High-fructose diet-induced hypertriglyceridemia is associated with enhanced hepatic expression of ACAT2 in rats

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Short title: High fructose-induced hepatic ACAT2 expression

Summary

High levels of fructose induce hypertriglyceridemia, characterized by excessive levels of triglyceride-rich lipoproteins such as very low-density lipoprotein (VLDL); however, the underlying mechanisms are poorly understood. The aim of this short communication was to examine hepatic changes in the expression of genes related to cholesterol metabolism in rats with hypertriglyceridemia induced by high-fructose or high-glucose diets. Rats were fed a 65% (w/w) glucose diet or a 65% (w/w) fructose diet for 12 days. Serum levels of triglycerides, total cholesterol, and VLDL+LDL-cholesterol, hepatic levels of triglycerides and cholesterol, and ACAT2 expression at the gene and protein levels were significantly higher in the fructose diet group compared to the glucose diet group. The hepatic levels of *Abcg5/8* were lower in the fructose group than in the glucose group. Serum high-density lipoprotein (HDL)-cholesterol and hepatic expression levels of *Hmgcr*, *Ldlr*, *Acat1*, *Mttp*, *Apob*, and *Cyp7a1* did not differ significantly between groups. These findings suggest that high-fructose diet-induced hypertriglyceridemia is associated with increased hepatic ACAT2 expression.

Key Words

ACAT2, Fructose, Hypertriglyceridemia, Liver, Rats

Fructose is mainly consumed as dietary sugars (e.g., sucrose or high-fructose corn syrup) and its excessive consumption is closely related to metabolic diseases such as fatty liver and obesity (Herman and Samuel 2016). Several animal studies including ours have demonstrated that fructose, compared to that with glucose, potently induces the *de novo* synthesis of fatty acids and triglyceride accumulation in the liver (Koo *et al.* 2008, Janevski *et al.* 2012, Shimada *et al.* 2017, 2019) most likely due to bypassing the main rate-limiting step in glycolysis. Excessive triglycerides are assembled with cholesterol and apolipoproteins in the liver and are secreted as very low-density lipoprotein (VLDL) particles into the blood. Thereby, excessive intake of fructose, compared to that of glucose, leads to hypertriglyceridemia. However, the mechanisms through which fructose induces hypertriglyceridemia including VLDL secretion are poorly understood. Little evidence is available regarding the relationship between a high-fructose or a high glucose diet and hepatic cholesterol metabolism. Koo *et al.* (2008) showed that high levels of fructose, as opposed to glucose, do not enhance the expression of 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) and microsomal triglyceride transfer protein (*Mttp*) genes in rat livers despite hypertriglyceridemia induced by high fructose. These genes encode the rate limiting enzyme for cholesterol synthesis (HMGCR) and a protein involved in the assembly and secretion of apolipoprotein B (APOB)-containing lipoproteins that stabilize

VLDL (MTTP). Therefore, we hypothesized that high fructose-induced hypertriglyceridemia might affect other genes related cholesterol metabolism in the liver.

In this study, we examined changes in the hepatic expression of genes related to cholesterol synthesis/uptake/secretion/catabolism/excretion in rats with hypertriglyceridemia induced by a high-fructose diet, as compared to that with a high-glucose diet.

Five-week-old male Wistar rats (SLC, Shizuoka, Japan) were assigned to a glucose diet group or a fructose diet group (n = 7 per group). The composition of diets was as follows: 20.0% (w/w) casein, 65.0% carbohydrate (glucose or fructose), 5.0% corn oil, 5.0% cellulose, 3.5% AIN93G mineral mixture, 1.0% AIN93 vitamin mixture, 0.3% L-cystine, and 0.2% choline chloride. The animals were allowed free access to diets and water for 12 days. At the end of the feeding period, the rats were fasted for 7 h and euthanized by cardiac puncture under isoflurane anesthesia. Blood and liver tissues were collected for subsequent assays. All animal care and experimental procedures were approved by the Gifu University Animal Care and Usage Committee.

Blood was centrifuged at 1,710 × *g* for 15 min at 4 °C to prepare serum. Serum concentrations of triglycerides, total cholesterol, and high-density lipoprotein (HDL)-cholesterol were measured using commercial kits (Triglyceride E-test Wako, Cholesterol

E-test Wako, and HDL-Cholesterol E-test Wako, respectively; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Serum VLDL+low-density lipoprotein (LDL)-cholesterol was determined as follows: total cholesterol − HDL-cholesterol.

Total lipids from liver tissues were extracted by the Bligh and Dyer method (Bligh and Dyer 1959) with some modifications. Liver tissues were homogenized with 0.1 M KCl and mixed with chloroform and methanol at a ratio of 1:1:1. The homogenates were centrifuged at 800 × *g* for 10 min at room temperature. Then, the lower organic phase was collected and dried as lipid extracts. The levels of triglycerides and cholesterol in the lipid extracts were dissolved in 2-propanol and measured using a commercial kit (Triglyceride E-test Wako and Cholesterol E-test Wako, respectively; FUJIFILM Wako Pure Chemical Corporation).

Hepatic total RNA was extracted and converted to cDNA using commercial kits (RNeasy Mini kit; Qiagen, Tokyo, Japan and ReverTra Ace qPCR RT kit; Toyobo, Osaka, Japan, respectively). PCR amplification was performed in a final volume of 20 μL, which contained SYBR Green (TB Green Premix Ex Taq; Takara, Shiga, Japan), 0.2 μM of each primer, Rox reference dye, and 20 ng of cDNA, using a real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan). The primers used were listed in Table 1. Relative mRNA levels were determined by the ΔΔCt method (Livak

and Schmittgen 2001), using ribosomal protein, large, P0 (*Rplp0*) as a normalization control.

Liver tissues were homogenized in radioimmunoprecipitation buffer (50 mM tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% Nonidet P-40) containing protease inhibitors (Complete Mini; Sigma-Aldrich Japan, Tokyo, Japan). The homogenates were centrifuged at 16,100 × *g* for 30 min at 4 °C to prepare lysates. Protein concentrations in the lysates were determined by a bicinchoninic acid assay (BCA protein assay kit; Takara). The lysates containing an equal amount of protein (40 μg) were separated by 12.5% SDS-PAGE, and then transferred onto PVDF membranes. After transfer, the membranes were washed in phosphate-buffered saline (PBS) with 0.1% tween 20 (PBS-T) and blocked in 3–5% skimmed milk in PBS-T for 1 h at room temperature. Thereafter, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-ACAT2 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-aldolase, fructose-bisphosphate B (ALDOB) (GeneTex, Irvine, CA, USA), and anti-TATA-binding protein (TBP) (GeneTex). Subsequently, the membranes were washed in PBS-T and incubated for 1 h at 4 °C with the secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology Japan, Tokyo, Japan). After washing with PBS-T, the signals were detected by enhanced chemiluminescence

(Immnostar LD; FUJIFILM Wako Pure Chemical Corporation) using an imaging system (LAS3000; Fujifilm, Tokyo, Japan). Relative protein levels were normalized using TBP as a loading control.

Values are expressed as means ± SEM. Differences between the two groups were evaluated by the Student's *t*-test. $P < 0.05$ indicated statistical significance.

Body weight, food intake, and serum HDL-cholesterol levels did not differ between groups. Hepatic triglyceride and cholesterol levels and serum levels of triglycerides, total cholesterol, and VLDL+LDL-cholesterol were significantly higher in the fructose group than in the glucose group (Table 2).

Hepatic expression levels of *Hmgcr*, LDL receptor (*Ldlr*), acetyl-coenzyme A acetyltransferase 1 (*Acat1*), *Mttp*, *Apob*, and cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*) were not different between groups. These genes encode proteins involved in LDL update from the blood (LDLR) and cholesterol esterification (ACAT), and the rate limiting enzyme for cholesterol catabolism and bile acid synthesis (CYP7A1). Hepatic levels of ATP binding cassette subfamily G member 5 (*Abcg5*) were significantly lower ($P < 0.05$) in the fructose group and *Abcg8* was also lower ($P = 0.057$). ABCG5 and ABCG8 form a heterodimer and are involved in the excretion of cholesterol in bile. In contrast, hepatic expression of ACAT2 and ALDOB was significantly higher at the

gene and protein levels in the fructose group (Fig. 1A, 1B). ALDOB is a key enzyme involved in fructolysis and is induced by high levels of fructose (Koo *et al.* 2008, Shimada *et al.* 2017).

High levels of fructose induce hypertriglyceridemia. In this study, we observed higher levels of serum triglycerides and VLDL+LDL-cholesterol in rats fed a high-fructose diet compared to those in animals fed a high-glucose diet. These results were consistent with those of previous studies using rats (Koo *et al.* 2008, Shimada *et al.* 2017). Interestingly, in rats fed a high-fructose diet, we observed an increase in the hepatic expression of ACAT2 at both gene and protein levels. This is the first study to show that feeding rats a high-fructose diet induces hepatic expression of ACAT2 and hypertriglyceridemia. ACAT2 is predominantly expressed in livers and intestines, whereas ACAT1 is ubiquitously expressed (Anderson *et al.* 1998, Cases *et al.* 1998, Oelkers *et al.* 1998). Especially in livers, ACAT2 is localized to hepatocytes, which play a main role in nutritional metabolism, whereas ACAT1 is localized in Kupffer cells, which play a key role in the removal of foreign bodies (Parini *et al*. 2010). In addition, Liang *et al*. (2004) reported that the assembly/secretion of VLDL is more strongly induced in rat hepatocytes overexpressing human *Acat2* than those expressing *Acat1*. Moreover, several studies showed that *Acat2* deficiency or inhibition prevents

atherosclerosis (Willner *et al*. 2003, Ohshiro *et al*. 2011), whereas *Acat1* deficiency had no effect, in atherosclerotic model mice (Accad *et al*. 2000, Fazio *et al*. 2001). Considering our results and aforementioned reports, the high fructose-induced hepatic expression of ACAT2, but not ACAT1, might lead hypertriglyceridemia and promote subsequent vascular diseases such as atherosclerosis via lipid metabolic abnormalities in the liver. However, it is unclear how fructose enhances the expression of *Acat2* in the liver. The present data indicate that feeding rats a high-fructose diet also induces hepatic expression of ALDOB and ACAT2 at the gene and protein levels. In addition, several studies have demonstrated that the transcription factor hepatocyte nuclear factor 1 (HNF1) regulates both *Aldob* and *Acat2* (Gregori *et al.* 2002, Pramfalk *et al.* 2005). Therefore, high levels of fructose might increase *Acat2* expression via the activation of HNF1 in rat livers. Further studies should investigate whether high fructose levels induce the recruitment of HNF1 to the *Acat2* promoter.

It should be noted that high-fructose feeding resulted in higher cholesterol and lower *Abcg5/8* expression in the liver. Su *et al.* (2012) reported that higher levels of plasma cholesterol and triglycerides are observed in *Abcg5/8*-knockout mice fed a high-fat diet. Considering the role of ABCG5/8 in the excretion of cholesterol from the liver to bile, it is likely that a fructose-induced increase in hepatic cholesterol is partially

responsible for the lower *Abcg5/8* expression, which might lead to subsequent hyperlipidemia. This hypothesis requires further investigation.

In conclusion, our results suggest that hypertriglyceridemia induced by a high-fructose diet is associated with increased ACAT2 expression at both gene and protein levels in the liver. This finding has implications for the prevention and treatment of metabolic diseases such as fatty liver and obesity.

Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgments

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Table 1. Sequences of primers

	Forward	Reverse
Hmgcr	5’-TCTGCAGTACCTGCCTTACAGA-3’	5’-ATCACGTTCTCACAGCAAGC-3’
Ldlr	5’-CCCAACCTGAAGAATGTGGT-3’	5’-TGGCATCATAGGACAAGCTG-3’
Acat1	5’-GTCCATGGAGGAGCTGTTTC-3’	5’-AAGGCATGAGCCAAGTGAAC-3’
Acat2	5’-CCAAAGTGGCTCCAGAAGAG-3’	5’-CCACACTGGCTTGTCGAGTA-3’
Mttp	5’-TCTTCCAGTACATCGGAAAGG-3’	5’-GTGGCTGCAATTAAGCCTTC-3’
Apob	5’-GATGGAGATGGGAGATGAGGT-3’	5’- GGGCTCCTCATCAACAAGAG-3’
Cyp7a1	5’-CAAGACGCACCTCGCTATTC-3’	5’-CTGCTTTCATTGCTTCAGGA-3’
Abcg5	5’-GAATGTGTCCTTCAGCGTCA-3’	5’-GCTGGCATGATTTGATGTTC-3’
Abcg8	5’-AACCCTGCTGACTTCTACGTG-3’	5’-TGCAAGTAATCGAGCCTTCTC-3’
Aldob	5’-TTGCCAATGGGAAGGGTA-3’	5’-ATCCTCTGTAGGCGGTTTCC-3’
Rplp0	5’-CGAGAAGACCTCTTTCTTCCAA-3’	5’-AGTCTTTATCAGCTGCACATCG-3’

Table 2. Physiological and biochemical parameters of rats fed a glucose diet or a fructose diet for 12 days

	Glucose	Fructose
Body weight		
Initial (g)	122 ± 3	120 ± 3
Final (g)	171 ± 4	164 ± 3
Food intake (g/d)	12.9 ± 0.4	12.0 ± 0.3
Liver		
Weight (g)	7.23 ± 0.26	8.60 ± 0.35**
Triglycerides (mg/g liver)	51 ± 23	135 ± 19*
Cholesterol (mg/g liver)	7.06 ± 1.05	9.81 ± 0.59*
Serum		
Triglycerides (mg/100 ml)	130 ± 8	254 ± 29**
Total cholesterol (mg/100 ml)	75 ± 3	103 ± 4**
VLDL+LDL-cholesterol (mg/100 ml)	37.6 ± 2.4	58.5 ± 4.9**
HDL-cholesterol (mg/100 ml)	37.8 ± 3.5	49.9 ± 2.9

Values are expressed as means ± SEM (n = 7). $^{**}P < 0.01$, $^{*}P < 0.05$.

Figure legend

Fig. 1. Hepatic expression of cholesterol metabolism-related genes and proteins in rats fed a glucose or fructose diet.

(A) Expression of cholesterol metabolism-related genes. (B) Expression of ACAT2 and ALDOB proteins. Values are expressed as means ± SEM (n = 7). $^{**}P < 0.01$, $^{*}P < 0.05$.