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Fructose consumption induces hypomethylation of hepatic mitochondrial DNA in rats

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ABSTRACT

Aims: Fructose may play a crucial role in the pathogenesis of metabolic syndrome (MetS). However, the pathogenic mechanism of the fructose-induced MetS has not yet been investigated fully. Recently, several reports have investigated the association between mitochondrial DNA (mtDNA) and MetS. We examined the effect of fructose-rich diets on mtDNA content, transcription, and epigenetic changes.
Main methods: Four-week-old male Sprague-Dawley rats were offered a 20% fructose solution for 14 weeks. We quantified mRNAs for hepatic mitochondrial genes and analyzed the mtDNA methylation (5-mC and 5-hmC) levels using ELISA kits.
Key findings: Histological analysis revealed non-alcoholic fatty liver disease (NAFLD) in fructose-fed rats. Hepatic mtDNA content and transcription were higher in fructose-fed rats than in the control group. Global hypomethylation of mtDNA was also observed in fructose-fed rats.
Significance: We showed that fructose consumption stimulates hepatic mtDNA-encoded gene expression. This phenomenon might be due to epigenetic changes in mtDNA. Fructose-induced mitochondrial epigenetic changes appear to be a novel mechanism underlying the pathology of MetS and NAFLD.

1. Introduction

Fructose, a simple sugar that is naturally contained in fruit and vegetables, is commonly used as a sweetener in food and beverages. Fructose consumption has gradually increased in the United States since the 1950s [1]. This increasing rate of caloric intake from fructose is considered to be a contributing factor to the increase in obesity in developed countries. In fact, mounting epidemiological and experimental evidence supports that elevated fructose intake alone can be a risk factor for metabolic syndrome, dyslipidemia, insulin resistance, obesity, and type 2 diabetes [2, 3, 4]. Therefore, social concern regarding disorders of fructose metabolism has been growing for the last decade in developing countries. However, the pathogenic mechanism of fructose-induced MetS-related phenotypes has not yet been investigated fully.

Mitochondrial DNA (mtDNA) is double-stranded, circular DNA located in the mitochondrial matrix. Because the mitochondrial genome encodes many proteins essential for ATP production (e.g., ATP synthase and NADH dehydrogenase), expression of mtDNA may be connected with pathological states in the development of the MetS and NAFLD. Indeed, Carabelli et al. reported that a high-fat-induced rat model of NAFLD showed a significant increase in hepatic mtDNA levels, which positively correlated with triglyceride content in the rat liver [5]. Similarly, Nagai et al. demonstrated that rat hepatic mtDNA content increased in high-fructose-fed rats [6]. Furthermore, it has been demonstrated that fructose consumption upregulates the expression of mtDNA-encoded genes among them those being responsible for ATPase [6], suggesting that alteration of mtDNA content and gene expression is involved in the pathogenic mechanism of MetS-related phenotypes.

Epigenetic regulation of gene expression has been suggested to play a critical role in various biological processes [7]. Epigenetic mechanisms regulate the structure and function of chromatin through two typical mechanisms that lead to histone modification and/or modifications of DNA cytosine [7]. Histone modification is a covalent post-translational modification to histone proteins, such as histone methylation, acetylation, phosphorylation, and ubiquitylation [8]. These histone modifications play fundamental roles in most biological processes that are

Abbreviations: MetS, metabolic syndrome; mtDNA, mitochondrial DNA; NAFLD, non-alcoholic fatty liver disease; mt-ND6, mitochondrial NADH dehydrogenase 6; NASH, non-alcoholic steatohepatitis; HDLC, high-density lipoprotein cholesterol; TET, ten-eleven translocation.

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involved in gene expression. With respect to DNA cytosine, 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) are major molecules of epigenetic DNA modification that play an important role in the control of gene expression [9]. The conversion of 5-mC to 5-hmC precedes demethylation and seems to play a direct role in the regulation of gene expression. Over the past decade, 5-mC modifications have been increasingly recognized as a key process in the pathogenesis of complex disorders, including cancer, diabetes, and cardiovascular disease. For example, Zhao et al. (2012) reported that DNA methylation status is associated with insulin resistance [10]. Altered DNA methylation status has also been observed in several cancers [11]. The conversion of 5-mC to 5-hmC is a newly discovered epigenetic modification that is presumably generated by oxidation of 5-mC by the TET family of cytosine oxygenases [12]. Recent studies have investigated the epigenetic functions of 5-hmC, a hydroxylated and methylated form of cytosine. Similar to 5-mC, 5-hmC appears to be involved in the pathogenesis of various diseases, such as cancer [13]. Ficz et al. (2014) reported that cancer cell lines and primary tissues, such as melanoma and carcinoma, have lower 5-hmC levels than normal tissues [14]. Increased 5-hmC levels were also observed in Alzheimer's disease patients. These associations between 5-hmC levels and various diseases suggest that deeper insights into epigenetic mechanisms will increase our understanding of disease pathologies.

Since mitochondria do not contain histones, mtDNA is associated with different proteins into aggregates called nucleoids [15]. Therefore, DNA methylation/hydroxymethylation rather than histone modifications appear to be important for mitochondrial epigenetics. Indeed, DNA methylation regulates the expression of mtDNA-encoded genes [16, 17]. mtDNA methylation status may be involved in pathogenesis of MetS via dysfunctional regulation of crucial metabolic proteins. For example, Pirola et al. (2013) reported hypermethylation of mtDNA in nonalcoholic steatohepatitis (NASH) patients and also found that liver mt-ND6 mRNA expression significantly decreased in these patients, suggesting an association between mtDNA methylation and mitochondrial transcription [18]. More recently, Pirola et al. also suggested the possibility that 5-hmC might be involved in the pathogenesis of NAFLD by regulating liver mitochondrial biogenesis [19]. These results suggest that epigenetic modification of mtDNA is involved in the pathogenesis of NASH and NAFLD.

Although many studies over the past few decades have investigated the pathogenic mechanism of fructose-induced metabolic disorders [18, 20, 21], the role of epigenetic modifications of mtDNA has not yet been investigated. Thus, we hypothesized that the pathogenesis of metabolic disorders induced by fructose is related to the mtDNA methylation/hydroxymethylation status. In this study, we showed that fructose consumption induced hypomethylation (5-mC and 5-hmC) of mtDNA. These data suggest the possibility that altered mtDNA methylation levels are related to fructose-induced MetS.

2. Materials and methods

2.1. Animals

The study was approved by the Fujita Health University's Animal Ethics Committee. 4-Week-old male Sprague-Dawley rats (SLC, Shizuoka, Japan) were housed in environmentally controlled conditions at room temperature (23 °C ± 3 °C) under a 12:12 h light-dark cycle. After acclimatization for 1 week, the animals were divided into two experimental groups. The control group (Cont, $n = 6$) received distilled water for 14 weeks, while the fructose-fed group (Fru, $n = 6$) received a 20% fructose solution for the same period. All animals had ad libitum access to their respective treatment solutions and standard chow (MF, Oriental Yeast, Tokyo, Japan) at all times [20, 22]. Composition of MF diet was reported previous study [22]. Body weight, food intake, and the consumption of the treatment solutions were recorded once per week.

At the end of the study, access to food was withdrawn from all animals at 3 h before dissection. Blood was collected by cardiac puncture and centrifuged at 1700 *g* for 10 min. Serum was collected and stored at −80 °C until use. After collecting blood, all animals were perfused with saline. Liver tissues were harvested and stored at −80 °C until use.

2.2. Serum and hepatic lipid analyses

Hepatic lipid was extracted using the Folch method as previously described [23]. Triglyceride and total cholesterol from extracted lipids and serum were measured with a BiOLis 24i chemistry analyzer (Tokyo Boeki Medical System, Tokyo, Japan) using the triglyceride E-Test Wako kit and the cholesterol E-Test Wako kit (Wako, Osaka, Japan). High-density lipoprotein cholesterol (HDL—C) and low-density lipoprotein cholesterol (LDL-C)/very-low-density lipoprotein cholesterol (VLDL-C) were measured using the EnzyChrom AF HDL and LDL/VLDL Assay kit (BioAssay Systems, CA, USA).

2.3. Oil red O staining

Oil red O staining was performed to analyze fat accumulation in the liver (Fiorini et al., 2004). Frozen hepatic sections (20-μm) were postfixed in a 10% formaldehyde solution for 30 s. The sections were dipped in 60% isopropanol and stained in Oil Red O working solution (60% undiluted solution of Oil Red O in isopropanol) at 50 °C for 15 min. After the sections were rinsed in 60% isopropanol, they were counterstained with Mayer's hematoxylin for 10 min and mounted with Aquatex (Merck, NJ, USA).

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from liver tissues using the guanidine isothiocyanate-phenol-chloroform method as previously described (Yamada et al., 2009). Total RNA (2 μg) was reverse-transcribed into cDNA at 25 °C for 10 min, 37 °C for 60 min, and 70 °C for 15 min using the following reagents: dNTPs (Takara, Otsu, Japan), recombinant RNase inhibitor (Takara), random hexadeoxyribonucleotide (pd (N) 6) primers (Takara), 5X first strand buffer (Invitrogen, Tokyo, Japan), 100 mM DTT (Invitrogen), and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen).

2.5. Quantitative assessment of mRNA expression

Real-time PCR for quantitative assessment of mRNA expression was performed on a PRISM-7900HT instrument (Applied Biosystems, CA, USA) using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan): 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 1 min. Primers for target genes were designed by Universal ProbeLibrary Assay Design Center (Roche Diagnostics GmbH, Mannheim, Germany). All primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). The primers for target genes are as follows; mt-ND1, 5′-ATGGCCTTCCTCACCCTAGT-3′ (forward) and 5′-GTTAGGGGGCGTATGGGTTC-3′ (reverse); mt-ND2, 5′-CCCAAGGAATTC CCCTACACA-3′ (forward) and 5′-GGCGCCAACAAAGACTGATG-3′ (reverse); mt-ND3, 5′-GGAACATACCAAGGCCACCA-3′ (forward) and 5′-TCGTGGGTAGGAACTAGGCT-3′ (reverse); mt-ND4, 5′-CTCTCCCTCA CAACACACCC-3′ (forward) and 5′-GGAGCTTCTACGTGGGCTTT-3′ (reverse); mt-ND4L, 5′-ACTCTCCTCTGCCTAGAAGGAA-3′ (forward) and 5′-AAACCTACTGCTGCTTCGCA-3′ (reverse); mt-ND5, 5′-AGTTGT GGCAGGGATCTTCC-3′ (forward) and 5′-TCGTTTTGGGTGAGAGCACA-3′ (reverse); mt-ND6, 5′-TTGGGGTTGCGGCTATTTAT-3′ (forward) and 5′-ATCCCCGCAAACAATGACCA-3′ (reverse); mt-CO1, 5′-GCTTCGTCCACTGA TTCCCA-3′ (forward) and 5′-GCAAAGTGGGCTTTTGCTCA-3′ (reverse); mt-CO2, 5′-ACAAGACGCCACATCACCTA-3′ (forward) and 5′-TGGGCGTC TATTGTGCTTGT-3′ (reverse); mt-CO3, 5′-GGAACATACCAAGGCCACCA-3′ (forward) and 5′-TCGTGGGTAGGAACTAGGCT-3′ (reverse); mt-ATP6, 5′-