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Dietary fructose induces a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver

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ABSTRACT

Dietary fructose has been suspected to contribute to development of metabolic syndrome. However, underlying mechanisms of fructose effects are not well characterized. We investigated metabolic outcomes and hepatic expression of key regulatory genes upon fructose feeding under well defined conditions. Rats were fed a 63% (w/w) glucose or fructose diet for 4 h/day for 2 weeks, and were killed after feeding or 24-hour fasting. Liver glycogen was higher in the fructose-fed rats, indicating robust conversion of fructose to glycogen through gluconeogenesis despite simultaneous induction of genes for de novo lipogenesis and increased liver triglycerides. Fructose feeding increased mRNA of previously unidentified genes involved in macronutrient metabolism including fructokinase, aldolase B, phosphofructokinase-1, fructose-1,6-bisphosphatase and carbohydrate response element binding protein (ChREBP). Activity of glucose-6-phosphate dehydrogenase, a key enzyme for ChREBP activation, remained elevated in both fed and fasted fructose groups. In the fasted liver, the fructose group showed lower non-esterified fatty acids, triglycerides and microsomal triglyceride transfer protein mRNA, suggesting low VLDL synthesis even though plasma VLDL triglycerides were higher. In conclusion, fructose feeding induced a broader range of genes than previously identified with simultaneous increase in glycogen and triglycerides in liver. The induction may be in part mediated by ChREBP.
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1. Introduction

A moderate amount of fructose from fresh fruits and vegetables has long been a component of human diets. However, abundant production of refined sweeteners has dramatically increased fructose intake. An estimated average consumption of added sugars (refined cane and beet sugar, corn sweeteners, edible syrups, and honey) in the United States is 126 g/capita/day in 2000 based on food supply data [1]. Popkin and Nielsen also estimated that more than 30% of carbohydrates consumed came from added sugars in the United States [2].

In humans, high fructose in diets reduces insulin sensitivity [3] and elevates plasma triglycerides in both fed and fasting conditions [4]. In animals, diets high in fructose cause multiple symptoms of metabolic syndrome such as insulin resistance [5–7], impaired glucose tolerance [8], hyperinsulinemia [9], hypertension [9], and hypertriglyceridemia [7,8]. Thus, high consumption of dietary fructose, primarily from sucrose and high-fructose corn syrup, has been implicated as a contributing factor to the development of obesity and accompanying metabolic abnormalities such as insulin resistance and hypertriglyceridemia.

Metabolism of dietary fructose, which occurs mainly in liver, differs from that of glucose. Hepatic glucose metabolism is acutely regulated by phosphofructokinase I (PFK I), a key regulatory step of glycolysis. In contrast, fructose enters the glycolytic pathway at the triose level, bypassing PFK I. This difference in initial metabolism of fructose not only acutely affects carbohydrate metabolism by changing supply of intermediate metabolites, but also induces metabolic adaptation including changes in gene expression. Previous studies reported increased mRNA expression of glycolytic (liver-type pyruvate kinase, PK) [10], lipogenic (fatty acid synthase, FAS) [11], and gluconeogenic enzymes (glucose-6-phosphatase, G6Pase; phosphoenolpyruvate carboxykinase, PEPCK) [12,13].

However, several confounding factors must be resolved to define the differential effect between fructose and glucose on gene expression

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Abbreviations: ACC, acetyl-CoA carboxylase; AOX, acyl-CoA oxidase; ChREBP, carbohydrate responsive element binding protein; CPT1a, carnitine palmitoyltransferase 1a; FAS, fatty acid synthase; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase; FAT/CD36, fatty acid translocase; GK, glucokinase; GKRP, glucokinase regulatory protein; GPAT, glycerol-3-phosphate acyltransferase; G6PD, glucose-6-phosphate dehydrogenase; GS, glycogen synthase; HMGCS, mitochondrial 3-hydroxy-3-methylglutaryl (HMG) CoA synthase; KHK, ketohexokinase; Mlx, Max-like factor X; MTP, microsomal triglyceride transfer protein; NEFA, non-esterified fatty acid; PEPCK, phosphoenolpyruvate carboxykinase; PFK I, phosphofructokinase I; PK, pyruvate kinase (liver-type); SREBP1c, sterol regulatory element binding protein 1c

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and metabolic consequences. First, genes involved in carbohydrate metabolism are strongly regulated by hormones such as insulin, glucagon and glucocorticoids, and are sensitive to nutritional status [14]. Many previous studies were conducted without clearly defining fed and fasted conditions [15,16]. Second, existing studies typically investigated the effect of fructose on expression of only one or two genes at a time, thus were unclear in the scope of the fructose effect [10,12,13]. Third, previous studies vary widely in the ratio of fructose and other carbohydrate sources in diets [15,17]. A small amount of fructose in the presence of high levels of glucose increased the net hepatic glucose uptake and the glycogen synthesis [18–20]. It is unknown whether or not fructose alone can increase glycogen synthesis in the absence of concomitant administration of glucose. Also, there is likely a difference between acute and chronic effects of fructose administration because a temporary decrease in food intake was reported when a diet is switched from high glucose to high fructose [21]. Another question yet to be addressed is the underlying mechanism that mediates fructose effects on gene expression. Carbohydrate response element binding protein (ChREBP), a transcription factor of the bHLHLZ family, induces lipogenic genes such as PK [22], S14 [23], acetyl-CoA carboxylase (ACC) [24] and FAS [25] in response to high glucose. However, a recent study showed that ChREBP-null mice were intolerant to high sucrose/fructose diet, suggesting a critical role of ChREBP in fructose metabolism [26].

Therefore, the objectives of the present study were to investigate 1) the differential effect of dietary fructose and glucose on gene expression and on carbohydrate and lipid metabolism in well defined conditions, and 2) a possible role of ChREBP in the process. We achieved this objective by analyzing key regulatory genes and metabolic outcome in clearly defined metabolic conditions: 1) in both fasted and fed states, 2) after adapting to a diet for 2 weeks, and 3) using a fructose-only diet to exclude potential confounding effects of fructose on the dietary glucose metabolism.

2. Materials and methods

2.1. Animals and diets

Twenty-four Sprague–Dawley male rats (5 weeks old, Harlan, Indianapolis, IN) were acclimated for 2 days and adapted to a 4 h/day feeding protocol for 1 week with a standard laboratory diet. This meal feeding regimen synchronizes metabolic conditions of animals, and has been used to study regulation of metabolism by nutrients [27,28]. The animals were individually housed with controlled temperature and lighting (22± 2 °C and a 12-hour light/dark cycle). After adaptation, the rats were fed with either a glucose (n=12) or a fructose (n=12) pelleted diet for 2 weeks. Glucose and fructose diets (Dyets Inc., Bethlehem, PA) were formulated by replacing the carbohydrate source in AIN-93G diet with glucose (anhydrous dextrose) and fructose, respectively. Food consumption was measured every day for each rat. After 2 weeks, half of each group (n=6) was fasted for 24 h before euthanizing. The other half of the animals were killed at the end of a 4 h-feeding. All procedures under protocol were reviewed and approved by the University of Illinois Laboratory Animal Care Advisory Committee.

2.2. Blood glucose analysis

Blood was collected from the saphenous vein of conscious rats using microhematocrit tubes. Blood glucose was measured using One Touch BASIC Diabetes Monitoring System (Lifescan Inc., Milpitas, CA).

2.3. Processing and analysis of blood

After blood sampling for glucose analysis, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/ml). The abdomen was opened, and ~6 ml blood was drawn from vena cava in a syringe containing 120 µl of 50 mM EDTA (final 1 mM). After centrifugation, plasma was stored at −80 °C. Plasma insulin and glucagon were measured using commercial radioimmunoassay kits (Linco Research, St. Charles, MO). Concentration of plasma non-esterified fatty acids (NEFA) was measured using a commercial kit (Wako, Richmond, VA).

The concentration and distribution of triglycerides among the very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions in plasma were measured by use of a modified lipoprotein cholesterol autoprofiler method [29]. This method involves 1) rapid separation of VLDL, LDL and HDL particles by short-spin density gradient ultracentrifugation in a rotor with swingout buckets (Beckman SW 50.1, Beckman Coulter Inc., Fullerton, CA); 2) continuous online mixing of the effluent from density gradient tubes with enzymatic triglyceride assay reagent (Roche Diagnostics, Indianapolis, IN); 3) online incubation and continuous flow measurement of absorbance; and 4) computerized calculation of triglycerides associated with VLDL, LDL and HDL peaks.

2.4. Histochemistry

The liver was removed and weighed. For histochemical evaluation of glycogen in the liver, one ~5 mm thick slice from the left lateral lobe, right medial lobe and caudal lobe of the liver were immersion fixed in absolute ethanol for 24 h, then processed through graded alcohols and imbedded in paraffin. Sections 3 µm thick were stained with periodic acid Schiff (PAS) reagent for visual examination under the microscope.

2.5. Determination of liver glycogen content

Liver glycogen content was measured by a phenol–sulfuric acid colorimetric assay as described previously [30]. Briefly, powdered frozen liver samples were dissolved in 30% (wt/vol) KOH saturated with Na_2SO_4 and then boiled for 15 min to get a homogenous solution. Then samples were cooled on ice for 30 min, and 95% ethanol was added to precipitate glycogen. After centrifuging at 840 ×g, glycogen pellets were resuspended in distilled water. Samples were incubated in the 5% phenol solution and 96% sulfuric acid solution at room temperature for 10 min, and the absorbance was read at 490 nm.

2.6. Fatty acid analysis

Gas chromatography was used to quantify total concentration of triglyceride and NEFA in the liver. The detailed procedure has been reported previously [28]. Briefly, total liver lipids were extracted using chloroform/methanol (2:1, vol:vol) that contained 50 µg each of C17:0, free fatty acid and triglyceride as internal standards. The lipid extract was then separated by thin layer chromatography into free fatty acids and triglycerides. Each fatty acid fraction was methylated using Methanolic HCl (Supelco, Bellefonte, PA). The amount of individual fatty acid was determined by comparing gas chromatography peaks with a peak of internal standard (17:0).

2.7. RNA analysis

Remaining liver was frozen immediately in liquid nitrogen and subsequently stored at −80 °C. Total RNA from liver tissue was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality and concentration were determined by agarose gel electrophoresis and spectrophotometry, respectively. RNA expression of target genes was measured using a real-time quantitative PCR method with

Table 1
Primer sequences used for real-time PCR

Gene		Genbank
KHK	Sense ACGGATCGCAGGTGCCTAT Antisense AGCACAGTGCAGGAGTTGGA	NM031855
AldolaseB	Sense GCCACCTCACACAGCTTCTG Antisense TCGGTGAGCCATGATGACA	NM012496
GK	Sense TGTGAGGCACGAAGACCTAGAC Antisense TCCTACGATGTTGTTCCCTTCTG	NM012565
GKRP	Sense AAACGATATCAGCATGTGATCGA Antisense GCCGCTTCATACCCTGACA	NM013120
PFKI	Sense CCATGGATGAGAAGAGGTTTGAC Antisense GGTGGGCGAGGAGCTTGTA	BC061791
PK	Sense GGGTGTGAACTTGCCAAACA Antisense CCGAAGCGCAGATCCAAA	NM012624
G6Pase	Sense TTCCCATCTGGTTCCACATTC Antisense CGAGGTTGAACCAGTCTCCAA	NM013098
FAS	Sense TCGGCGAGTCTATGCCACTATT Antisense ACAGAGAACGGATGAGTTGTTCCT	NM017332
GPAT	Sense GACGAAGCCTTCCGAAGGA Antisense GACTTGCTGGCGGTGAAGAG	AF021348
CPT1a	Sense GGCTCTGGGTGGCAGTCAT Antisense CCGTGTTCTGCAAACATCCA	NM031559
HMGCoAS	Sense ACCCTCGATGACGTGCAATAT Antisense AGCCGAGCTAGGGATTTCTG	NM173094
AOX	Sense AAGAACTCCAGATAATTGGCACCTA Antisense TTTCCAAGCCTCGAAGATGAG	NM017340
SREBP1c	Sense CGACGGAGCCATGGATTG Antisense CAAATAGGCCAGGGAAGTCACT	L16995
ChREBP	Sense CAGATGCGGGACATGTTTGA Antisense AATAAAGGTCGGATGAGGATGCT	AB074517
Mlx	Sense CCGCAGACTCTACGGGAGATT Antisense TGGCTGCTCTTCGGGTTTC	NM001034112
G6PD	Sense TTCCGGGATGGCCTTCTAC Antisense TTTGCGGATGTCATCCACTGT	NM017006

SYBR Green fluorescence dye (Applied Biosystems, Foster City, CA), as described previously [30]. Briefly, 2 μg purified RNA was reverse transcribed into complementary DNA. Published primer sequences were used for phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) [31], microsomal triglyceride transfer protein (MTP) [32], and fatty acid translocase/CD36 (FAT/CD36) [33]. Primers for other genes were designed using the Primer Express software (Applied Biosystems) and listed in Table 1. All primers were purchased from MWG Biotech (High Point, NC). Real-time PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). Amplification efficiency of each primer set was verified before sample analysis. The mRNA abundance relative to 18 S rRNA was determined using comparative critical threshold method according to manufacturer's instruction.

2.8. Assay of hepatic glucose 6 phosphate dehydrogenase (G6PD) enzyme activity

About 0.5 g of liver was homogenized in 5 ml of a 0.25 M sucrose solution containing 1 mM of EDTA in 10 mM of Tris–HCl buffer (pH 7.6). After precipitating a nuclei fraction by centrifuging at 2450 ×*g* for 10 min, the supernatant was centrifuged at 105,000 ×*g* for 60 min at 4 °C to obtain a cytosolic fraction. Protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. The enzyme activities of G6PD (EC1.1.1.49) in supernatant were determined with a method described by Dror [34]. The reaction solution contained 0.16 M of Tris–HCl buffer (pH 7.6), 30 mM of $MgCl_2$, 3.3 mM of glucose-6-phosphate, 1.6 mM of oxidized $NADP^+$, and 0.5 U of type VI 6-phosphogluconate dehydrogenase/ml. Reaction was started by adding 6-phosphogluconate dehydrogenase, and appearance of NADPH was measured with a spectrophotometer at 340 nm.

2.9. Data analysis and statistics

Data were analyzed by one-way ANOVA coupled with Fisher's post-hoc test using Statview 5.0.1 (SAS Institute, Cary, NC). All data are reported as means ± standard deviation (SD). Significance was set at $p < 0.05$.

3. Results

3.1. Food intake and growth

As shown in Fig. 1, the fructose group ate 32% less food than the glucose group in the first day of switching diets from a high-starch laboratory diet. On the second day, however, the food intake of the fructose group increased to the level of the glucose group, indicating that the rats were able to adapt to the high-fructose diet in 1 day. The final weight gain of the two groups for the two-week period did not reach a significant difference (Table 2), showing that the high-fructose diet was well tolerated. The relative liver weights (liver weight (g)/body weight (g)) of the fructose groups were significantly higher than those of the glucose groups, indicating hepatomegaly as a response to a high-fructose diet (Table 2).

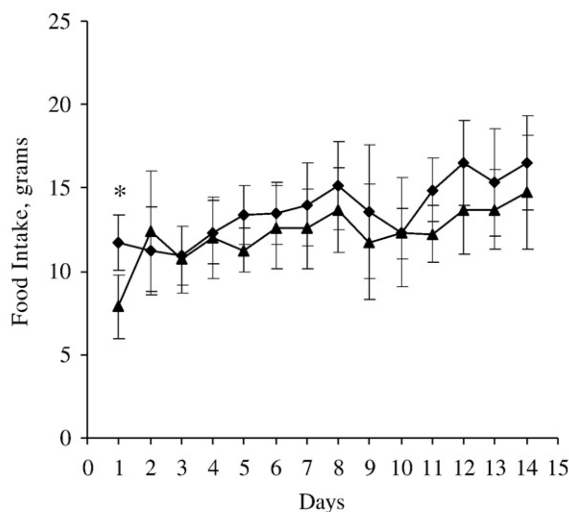

Fig. 1. Food intake of fructose-fed animals was lower in the first day. Animals were fed either a glucose diet (diamond) or a fructose diet (triangle) for 2 weeks, and food intake was measured daily. Values are means, and error bars denote standard deviation. For each time point, $n = 12$ for each group. *significantly different ($p < 0.05$).

Table 2
Weight gain and liver characteristics

	Glucose		Fructose	
	Fed	Fasted	Fed	Fasted
Weight gain (g)	102 ± 27		91 ± 41	
Liver weight (g)	9.1 ± 1.2^{ab}	6.7 ± 0.5^{c}	10.0 ± 1.1^{a}	8.2 ± 1.8^{bc}
Relative liver weight (%) (liver weight/body weight)	3.5 ± 0.3^{b}	2.6 ± 0.4^{c}	4.2 ± 0.5^{a}	3.3 ± 0.8^{b}
Glycogen (mg/g liver)	13.2 ± 5.2^{b}	0.7 ± 0.5^{c}	22.8 ± 7.0^{a}	1.0 ± 0.5^{c}
Triglyceride (μmol/g liver)	5.1 ± 0.7^{c}	11.7 ± 1.2^{a}	9.2 ± 2.1^{b}	5.3 ± 0.6^{c}
Non-esterified fatty acids (nmol/g liver)	139 ± 51^{b}	304 ± 96^{a}	156 ± 45^{b}	172 ± 60^{b}

Animals were fed either the glucose diet or the fructose diet for 2 weeks. Half of the animals were killed at the end of 4 h-feeding, and the other half were killed after fasting for 24 h. Liver glycogen content was measured by a phenol–sulfuric acid colorimetric assay. Liver triglycerides and non-esterified fatty acids were measured by gas chromatography. Values are expressed as the means ± standard deviation ($n = 6$). The different superscripts denote statistical difference ($p < 0.05$).

3.2. Liver and plasma metabolic profiles

Liver slices were stained by the periodic acid Schiff (PAS) method to evaluate the magnitude and distribution of hepatic glycogen storage. Compared with the glucose-fed group, the fructose-fed group showed more intense staining, primarily in the periportal region (Fig. 2). Quantitation of liver glycogen was consistent with this histological observation, being 1.7 times higher in the fructose-fed animals than in the glucose-fed animals (Table 2). This increased glycogen deposition indicates a robust conversion of fructose to glucose-6-phosphate, and a larger metabolic role of liver in disposing dietary carbohydrate in the fructose group than in the glucose group, in which skeletal muscle and

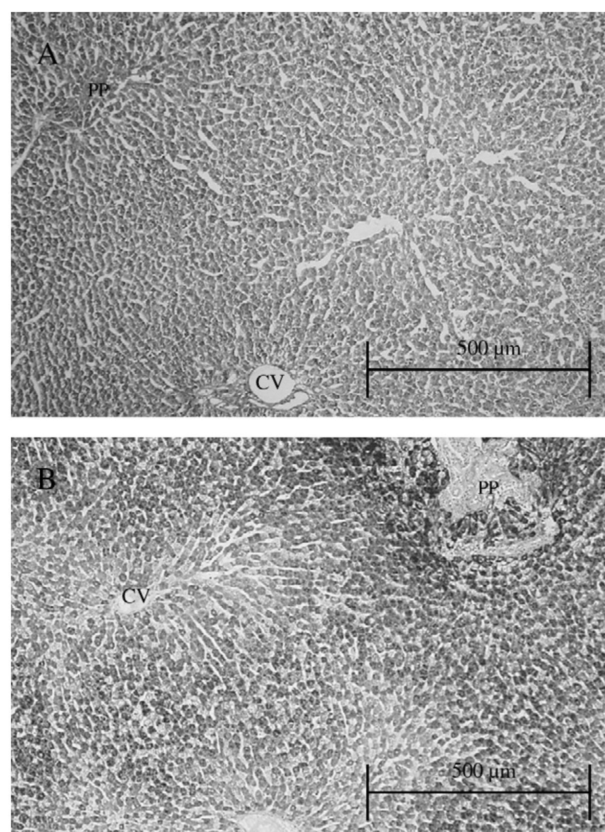

Fig. 2. Glycogen store was increased in the periportal area of fructose-fed animals. Animals were fed either a glucose diet or a fructose diet for 2 weeks, and were euthanized at the end of a 4 h-feeding. Liver glycogen was stained by periodic acid Schiff. A, liver from the glucose-fed animals; B, fructose-fed animals; PP, periportal area; CV, central venous area.

Table 3
Blood glucose and plasma triglycerides, non-esterified fatty acids, insulin, and glucagon concentrations

	Glucose		Fructose	
	Fed	Fasted	Fed	Fasted
TG (mg/dl plasma)	68±17[b]	25±3[c]	132±56[a]	34±12[c]
VLDL	52.2±12.8[b]	6.8±0.9[d]	101.7±42.9[a]	14.6±5.1[c]
LDL	4.3±1.1[b,c]	3.7±0.5[c]	16.6±2.2[a]	6.3±2.2[b]
HDL	11.1±2.6[ab]	14.2±1.9[a]	8.8±1.1[b]	13.4±4.7[a]
NEFA (mEq/L plasma)	0.04±0.04[a]	0.27±0.09[b]	0.12±0.13[a]	0.22±0.05[b]
Glucose (mg/dl whole blood)	84±14[a]	45±10[c]	63±12[b]	50±9[b,c]
Insulin (ng/ml plasma)	5.6±1.7[b]	1.1±1.0[c]	9.4±3.6[a]	1.3±1.0[c]
Glucagon (pg/ml plasma)	90±27[a,b]	64±30[b,c]	120±54[a]	47±14[c]
Insulin/glucagon ratio	77±39[a]	28±32[b]	91±19[a]	35±33[b]

Animals were treated as described in Table 2. At the end of 2 weeks, blood was drawn from conscious rats for blood glucose determination. Plasma was prepared from the blood collected via cardiac puncture after anesthesia, and was used for all other analyses. Values are expressed as the means±standard deviation (n=6). The different superscripts denote statistical difference ($p<0.05$).

adipose tissue significantly contribute to disposal of dietary glucose. In fasted condition, both histology and quantitation showed depletion of glycogen in both glucose and fructose groups (Table 2, histology data not shown).

The glucose groups showed lower liver triglycerides in a fed state than in a fasted state (Table 2), a consistent observation by others [35], whereas the fructose groups showed a reversed pattern (Table 2). The hepatic triglyceride concentration was higher in the fructose group than in the glucose group in the fed condition, suggesting increased fatty acid and triglyceride synthesis by liver in the fructose group in the fed state. In contrast, both triglycerides and NEFA were significantly lower in the fructose group than in the glucose group when animals were fasted, suggesting decreased uptake and/or increased utilization of fatty acids in the liver of the fructose group in the fasted condition (Table 2).

Table 3 shows that blood glucose concentrations from conscious animals were lower in the fructose group than in the glucose group in the fed condition, whereas there was no difference in blood glucose levels between fasted groups. On the other hand, the plasma insulin was significantly higher in the fructose group than in the glucose group in the fed condition. Plasma glucagon did not reach statistical significance between the fructose and glucose groups (Table 3). However, the fructose group showed much higher glucagon in the fed state than in the fasted state (2.6×, $p<0.05$), whereas the glucose group showed a smaller change (1.4×, nonsignificant) (Table 3). Our previous study also showed an increase in glucagon after feeding a moderately-high protein diet [36], suggesting glucagon may play a role in inducing gluconeogenic genes when a diet contains high fructose or high protein. Plasma VLDL and LDL triglycerides were higher in the fructose group in both fed and fasted conditions, whereas no difference was observed in plasma NEFA between the fructose and glucose groups (Table 3).

3.3. Gene regulation by dietary fructose

Compared with the glucose-fed group, the fructose-fed group had higher mRNA of fructose metabolizing enzymes, ketohexokinase (KHK, fructokinase) and aldolase B (Fig. 3 A). Fig. 3 B shows genes for the glycolysis pathway. Phosphofructokinase I (PFK I) and PK mRNA were induced more in the fructose-fed group than in the glucose-fed group, whereas there was no difference in the mRNA expression of glucokinase (GK) and glucokinase regulatory protein (GKRP) between the fructose and glucose groups. There was no difference in the glycogen synthase RNA among 4 groups (data not shown). However, glycogen synthase is acutely activated by insulin. Thus, the increased glycogen deposition (Table 2) and plasma insulin (Table 3) in the fructose-fed group suggest a higher glycogen synthase activity in the fructose-fed group than in the glucose-fed animals. Among the gluconeogenic genes, the fructose-fed group had higher

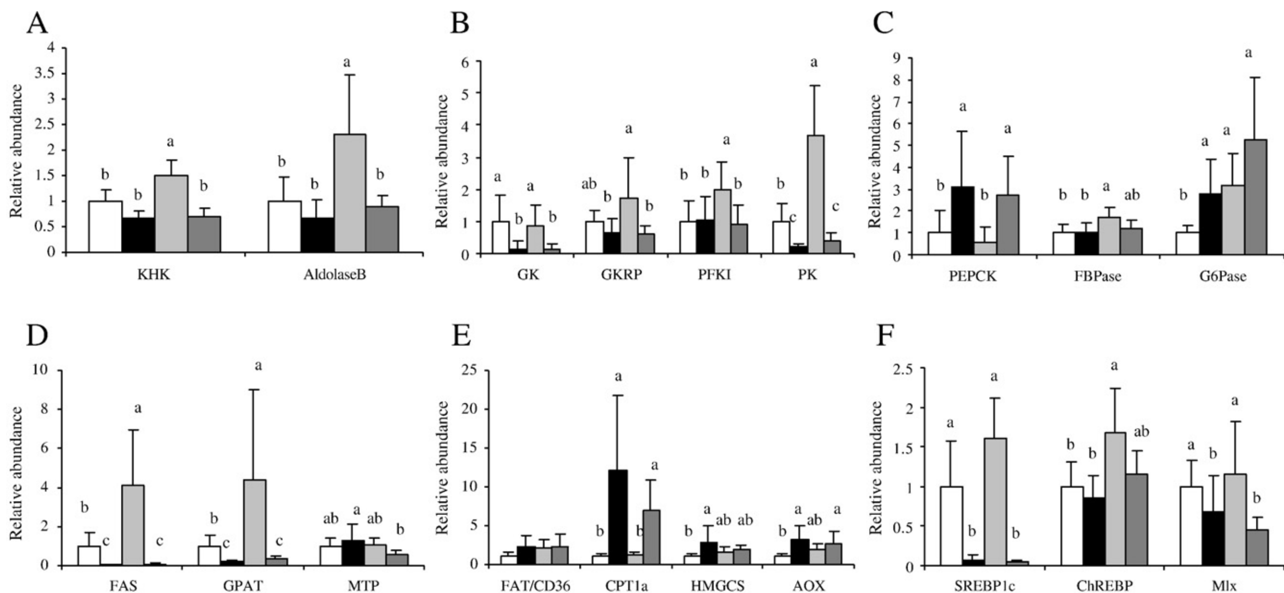

Fig. 3. Regulation of hepatic genes involved in carbohydrate and lipid metabolism by dietary fructose. Animals were fed either a glucose diet or a fructose diet for 2 weeks. Half of the animals were killed at the end of 4 h-feeding, and the other half were killed after fasting for 24 h. RNA abundance related to ribosomal 18 S RNA was determined using real-time PCR. The data were expressed as a relative change from the glucose-fed group. Glucose-fed group, white bar; glucose-fasted group, black bar; fructose-fed group, light gray bar; and fructose-fasted group, dark gray bar. Results are expressed as means, and error bars show standard deviation from five to six animals. Different superscript denotes statistical difference ($p<0.05$). A, Fructose metabolism: ketohexokinase (KHK), aldolase B. B, Glycolysis: glucokinase (GK), glucokinase regulatory protein (GKRP), phosphofructokinase I (PFK I), pyruvate kinase (PK). C, Gluconeogenesis: phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase). D, Lipogenesis: fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), and microsomal triglyceride transfer protein (MTP). E, Fatty acid transporter/oxidation: fatty acid translocase (FAT/CD36), carnitine palmitoyltransferase 1a (CPT1a), mitochondrial 3-hydroxy-3-methylglutaryl (HMG) CoA synthase (HMGCS), and acyl-CoA oxidase (AOX). F, Transcription factors: sterol regulatory element binding protein 1c (SREBP1c), carbohydrate regulatory protein (ChREBP), and Max-like factor X (Mlx).

mRNA expressions of FBPase and G6Pase than the glucose-fed group, but not PEPCK mRNA (Fig. 3 C).

Fig. 3 D shows genes involved with the lipogenesis pathway. Compared to the glucose-fed group, the fructose-fed group had significantly higher mRNA expressions of FAS, a key lipogenic enzyme, and glycerol-3-phosphate acyltransferase (GPAT), a regulatory enzyme of glycerolipid synthesis. Interestingly, mRNA of MTP, a transporter that has a regulatory role in VLDL synthesis, was significantly lower in the fructose group in fasted condition (Fig. 3 D) although plasma VLDL was higher (Table 3). There was no significant difference in the gene expression of fatty acid oxidation enzymes between the glucose-fasted and fructose-fasted animals (Fig. 3 E).

Among transcription factors, ChREBP mRNA was significantly induced by fructose feeding, whereas sterol regulatory element binding proteins-1c (SREBP1c) gene did not reach statistical significance ($p=0.06$) although the magnitude of induction by fructose was similar to ChREBP (Fig. 3 F). The fructose diet did not affect the expression of Max-like factor X (Mlx), the heterodimeric partner of ChREBP (Fig. 3 F).

3.4. Enzyme activity and mRNA expression of glucose-6-phosphate dehydrogenase (G6PD)

We measured mRNA and activity of G6PD, the regulatory enzyme of the pentose phosphate pathway, in the liver to determine whether a fructose effect on G6PD was at the gene expression level (Fig. 4). G6PD activity was higher in the fructose group than in the glucose group in both fed and fasted conditions (Fig. 4 A). In contrast, there were large variations in mRNA expression within each group, and no significant difference was observed among groups (Fig. 4 B). Neither was there correlation between the mRNA expression and the enzyme activity of G6PD ($r^2=0.0146$), indicating that the induction of G6PD by dietary fructose is at the translational or post-translational level. Moreover, the lack of effects of fasting and feeding on the G6PD activity (Fig. 4 A) suggest a slow turnover rate of the enzyme.

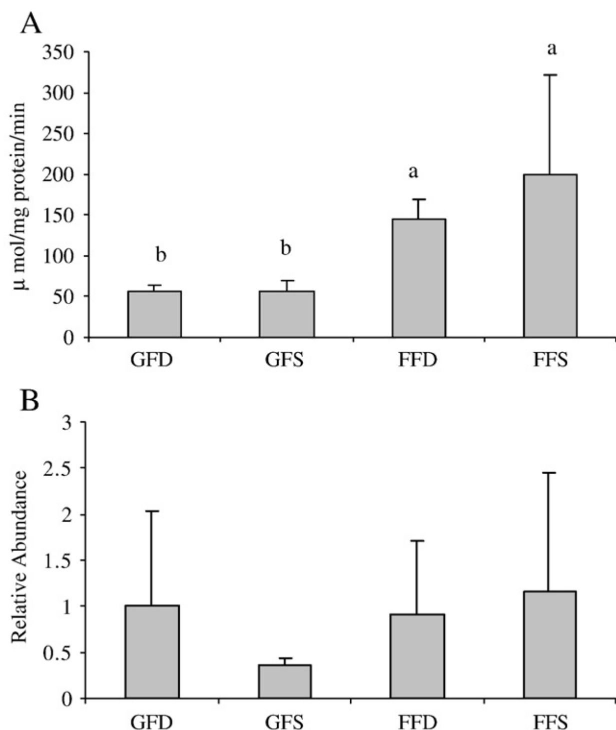

Fig. 4. Enzyme activity of glucose-6-phosphate dehydrogenase (G6PD) was induced in the fructose groups, whereas mRNA expression of G6PD did not differ among groups. Animals were fed either the glucose diet or the fructose diet for 2 weeks. Half of the animals were killed at the end of 4 h-feeding, and the other half were killed after fasting for 24 h. A, cytosolic fraction was used for enzymatic assay. G6PD activity was determined by measuring $NADP^+$ reduction. B, RNA abundance related to ribosomal 18S RNA was determined using real-time PCR. Results are expressed as means, and error bars show standard deviation ($n=6$). Different superscript denotes statistical difference ($p<0.05$). GFD, glucose-fed group; GFS, glucose-fasted group; FFD, fructose-fed group; FFS, fructose-fasted group.

4. Discussion

4.1. A unique pattern of gene expression by fructose feeding

The liver plays the major role in fructose metabolism. The current study provided a well defined view of fructose effects on metabolic outcome and the expression of genes involved in the metabolic pathways of fructose. As summarized in Fig. 5 A, dietary fructose in the fed state induces a broader range of hepatic genes involved in the pathways for fructose metabolism than previously identified.

KHK and Aldolase B are required specifically for fructose metabolism in liver before fructose metabolites enter the glycolysis/gluconeogenic pathways. Studies reported that aldolase B mRNA was induced by insulin and dexamethasone, and repressed by glucagon in rat hepatocytes [37,38]. However, our study showed that fasting and refeeding had only small and nonsignificant effects on KHK and aldolase B gene expression in the glucose group, whereas these genes were significantly induced in the fructose-fed group (Fig. 3 A). The underlying mechanism of this fructose effect is yet to be elucidated. Although fructose does not stimulate insulin secretion, chronic fructose feeding causes insulin resistance, resulting in higher plasma insulin [21]. Thus, increased plasma insulin in the fructose-fed group (Table 3) may have contributed to the induction of KHK and aldolase B genes. However, presence of an unidentified insulin independent mechanism cannot be ruled out.

Among genes of key regulatory enzymes of gluconeogenesis, mRNA of FBPase and G6Pase were induced by dietary fructose (Fig. 3 C, Fig. 5 A). The induction of FBPase mRNA by fructose (Fig. 3 C) is particularly interesting because the FBPase expression was unaffected by feeding cycles [39] (Fig. 3C, glucose groups). When fed with glucose, dietary fructose has been shown to stimulate glycogen synthesis from glucose [18–20]. However, because no dietary glucose was present in our fructose diet, the substrate for glycogen synthesis in the fructose-fed group likely came from dietary fructose through the gluconeogenic pathway. Thus, the induction of FBPase is consistent with the robust accumulation of glycogen in the fructose-fed animals in this study (Fig. 2, Table 2).

Contrary to the response of G6Pase and FBPase, the expression of PEPCK, another key enzyme of gluconeogenesis, was not increased by the fructose diet either in fasting or in fed condition compared with the glucose group (Fig. 3 C). Unlike G6Pase and FBPase, PEPCK enzyme has no known acute regulation, and is primarily regulated at transcriptional level [40]. Because simultaneous induction of PK and PEPCK could result in a futile cycle, the lack of PEPCK induction by fructose is physiologically suitable when PK was highly induced by fructose in this study (Fig. 5).

It is noteworthy that fructose showed a differential effect on PEPCK and G6Pase genes in our study (Fig. 3 C) because these two genes largely share common transcriptional regulatory mechanisms. Both genes are induced by glucagon acting via cAMP [41] and glucocorticoids [42,43], and repressed by insulin [44]. Both genes require interaction of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) with hepatocyte nuclear factor 4α [45]. Massillon et al. showed that xylitol infusion stimulated G6Pase transcription, whereas it decreased PEPCK expression and suggested an involvement of the pentose phosphate pathway in the differential regulation of G6Pase and PEPCK [46]. Consistent with their findings, our study showed that the fructose diet increased G6PD activity, a rate-limiting enzyme of the pentose phosphate pathway (Fig. 4). Further studies will be warranted to elucidate the underlying mechanism of the differential effects of dietary fructose on G6Pase and PEPCK.

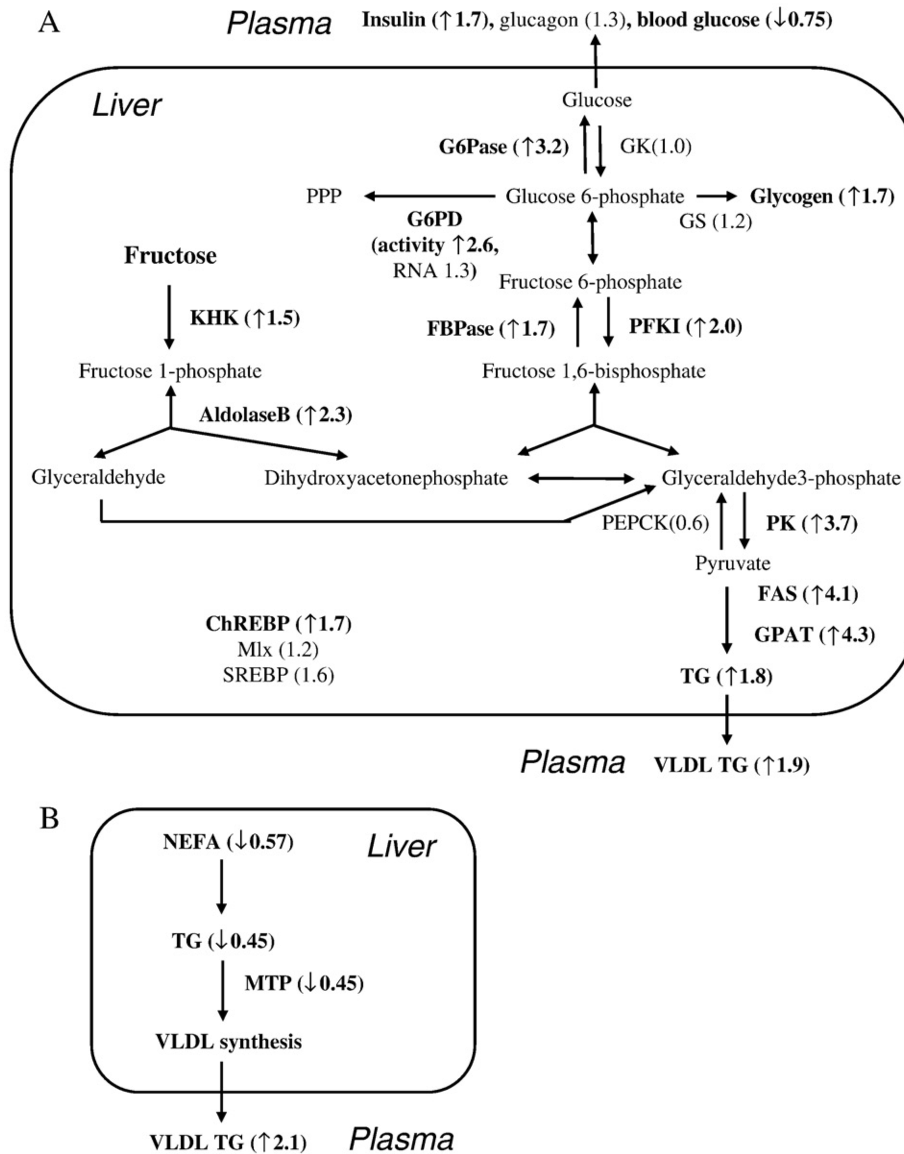

Fig. 5. Summary of changes in gene expression and metabolic outcomes by dietary fructose. A, fed, and B, fasted animals. Numbers represent relative changes of the fructose group from the glucose group. Bold letters denote statistically significant ($p<0.05$).

To our surprise, the mRNA of liver PFK I, a key regulatory enzyme of the glycolysis pathway (Fig. 5), was about two times higher in the fructose-fed group than in the glucose-fed group (Fig. 3 B). This paradoxical induction of both PFK I and FBPase genes by fructose was also reported in the small intestine of neonatal rats [47]. Because PFK I is under strong regulation by an allosteric activator fructose-2,6-bisphosphate [48], it is yet to be elucidated whether this simultaneous induction of PFK I and FBPase genes actually increases enzyme activities, and thus results in futile cycling (Fig. 5). Alternatively, upregulation of FBPase and PFK I genes may be zone specific, expressed primarily in the periportal area and in the perivenous area in liver, respectively, as reported by Jungermann [49].

4.2. A possible role of ChREBP in mediating the fructose effects

ChREBP induces lipogenic genes such as PK [50], ACC [24], FAS [51] and possibly GPAT [52] in response to high carbohydrate (glucose or starch) diets. In our study, the mRNA of PK, FAS, and GPAT were highly induced when dietary glucose was replaced with fructose (Fig. 3 B, D). Although ACC and FAS genes are induced by both ChREBP and SREBP-1 [24,51,53,54], PK is responsive to only ChREBP [55], suggesting ChREBP as a possible mediator of the fructose effect on these genes. Moreover, a recent study with primary cultures of hepatocytes identified genes dependent on Mlx, an obligatory dimerization partner of ChREBP. The Mlx responsive genes included KHK, aldolase B, G6Pase, FBPase and PFK I [56]. These genes were also induced in our study by dietary fructose (Fig. 3 A, B, C), further suggesting involvement of ChREBP in regulation of gene expression by fructose. This suggested involvement of ChREBP in mediating the fructose effect is further supported by a report that ChREBP-null mice were intolerant of a high-fructose diet [26], and our observation that ChREBP mRNA itself was also responsive to dietary fructose (Fig. 3 F).

Activity of ChREBP is regulated by xylulose 5-phosphate, an intermediate metabolite of the pentose phosphate pathway. When the pentose phosphate pathway is active, xylulose 5-phosphate increases and activates a protein phosphatase 2A [57,58], which in turn dephosphorylates and activates the ChREBP [59]. The present study showed that 2 weeks of fructose feeding increased the activity of G6PD, the regulatory enzyme of the pentose phosphate pathway (Fig. 4). Pagliassotti et al. also reported that sucrose feeding produced a four-fold

increase in xylulose 5-phosphate in liver [13]. Taken together, induction of genes by dietary fructose observed in our study is likely to be mediated at least in part by ChREBP although changes in hormonal milieu (Table 3) may also have a role.

Although mRNA processing is likely the major regulatory step of G6PD activity [60], no correlation was observed between the mRNA and the activity of G6PD in this study (Fig. 3). Thus, the mechanism by which fructose increases G6PD activity is yet to be elucidated.

4.3. Changes in fasting lipid metabolism in the fructose group

Differences in gene expression and metabolic outcome between the glucose and fructose groups became much less in a fasted state than in a fed state (Fig. 5 A, B). Nevertheless, lipid metabolism showed a clear difference between the two dietary treatments (Fig. 5 B). In the fasted state, the fructose group showed significantly lower NEFA, triglycerides (Table 2) and MTP mRNA (Fig. 3 D) in liver than the glucose group. MTP is rate-limiting for VLDL secretion [61], and its activity is mainly regulated at transcription [32,62]. Thus, decreased substrates (NEFA and triglycerides) and MTP mRNA suggest a reduction in VLDL synthesis in the fructose group when the animals were fasted. In contrast, plasma VLDL triglycerides in the fructose group were higher in the fasted state than in the glucose group (Table 3). Thus, our data suggest that elevated VLDL TG in the fasted fructose group may be due to decreased VLDL clearance, not increased secretion. Consistent with this observation, Hirano et al. reported a slow VLDL catabolism in fructose-fed rats [63]. Because hepatic glycogen stores were highly increased by fructose feeding, the extrahepatic tissues in the fructose group may draw more energy from plasma glucose and less from VLDL TG during fasting, resulting in slow VLDL clearance. Determining VLDL turnover and respiratory quotient will be required to test this hypothesis.

5. Conclusions

In conclusion, we have shown in this study that 1) dietary fructose induced a broader range of genes in a fed state than previously identified including induction of KHK, aldolase B, FBPase, PFK I and ChREBP, but not PEPCK, 2) G6PD activity was higher in the fructose group than in the glucose group in both fed and fasted states, suggesting activation of ChREBP, 3) genes induced by dietary fructose overlap with genes induced by ChREBP/Mlx, suggesting ChREBP as a likely mediator of gene induction by fructose, 4) fructose was robustly converted to glucose and stored as glycogen in liver without dietary glucose, and 5) high dietary fructose resulted in low liver triglycerides and MTP with high plasma VLDL triglycerides in the fasted fructose group, suggesting a possible shift of energy source of extra hepatic tissues from triglycerides to glucose. Underlying mechanisms of these fructose effects are yet to be elucidated.

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