes associated with hepatic gluconeogenesis, were increased in the livers of 6-week-old rats and were significantly enhanced in 19-week-old rats.

Hepatic expression of ATF3 is enhanced in ZDF rats and NAFLD participants

Stress-inducible ATF3 levels were significantly increased in the livers of 6-week-old ZDF rats with mild steatosis and were further significantly increased in 19-week-old ZDF rats with severe steatosis and T2D (Fig. 1A). Hepatic expression of 4-hydroxy-2-nonenal (4-HNE), a marker of oxidative stress, showed a similar trend to that of ATF3 expression in the liver tissues of ZDF rats (Fig. 1B). In particular, ATF3 expression was positively correlated with hepatic TG accumulation and liver weight (Fig. S1E, F), suggesting an explicit role of ATF3 in the progression of hepatic steatosis. For all functional elements of ATF3, we conducted literature-based functional connectivity analyses using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database resource. *ATF3*-centered regulatory networks were significantly associated with NAFLD pathway-associated terms, including apoptosis-related gene networks in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (Fig. 1C). Next, to confirm the biological significance of ATF3 in the progression of hepatic steatosis and the development of T2D in participants with NAFLD, we conducted gene expression profiling analyses using human liver samples from different phases in the accessible public domain (GSE48452). Hepatic *ATF3* mRNA expression levels were significantly higher in participants with an NAFLD activity score (NAS) >5 compared to that of those