Supporting Information

Ishimoto et al. 10.1073/pnas.1119908109

SI Materials and Methods

Animal Study. Ketohexokinase A and C knockout (KHK-A/C KO) mice, which were of C57BL/6 background and were lacking both ketohexokinase A and ketohexokinase C, and ketohexokinase A knockout (KHK-A KO) mice were generated as described previously (1). KHK-A/C knockout homozygous mice, KHK-A knockout homozygous mice and wild-type (WT) litter mates (male, 8 wk-old) were used. They were maintained in temperature- and humidity-controlled specific pathogen-free condition on a 12-h dark/12-h light cycle, and allowed ad libitum access to normal laboratory chow (Harlan Teklad; no. 2918). WT mice, KHK-A/C KO mice and KHK-A KO mice were assigned to one of three groups (n = 8–9) respectively, matching mean body weight among the groups. Mice had free access to water containing 15% fructose, 30% (wt/vol) fructose or tap water for 25 wk. Fructose water was prepared by dissolving D-(−)-fructose (Sigma-Aldrich) in tap water. Body weight was measured every week, and energy intake from both normal chow and fructose water was measured 3 times per week. Systolic blood pressure was measured using a tail-cuff sphygmomanometer at 5, 10 and 15 wk (Visitech BP2000; Visitech Systems). At 19 wk, energy balance and fuel utilization were assessed with indirect calorimetry as described below, and body composition was measured by quantitative magnetic resonance with an Echo MRI-900 Whole Body Composition Analyzer (Echo Medical Systems). At 25 wk, mice were killed after 6 h fasting of food and fructose water. Blood were withdrawn, and tissues were taken and frozen in liquid nitrogen. All experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado.

Biochemical Analysis. Biochemical analysis for alanine aminotransferase, aspartate aminotransferase, urea nitrogen, total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, glucose and HbA1c was done with an automated chemistry analyzer (VetACE Clinical Chemistry System, Alfa Wassermann Diagnostic Technologies). The serum level of leptin and insulin was determined using a mouse leptin ELISA kit and mouse/rat insulin ELISA kit (Crystal Chem) respectively. Uric acid, phosphate, β-hydroxy butyrate concentration was measured with QuantiChrom uric acid assay kit, phosphate colorimetric assay kit (BioAssay Systems) and β-hydroxy butyrate assay kit (Cayman Chemical) respectively. Fructose levels in serum and urine were determined using EnzyChrom fructose assay kit (BioAssay Systems) which has a higher sensitivity (linear detection range, 12 μM to 1000 μM) that most available methods, and does not interfere with glucose and galactose, according to the manufacturer's report. This method shows higher serum fructose levels compared with other enzymatic assays for human or rodents (2, 3) or gas chromatography-mass spectrometry (4). Serum levels were consistently reproducible with repeated assay and fell within a tight standard linear curve, documenting that the assay is accurate for distinguishing fructose levels between groups.

Histopathology. For Oil Red O staining, 0.3% (wt/vol) Oil Red O solution (Sigma-Aldrich) was freshly prepared by mixing 0.5% (wt/vol) Oil Red O solution dissolved in isopropanol and distilled water at a ratio of 3:2, and then filtered. Liver sections (8 μm thick) were cut with a cryostat, fixed in 10% buffered formalin. Sections were rinsed with 60% isopropanol, stained with Oil Red O staining solution for 15 min, and then rinsed with 60% (vol/vol) isopropanol. Finally, sections were counterstained with hematoxylin, and then examined under a light microscope. For glycogen staining, periodic acid-Schiff stain was used. Liver sections (8 μm thick) were cut with a cryostat, fixed in methyl Carnoy's solution for 10 min and washed with deionized water. Sections were incubated in 0.5% (wt/vol) periodic acid solution for 10 min and washed with deionized water, then incubated in Schiff's reagent for 5 min and washed with deionized water. Finally, sections were dehydrated and then examined under a light microscope. Lack of periodic acid-Schiff (PAS) staining on adjacent sections pretreated with 0.5% (wt/vol) a-amylase solution (Sigma) for 20 min is considered to demonstrate glycogen.

Western Blotting. The liver tissues were homogenized in MAP kinase lysis buffer, and then centrifuged. Protein concentration was determined by the BCA protein assay (Pierce). Samples were subjected to SDS/PAGE (10% wt/vol) analysis, and then transferred to PVDF membranes. Membranes were incubated with specific primary antibodies to ketohexokinase (Atlas Antibodies), fatty acid synthase (Cell Signaling), and proteins were then revealed. Densitometry was done with Kodak Molecular Imaging software (Kodak).

Quantitative RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen), and then was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). Quantitative real time PCR was performed using i-Cycler and iQ SYBR Green Supermix (Bio-Rad). Primer pairs for mouse KHK-A and KHK-C described previously were used (1). Amplifications were performed for 1 cycle at 95 °C for 3 min and 45 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The results were normalized by β-actin expression.

Recombinant Protein and Kinetic Study. Recombinant proteins of human KHK-C and KHK-A were produced using the Profinity eXact fusion-tag system after transformation of BL21 (DE3) chemicompetent expression cells (Bio-Rad) with expression constructs. To purify KHK-C and KHK-A, the Bio-Scale Mini Profinity eXact cartridges were used on the Profinia protein purification instrument (Bio-Rad). Enzyme activity was measured at 37 °C in reactions containing 50 mM Pipes, 6 mM $MgCl_2$, 100 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 0.6 mM NADH, 10 units of pyruvate kinase, and 10 units of lactate dehydrogenase (5). Absorbance was measured at 340 nm. For measurement of ATP consumed in fructose metabolism, 100 ng or 1,000 ng of KHK-A or KHK-C was incubated with 5 mM fructose, 50 mM Pipes, 6 mM $MgCl_2$, 100 mM KCl, and 5 mM ATP at 37 °C. ATP levels were determined at various time points using ATP colorimetric assay kit (BioVision).

Ketohexokinase Activity Assay. KHK activity was measured by the method described previously (6, 7). In brief, the reaction mixture contained 50 mM imidazole buffer pH 7.0, 1.85 mM fructose, as substrate; 4 mM NaATP; 4 mM $MgCl_2$; 1M KAc, 20 mM *N*-acetylglucosamine, to inhibit the phosphorylation of fructose by hexokinase; 40 mM NaF; and 50 μg of liver protein. The mixture was incubated for 16 min at 37 °C, stopped by addition of 0.15 M $ZnSO_4$ and 0.1 M $Ba(OH)_2$. Samples were centrifuged and the residual fructose in the supernatant was measured according to Roe by addition of 0.1% alcoholic resorcinol and 30% HCl and absorbance read at 515 nm. Recombinant human KHK-C and recombinant KHK-C without NaATP and $MgCl_2$ were used as a positive and negative control, respectively.

Energy Balance. Measures of energy balance and fuel utilization were performed as we have described extensively in previous reports (8, 9). For measurements of energy balance, mice were acclimatized to the Colorado Nutrition Obesity Research Center animal satellite facility for several days before being placed in the eight-chamber metabolic monitoring system (Oxymax CLAMS-8M; Columbus Instruments). All mice remained in the chambers for at least 4 d, with at least 2 d of acclimatization to the new environment and at least 2 d for measurements of energy intake and expenditure. Forty-eight–h urine was collected in the chambers, and urinary urea nitrogen, creatinine, and fructose levels were determined. Metabolic rate (MR) was measured every 16 min and calculated with the Weir equation (MR = $3.941 \times vO_2 + 1.106 \times vCO_2 - 2.17 \times N$), where N = urinary nitrogen (10). MR averaged over the day was then extrapolated throughout the 24-h testing period to acquire estimates of total energy expenditure (TEE) described previously (8).

Statistical Analysis. All data are presented as the mean ± SEM. Independent replicates for each data point (n) are identified in Figs. S1–S3, Table S1, and Table S2. Data graphics and statistical analysis were performed using Prism 5 (GraphPad). Data without indications were analyzed by one-way ANOVA, Tukey's post hoc test. $P < 0.05$ was regarded as statistically significant.

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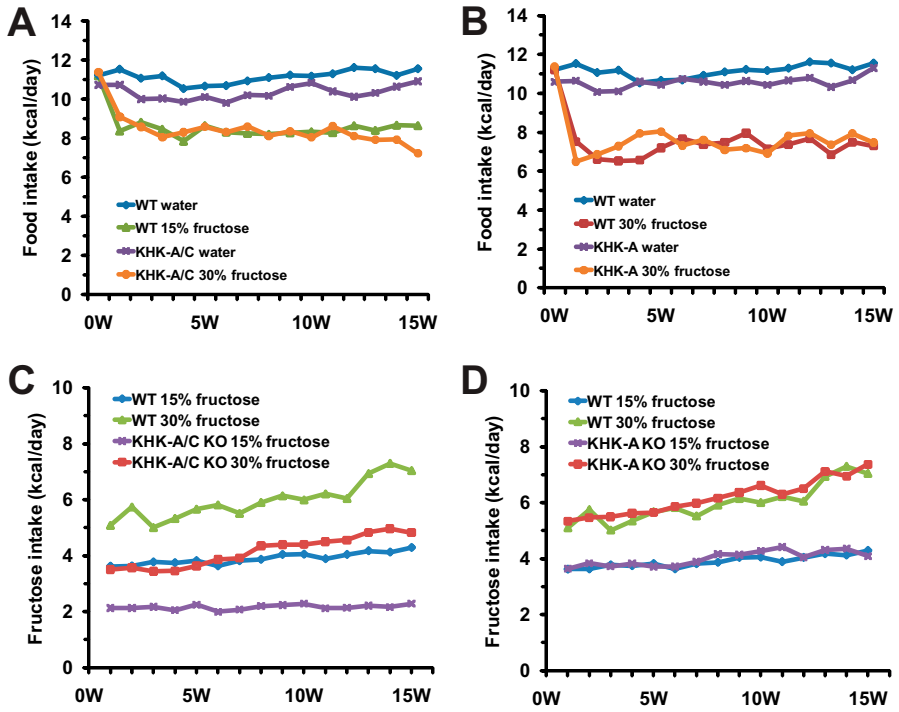

Fig. S1. WT mice, KHK-A/C KO mice, and KHK-A KO mice given ad libitum normal chow diet with 15 or 30% fructose water or tap water (n = 8–9). (*A* and *B*) Ad libitum energy intake of standard chow diet in WT mice and KHK-A/C KO mice (*A*) and in WT mice and KHK-A KO mice (*B*). (*C* and *D*) Ad libitum fructose intake in WT mice and KHK-A/C KO mice (*C*) and WT mice and KHK-A KO mice (*D*).

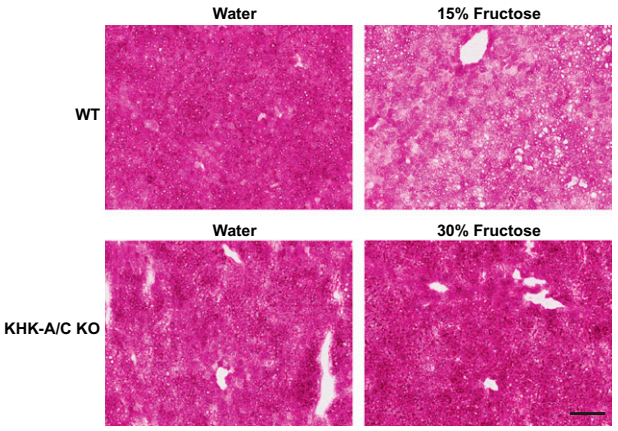

Fig. S2. WT mice and KHK-A/C KO mice given ad libitum normal chow diet with 15 or 30% fructose water, respectively, or tap water for 25 wk (n = 8–9). To observe the glycogen density in liver, periodic acid-Schiff stain was used. Representative images were shown. (Scale bar, 100 μm.)

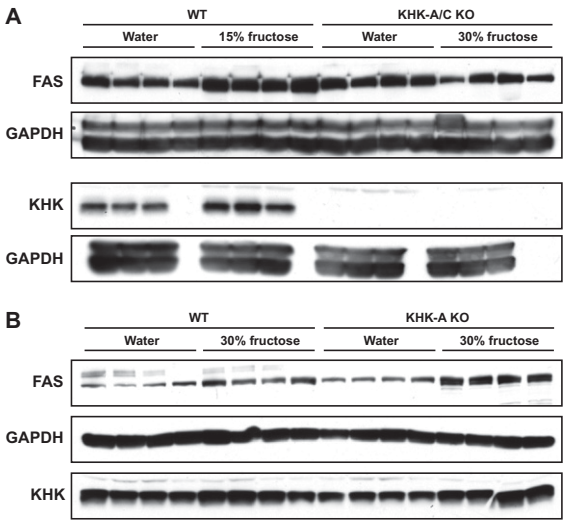

Fig. S3. WT mice, KHK-A/C KO mice, and KHK-A KO mice given ad libitum normal chow diet with 15 or 30% fructose water or tap water for 25 wk (n = 8–9). (*A*) Images of Western blot analysis of fatty acid synthase (FAS), ketohexokinase (KHK), and GAPDH in liver from WT mice and KHK-A/C KO mice (n = 3–4). (*B*) Images of Western blot analysis of FAS, KHK, and GAPDH in liver from WT mice and KHK-A KO mice (n = 4).

Table S1. Characteristics of 2-mo-old, male wild-type mice, KHK-A/C KO mice, and KHK-A KO mice

	WT (n = 6)	KHK-A/C KO (n = 5)	KHK-A KO (n = 6)	ANOVA
Body weight, g	23.4 ± 1.3	22.9 ± 1.4	23.0 ± 1.1	NS
Liver weight, g	0.89 ± 0.03	0.84 ± 0.08	0.90 ± 0.08	NS
Epididymal fat weight, g	0.38 ± 0.05	0.34 ± 0.07	0.32 ± 0.07	NS
Systolic blood pressure	101.1 ± 4.5	99.7 ± 8.5	104.2 ± 6.7	NS
AST, IU/l	36.0 ± 1.6	49.6 ± 7.8	37.4 ± 3.5	NS
ALT, IU/l	23.2 ± 1.1	25.0 ± 0.9	25.6 ± 1.0	NS
BUN, mg/dL	24.2 ± 1.7	26.6 ± 1.5	27.6 ± 2.7	NS
Creatinine, mg/dL	0.22 ± 0.02	0.20 ± 0.03	0.18 ± 0.04	NS
Uric acid, mg/dL	3.6 ± 0.4	3.6 ± 0.4	3.9 ± 0.2	NS
Glucose, mg/dL	197.7 ± 11.7	225.2 ± 12.8	225.4 ± 12.1	NS
Triglyceride, mg/dL	52.2 ± 2.3	42.6 ± 3.7	46.8 ± 2.0	NS
Total cholesterol, mg/dL	101.5 ± 4.2	98.0 ± 2.3	97.8 ± 3.4	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. NS, not significant.

Table S2. Characteristics of mice given ad libitum normal chow diet with 15 or 30% fructose water or tap water for 25 wk

	WT			KHK-A/C KO		KHK-A KO		WT 15% vs. KHK-A/C 30% (ANOVA)	WT 30% vs. KHK-A 30% (ANOVA)	KHK-A/C 30% vs. KHK-A 30% (ANOVA)
	Water (n = 9)	15% (n = 9)	30% (n = 9)	Water (n = 9)	30% (n = 8)	Water (n = 9)	30% (n = 8)			
Body weight and composition										
Body weight; 0W, g	23.1 ± 0.5	23.1 ± 0.4	23.2 ± 0.3	22.6 ± 0.5	23.0 ± 0.4	23.3 ± 0.3	23.3 ± 0.5	NS	NS	NS
Body weight; 25W, g	32.3 ± 2.0	35.2 ± 2.9*	36.1 ± 3.3*	30.0 ± 2.1	27.9 ± 1.8	31.5 ± 0.48	39.1 ± 1.94*	$P < 0.001$	NS	$P < 0.001$
ΔBody weight; 25W, g	9.2 ± 0.8	12.2 ± 0.7*	13.0 ± 0.9**	7.4 ± 0.5	5.3 ± 0.2**	8.2 ± 0.6	15.8 ± 1.5***	$P < 0.001$	NS	$P < 0.001$
Liver weight; 25W, g	1.24 ± 0.05	1.28 ± 0.05	1.42 ± 0.05*	1.08 ± 0.05#	1.08 ± 0.03	1.12 ± 0.03	1.57 ± 0.10***	$P < 0.05$	NS	$P < 0.001$
Lean mass; 19W, g	22.3 ± 0.9	23.0 ± 0.6	22.5 ± 0.6	20.8 ± 0.6	19.8 ± 0.4	21.8 ± 0.4	22.5 ± 0.7	$P < 0.05$	NS	$P < 0.05$
Fat mass; 19W, g	4.6 ± 0.6	6.7 ± 0.5*	7.7 ± 0.5**	4.4 ± 0.4	3.6 ± 0.3	5.6 ± 0.5	9.1 ± 1.0*	$P < 0.01$	NS	$P < 0.001$
Biochemical blood analysis										
AST, IU/l	60.3 ± 9.0	60.7 ± 13.4	48.1 ± 3.4	49.4 ± 6.1	44.1 ± 3.9	60.4 ± 10.4	49.1 ± 4.6	NS	NS	NS
ALT, IU/l	35.7 ± 6.7	34.0 ± 10.5	27.2 ± 2.6	27.4 ± 4.5	21.1 ± 1.9	34.8 ± 8.8	34.1 ± 8.5	NS	NS	NS
BUN, mg/dL	22.4 ± 1.5	21.9 ± 2.4	19.6 ± 0.6	25.6 ± 1.6	32.1 ± 1.6*	22.9 ± 1.3	19.6 ± 1.6	$P < 0.01$	NS	$P < 0.001$
Creatinine, mg/dL	0.19 ± 0.02	0.24 ± 0.03	0.23 ± 0.02	0.26 ± 0.02	0.26 ± 0.03	0.20 ± 0.02	0.26 ± 0.04	NS	NS	NS
Total cholesterol, mg/dL	128.2 ± 7.8	135.4 ± 4.8	149.9 ± 6.5	135.9 ± 8.5	79.9 ± 4.9***	126.8 ± 7.9	147.4 ± 10.4	$P < 0.001$	NS	$P < 0.001$
LDL cholesterol, mg/dL	57.1 ± 4.4	67.1 ± 4.3	84.3 ± 5.2*	54.6 ± 6.5	30.8 ± 2.1***	47.8 ± 4.9	76.1 ± 8.5*	$P < 0.001$	NS	$P < 0.001$
HDL cholesterol, mg/dL	59.4 ± 3.6	57.9 ± 3.8	63.1 ± 2.2	67.9 ± 3.4	39.2 ± 2.7***	47.0 ± 2.1	58.3 ± 3.1*	$P < 0.001$	NS	$P < 0.001$
Triglyceride, mg/dL	63.2 ± 4.2	62.6 ± 4.8	52.8 ± 2.4	80.7 ± 10.4	59.1 ± 6.5	60.4 ± 4.5	53.8 ± 3.3	NS	NS	NS
HbA1c, %	5.2 ± 0.04	5.6 ± 0.07**	5.3 ± 0.10	5.1 ± 0.13	4.9 ± 0.11	5.3 ± 0.06	5.2 ± 0.07	$P < 0.01$	NS	NS
Uric acid, mg/dL	4.2 ± 0.4	4.0 ± 0.7	3.4 ± 0.3	3.7 ± 0.3	3.0 ± 0.2	2.9 ± 0.2#	3.3 ± 0.3	NS	NS	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. respective water control (ANOVA). #$P < 0.05$ vs. WT water (ANOVA). NS, not significant.