Calcified Tissue International (2020) 106:541–552
https://doi.org/10.1007/s00223-020-00663-w

ORIGINAL RESEARCH

Bone Growth is Influenced by Fructose in Adolescent Male Mice Lacking Ketohexokinase (KHK)

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Received: 21 February 2019 / Accepted: 20 January 2020 / Published online: 29 January 2020

Abstract

Fructose is metabolized in the cytoplasm by the enzyme ketohexokinase (KHK), and excessive consumption may affect bone health. Previous work in calcium-restricted, growing mice demonstrated that fructose disrupted intestinal calcium transport. Thus, we hypothesized that the observed effects on bone were dependent on fructose metabolism and took advantage of a KHK knockout (KO) model to assess direct effects of high plasma fructose on the long bones of growing mice. Four groups ($n = 12$) of 4-week-old, male, C57Bl/6 background, congenic mice with intact KHK (wild-type, WT) or global knockout of both isoforms of KHK-A/C (KHK-KO), were fed 20% glucose (control diet) or fructose for 8 weeks. Dietary fructose increased by 40-fold plasma fructose in KHK-KO compared to the other three groups ($p < 0.05$). Obesity (no differences in epididymal fat or body weight) or altered insulin was not observed in either genotype. The femurs of KHK-KO mice with the highest levels of plasma fructose were shorter (2%). Surprisingly, despite the long-term blockade of KHK, fructose feeding resulted in greater bone mineral density, percent volume, and number of trabeculae as measured by μCT in the distal femur of KHK-KO. Moreover, higher plasma fructose concentrations correlated with greater trabecular bone volume, greater work-to-fracture in three-point bending of the femur mid-shaft, and greater plasma sclerostin. Since the metabolism of fructose is severely inhibited in the KHK-KO condition, our data suggest mechanism(s) that alter bone growth may be related to the plasma concentration of fructose.

Keywords Fructose · Ketohexokinase · Cyp24b1 · Cyp27a1

Introduction

During growth, adequate nutrition can ensure proper bone mass acquisition to reduce osteoporotic fracture risk later in life [1]. Fructose, a nutritive carbohydrate, has increasingly been consumed, as an element of the western diet, by

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00223-020-00663-w) contains supplementary material, which is available to authorized users.

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children in developed countries. Once consumed, most fructose absorption occurs in the lumen of the small intestine via transmembrane sugar transporters (GLUT2 and GLUT5), with adaptive expression of GLUT5 dependent on fructose consumption. Fructose travels to the liver via the portal vein and is metabolized by ketohexokinase (KHK). First-pass metabolism through the liver rapidly breaks down 50–75% of the load, reducing peripheral plasma fructose concentrations [2]. For an increasing number of adolescents, excessive fructose consumption may lead to non-alcoholic fatty liver disease, metabolic syndrome, obesity, insulin resistance, hypertension, atherosclerosis, and dyslipidemia and eventually, Type 2 Diabetes Mellitus [3]. Although these disorders have been associated with changes in bone growth and adaptation, the independent mechanism(s) by which fructose acts on bone are not fully understood.

Fructose may affect bone metabolism by disturbing the absorption, reabsorption, and excretion of essential vitamins and minerals necessary for healthy bone growth [1, 4]. In children and adolescents, fructose intake has been associated with increased fractures [5, 6]. While no studies have demonstrated a direct causal link, the literature on rodent models has suggested possible effects on bone cells and their precursors. For example, Felice et al. reported decreased number of active bone cells in vivo via histology, and after in vitro osteogenic cell culture of marrow from rats that drank 10% fructose water ad libitum, a level that induces the metabolic syndrome within weeks [4, 7-12]. In normal rodents, fructose affects vitamin D metabolism during periods of increased calcium demand (i.e., pregnancy, lactation, growth and calcium restriction). We have previously demonstrated that fructose reduced Vitamin D-dependent calcium transport in the gut during growth due to disruption of renal Vitamin D metabolism [4]. Vitamin D is activated into 1,25(OH) Vitamin D3 (1,25VitD3) by enzymes synthesized in the renal proximal tubule. As systemic calcium is tightly regulated, disruptions in calcium metabolism are detriments to bone [13]. However, chronic fructose consumption in humans and preclinical rodent models has not always produced detrimental effects (recently reviewed in [14, 15]).

While likely that high levels of ingestion are required to raise plasma fructose in normal individuals, people with mutations that result in lowered KHK protein expression, primarily in liver, small intestine, and kidney, are less able to handle fructose consumption and have elevated plasma concentrations [16]. Some of these people are diagnosed, most as adults, with the inborn metabolic disorder, Essential Benign Fructosuria (EBF) [17]. The KHK gene is mutually exclusively spliced into mRNA that result in isoforms A and C [18, 19]. KHK-C is primarily found in hepatocytes, enterocytes, renal proximal tubule cells, and white adipose tissue (WAT) cells and has the highest affinity for fructose of any hexokinase [20]. Fructolytic activity is accomplished peripherally without KHK-C by way of KHK-A or other hexokinases [14, 21, 22]. After breakdown of fructose by KHK, the following enzyme is aldolase B and the more serious disease of hereditary fructose intolerance (HFI) is due to deficiency of this enzyme's activity. Recently proposed and partially demonstrated in growing mice has been total blockade of KHK activity to treat HFI [23]. Logically, the blockade of such an important enzymatic activity would be expected to present its own sequelae. In this study, we begin to examine how blockade of KHK affects bone growth by utilizing KHK-KO that have both isoforms of KHK (KHK-A, KHK-C) deleted [21]. We hypothesized that in the absence of KHK, fructose feeding raises plasma levels and affects the ability of growing mice to build bone due to the direct effects of high levels in plasma. WT and KHK-KO mice were fed fructose to test effects on bone growth. We found plasma fructose rose to even greater levels than expected, long bones grew to shorter lengths, but unexpectedly, trabecular bone was increased and there was a potential benefit to cortical long bone mechanical properties when plasma fructose concentrations rose to high levels.

Materials and Methods

Genotyping

KHK-KO (background: C57BL6) mice were donated by R. J. Johnson, University of Colorado, and the generation of the model was previously described (Supplemental Methods) [21]. Both isoforms of KHK (KHK-A, KHK-C) were deleted, effectively abolishing fructose breakdown. Mice were introduced to diets at the commencement of the experiment at 4 weeks old.

Dietary and Experimental Design

The effects of fructose were examined in growing (4–12-week-old) male WT or KHK-KO mice (Supplemental Methods). Protocols were approved by the Institutional Animal Care and Use Committee (New Jersey Medical School, Rutgers). Two iso-caloric (equivalent kcal by weight), synthetic diets were designed, based on the standard American Institute of Nutrition (AIN)-93G formula containing other carbohydrates (Research Diets, New Brunswick, NJ; Table S1). Mice had ad libitum water access. After 8 weeks of feeding, mice were euthanized.

Tissue and Cell Collection

After sedation, blood was collected via cardiac puncture in tubes containing EDTA, and the inhibitors, protease (Pefabloc, Sigma) and DPP-IV (Millipore), at final concentrations

of 2 µg/mL and 0.02 µL/mL, respectively. Centrifugation was at 1300×*g* for 15 min and storage was at − 80° C. The small intestine was removed and flushed with cold PBS. Sections of duodenum and jejunum (Supplemental Methods), 1 cm each, were sampled and stored at -80° C in RNALater (Invitrogen, Thermo Fisher Scientific, Waltham, MA) for subsequent RNA extraction and gene expression analysis (Table S2). The right tibia was fixed in 10% neutral-buffered formalin for histomorphometry. The left femur was resected of muscle and placed in sterile ice-cold saline for flushing and cell culture and the right was wrapped in saline-soaked gauze and frozen for mechanical testing.

Plasma Analysis

Plasma fructose was determined by high-performance liquid chromatography (HPLC) of terminal blood draw as previously described (Supplemental Methods) [24]. Fluorescence was detected with GL-7453 (GL Sciences) set at an excitation and emission wavelength of 330 and 470 nm, respectively. Data were analyzed using EZChrom Elite version 3.1.5J (Agilent Technologies).

Plasma, bone, and gut markers were measured using the Milliplex multi-analyte profiling (MAP) kits MBNMAG-41K and MGTMAG-78K (EMDMillipore, Billerica, MA). Samples were incubated with antibody-coated magnetic beads overnight and then incubated with detection antibody (Supplemental Methods) and streptavidin–phycoerythrin 30 min for each subsequent incubation. Analysis was performed using MAGPIX® with xPONENT software.

Osteoblast Colony-Forming Assay

Left femur epiphyses were clipped, and the marrow flushed (Supplemental Methods). 2×10^6 cells were plated in 55-cm^2 petri dishes and pooled cell populations were grown in culture for 14 days. Cells were cultured in triplicate, with osteogenic media: α-MEM, supplemented with 10% fetal bovine plasma, 1% penicillin and 0.1% Amphotericin B, 50 µg/mL ascorbic acid, and 8 mM β glycerol phosphate. On day 5, media was changed, and non-adherent cells were removed. Osteoblast differentiation and activity were confirmed on day 14, when cells were fixed in methanol and stained for alkaline phosphatase (ALP) activity with a 2:1 NBT/BCIP (Sigma) dye buffered with 100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$.

Histomorphometry

Bones for histomorphometry were embedded in polymethyl-methacrylate (Sigma), trimmed, sectioned transversely at 25% of the length from proximal end (diaphysis) with a diamond saw (Isomet 5000, Buehler) and fine polished with silicon carbide abrasive paper and alumina slurries (successive particle diameters of 1.0 and 0.05 µm). Bone formation during the last week of feeding was assessed with dynamic histomorphometric measures using calcein labels that had been injected (i.p., 20 mg/kg of body weight, Invitrogen C-481) at 9 and 2 days prior to death to label bone-forming surfaces (osteoblast activity). Blocks were imaged with a reflective confocal microscope (A1, Nikon, Tokyo, Japan) at 20× and 60× magnification. Standardized bone parameter measurements were made on an interactive pen/tablet desktop workstation (Wacom Cintiq 21UX) using ImageJ software (Supplemental Methods) [25, 26]. Osteocyte lacunae between the calcein labels were point counted on 60× images.

µCT

Femurs were evaluated by µ-computed tomography (Bruker Skyscan 1172 120 µA, 80 keV, 0.5 mm aluminum filter) for density and morphology, at an isotropic voxel size of 8 µm (Supplemental Methods). Trabecular (Tb) and cortical (Ct) traits were measured at set distances from the distal growth plate. Two contiguous Tb regions of interest (ROIs) were hand drawn at the marrow/endosteal border. Starting 0.25 mm below the growth plate the distal ROI extended 1.75 mm and the distal ROI another 1.15 mm along the femur length. The Ct ROI began 2.15 mm distal to the growth plate and was 0.43 mm in depth along the femur.

Whole Bone Mechanical Testing

The mechanical properties of mid-diaphyseal femurs were quantified by loading to failure in 3-point bending at 0.05 mm/s until failure (Supplemental Methods) with an electro-mechanical actuator (Bose, Testbench, TA Instruments). The load–deflection curves were digitally sampled with the Bose WinTest software (Version X). Load–deflection curves were analyzed in MatLab (Version R2016b, Mathworks, Natick MA) for stiffness (the slope of the initial linear portion of the curve), strength (maximum load), post-yield deflection (deflection at failure minus deflection at yield, PYD), and work-to-failure (area under the curve prior to failure).

Real-Time RT-PCR

Total RNA was isolated from duodenum, jejunum, and kidney (Supplemental Methods). The primer list is detailed in Table S2. The relative abundance of each mRNA in each sample was then normalized according to the equation: Relative Quantity $RQ = 2^{-\Delta\Delta Ct}$ [27]. Ct values larger than 35 were removed (cut-off value > 35).

Analysis of Data

Researchers were blinded to specimen identity during sample collection, and subsequent analyses of samples were randomized in order to restrict biases (Supplemental Methods). Comparisons were analyzed with a two-way ANOVA and significant interactions and differences were determined by Tukey's post hoc method. Statistical significance is reported at $p < 0.05$, unless otherwise noted.

Results

Elevated Plasma Fructose with Feeding

After 8 weeks of feeding, plasma fructose was elevated in KHK-KO mice fed fructose (Fig. 1a). Plasma fructose concentrations were approximately 40-fold greater in KHK-KO mice fed 20% fructose compared with those fed the control diet of glucose. There were no genotype-dependent effects on plasma concentration in mice fed control diet. The fructose diet increased plasma fructose concentration in KO mice 4000% compared to WT fed glucose. KHK expression was increased in the duodenum and jejunum with fructose feeding in WT mice and, as expected, there was no measurable expression of KHK in KHK-KO (Fig. 1b). No differences were detected between groups in plasma concentrations of bone- and gut-derived cytokines after 8 weeks of feeding (Table S3).

Body weights were not different between any of the groups at weaning, and, as expected, body weight increased rapidly after weaning, during the 8 weeks of feeding (Fig. 2). Almost immediately after the onset of fructose feeding, a small but significant ($p < 0.05$) body weight reduction of 5–8% occurred in the KHK-KO mice. The reduced body weight persisted until 9 weeks of age (5 weeks of fructose feeding) at which point body weights equilibrated. At the end of the experiment, weights of individual organs were similar, except for the caecum which was enlarged in KHK-KO mice fed fructose (+200%, $p < 0.01$; Table S4).

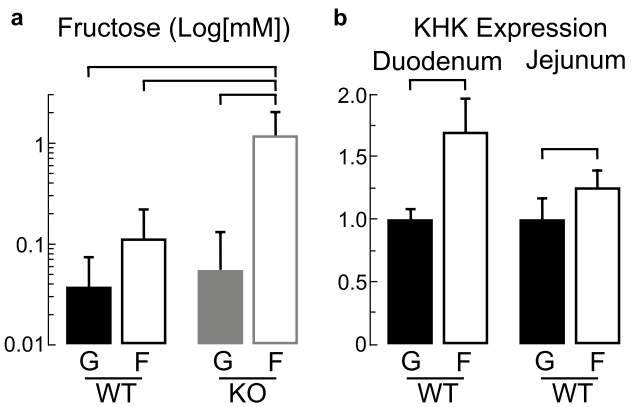

Fig. 1 **a** Plasma fructose concentration and **b** KHK expression (fold difference) in duodenum and jejunum for WT and KO (KHK not detectable in KO) mice fed for 8 weeks on a 20% glucose (G, control) or 20% fructose (F) diet started after weaning at 4 weeks of age ($n = 6$–8 mice per group; means ± SD). Bars indicate significant differences between groups determined by ANOVA and Tukey's post hoc test ($p < 0.05$)

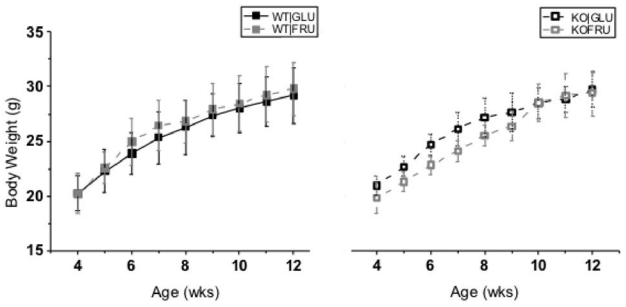

Fig. 2 Body weight for WT and KO mice fed for 8 weeks on a 20% glucose (G, control) or 20% fructose (F) diet started after weaning at 4 weeks of age ($n = 10$–12 mice per group; means ± SD). KO mice had lower body weight after the first week of feeding with fructose and recovered to the WT glucose level after the fifth week of feeding ($p < 0.05$, KO fructose vs. all other groups)

Effects of Fructose on Osteogenic Bone Cells

Osteogenic colonies detected by the presence of alkaline phosphatase demonstrated that cells from WT and KHK-KO mice fed fructose had significantly reduced OB-like colony counts compared to glucose-fed WT mice (Table 1). In contrast, the average colony size was similar between the 4 groups. Osteocyte lacunar density (#/mm^2) between double calcein labels was similar among groups (WT/Glucose: 991 ± 119; WT/Fructose: 968 ± 228; KO/Glucose: 978 ± 162; KO/Fructose: 1077 ± 295).

Reduced Femur Length in KHK-KO with Fructose

Longitudinal growth of the long bones was inhibited in KHK-KO mice by fructose feeding such that the lengths of femurs were reduced by 2.4% when compared to glucose-fed KHK-KO and by 2.9% when compared to WT fed fructose ($p < 0.05$; Fig. 3a). Despite these growth differences, food intake relative to body weight over the early period of feeding was slightly increased in the fructose-fed mice, regardless of genotype (Fig. 3b), though there were no differences at the conclusion of the study.

Table 1 Osteogenic potential of bone marrow cells measured by osteoblast colony formation unit (Ob-CFU) cultures

	WT		KHK-KO		*p* values		
	Glucose	Fructose	Glucose	Fructose	Diet	Genotype	Interaction
Count	1.00 ± 0.25^{a}	0.54 ± 0.35^{b}	$0.87 \pm 0.37^{a,b}$	$0.64 \pm 0.35^{a,b}$	0.002	ns	ns
Average size	1.00 ± 0.43	0.92 ± 0.43	1.13 ± 0.11	0.75 ± 0.12	ns	ns	ns
Total area	1.00 ± 0.57	0.48 ± 0.61	0.94 ± 0.30	0.46 ± 0.27	0.004	ns	ns

Primary bone marrow cells were cultured in osteogenic media for 14 days. Stained areas were normalized to WT glucose control and are represented as means ± SD. Comparisons by two-way ANOVA. Similar superscripts indicate values that are not significantly different from each other by Tukey's post hoc test

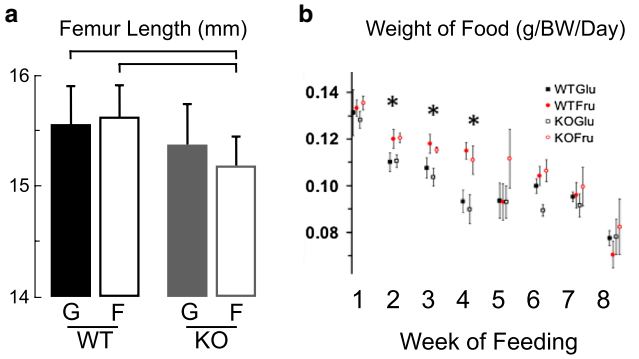

Fig. 3 **a** Femur lengths and **b** food consumption of WT and KO mice fed for 8 weeks on a 20% glucose (G, control) or 20% fructose (F) diet started after weaning at 4 weeks of age (panel **a**, $n = 10$–12 mice per group; panel **b**, $n = 3$–4 cages per group, means ± SD). Weight of daily food provided was normalized to the body weight (BW) of all mice in a cage. Fructose-fed mice ate more than glucose-fed mice in weeks 2–4 only. Significant differences between groups are determined by ANOVA with Tukey's post hoc test ($p < 0.05$)

Effects of Fructose on Metaphysis

Fructose had its greatest impact on the morphology of cancellous bone (Fig. 4). Fructose feeding affected the bone mineral density (BMD) and BV/TV of cancellous bone, more so in the KO than WT mice, and in the distal versus proximal ROIs. Cancellous BV/TV was greater with fructose feeding ($p < 0.05$) in KHK-KO by 91% and 61% in the distal and proximal ROIs, respectively. The increased volume was driven mainly by the increased Tb.N. The connectedness of the trabeculae was significantly increased by fructose with KHK-KO mice having higher connectivity in the distal trabecular ROI. This is reflected by the reduced pattern factor between the fructose-fed KHK-KO versus all other groups.

Effects of Fructose on Diaphysis

The endosteal perimeter of fructose-fed mice, both WT and KHK-KO, was significantly smaller (− 11%, $p < 0.05$) than glucose-fed controls (Table 2). However, there were no differences in MS, MAR, or BFR, measured in the final week of growth. Consistent with the effect of fructose feeding on endosteal perimeter, fructose feeding influenced ($p < 0.05$) the fraction of cross-sectional cortical bone area to total area (%Ct. Ar) measured by μCT without affecting total area in KHK-KO mice (13%, $p < 0.01$). Since there were no differences in total area or periosteal perimeter, the polar moment of inertia was unaffected. Three-point bending of femurs of WT and KHK-KO fed either glucose or fructose demonstrated similar structural mechanical properties. There were no significant differences in the load to failure, stiffness, and elastic or plastic displacement, regardless of diet or genotype (Table S5).

Expression of Bone Relevant Genes in Kidney and Gut

Expression of renal genes encoding for vitamin D metabolism was significantly different in KHK-KO fructose-fed mice, compared to all other groups (Fig. 5). The expression of Cyp27b1 (catalyzing the conversion of 25-vitamin D to 1,25-vitamin D) was significantly lower (~ 80%) in KHK-KO fed fructose. The gene that encodes the Vitamin D degrader (Cyp24a1) was significantly increased (~ 300%) in the KHK-KO mice fed fructose. Renal expression for the calcium-binding protein Calbindin D9K (CaBP9k) was decreased (> 50%) in fructose-fed KHK-KO compared to WT (Table 3). Expression of the apical calcium transporter, transient receptor potential vanilloid 5 (Trpv5), was significantly greater in KO mice compared to WT (~ 40%). Differences in renal gene expression were not associated with any variation in kidney weights (Table S4).

As expected, Glut5 in the duodenum and jejunum was significantly increased with fructose feeding in WT and not in KO. Intestinal expression of the calcium transporters Trpv6 and CaBP9k was unaffected by sugar type regardless of genotype. Jejunal expression of tryptophan hydroxylase 1 (Tph1), the rate-limiting enzyme for serotonin synthesis, was also not affected.

Relationships Between Plasma Fructose and Bone Parameters

To explore the large variation in plasma fructose levels in KHK-KO mice fed either glucose or fructose, we examined

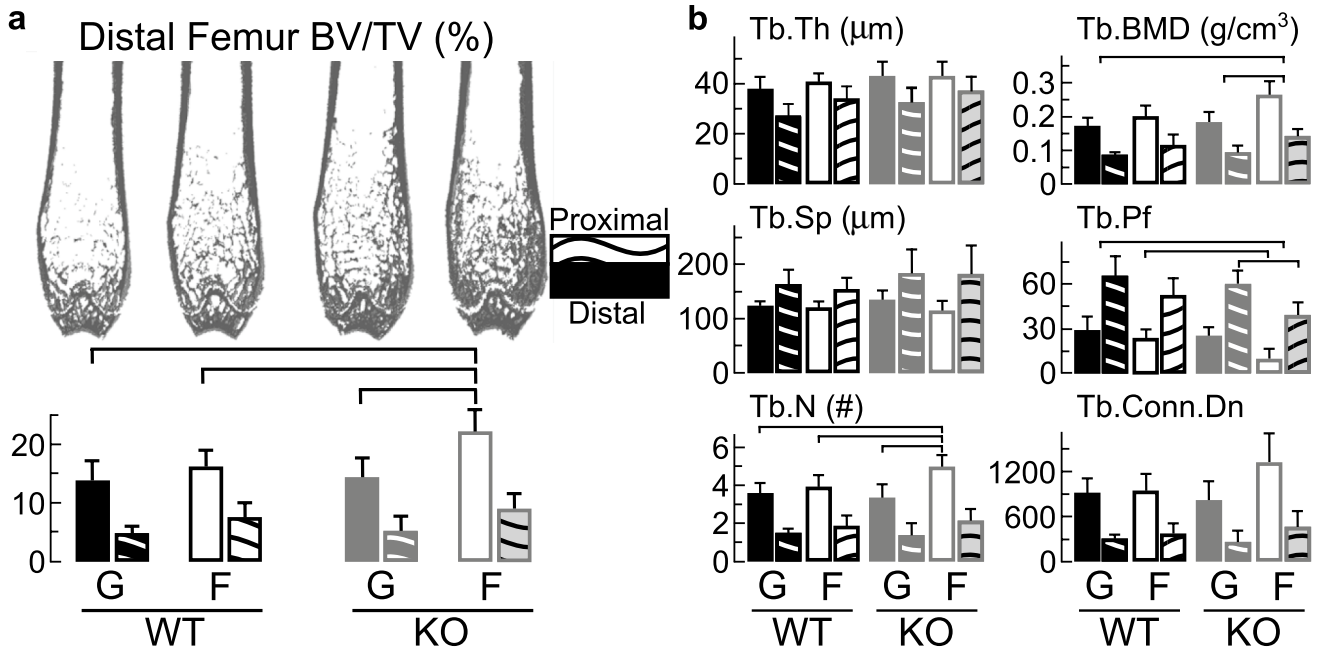

Fig. 4 **a** 3D coronal reconstructions of 100 μm thick sections imaged with μCT from distal femurs of WT and KO mice fed for 8 weeks on a 20% glucose (G, control) or 20% fructose (F) diet started after weaning at 4 weeks of age. Plotted are trabecular bone volume fraction (BV/TV) of distal (solid box plots) and proximal ROIs. **b** Distal and proximal trabecular (Tb) morphology (Th, Sp, N), composition (BMD) and connectivity (Pf, Conn.Dn). Significant differences between groups are determined by ANOVA with Tukey's post hoc test ($p < 0.05$; $n = 10$–12 mice per group; means ± SD)

Table 2 Femoral and tibial cortical measurements made by μCT, and histomorphometry ($n = 10$–12 mice per group; means ± SD)

	WT		KHK-KO		*p* values		
	Glucose	Fructose	Glucose	Fructose	Diet	Genotype	Interaction
μCT							
Femoral cortical							
Ct.BMD (g/cm^3)	0.98 ± 0.07	0.98 ± 0.06	0.99 ± 0.03	0.98 ± 0.09	ns	ns	ns
Ct.B.Ar (mm^2)	0.87 ± 0.06	0.96 ± 0.10	0.96 ± 0.12	1.03 ± 0.08	0.044	0.035	ns
Ct.M.Ar (mm^2)	1.82 ± 0.29	2.08 ± 0.28	2.21 ± 0.37	1.92 ± 0.24	ns	ns	0.023
Ct.Tt.Ar (mm^2)	2.69 ± 0.34	3.03 ± 0.38	3.17 ± 0.49	2.94 ± 0.29	ns	ns	0.048
Ct.Ar (%)	32.6 ± 2.1^{a,b}	31.6 ± 1.4^{a,b}	30.4 ± 1.5^{a}	34.2 ± 1.5^{b}	0.036	ns	0.001
Ct.MMI (mm^4)	0.66 ± 0.13	0.83 ± 0.20	0.87 ± 0.23	0.83 ± 0.12	ns	ns	ns
Ct.Th (μm)	97.0 ± 3.8	95.2 ± 2.8	101.7 ± 7.8	101.2 ± 11.3	ns	ns	ns
Histomorphometry							
Tibial endosteal							
Perimeter (mm)	4.51 ± 0.37 ^{a,b}	4.31 ± 0.45^{a}	5.01 ± 0.69^{b}	4.48 ± 0.24 ^{a,b}	0.047	ns	ns
MS (%)	0.69 ± 0.22	0.74 ± 0.11	0.68 ± 0.17	0.67 ± 0.16	ns	ns	ns
MAR (μm/day)	1.46 ± 0.34	1.62 ± 0.38	1.25 ± 0.40	1.71 ± 0.49	ns	ns	ns
BFR (μm^3/μm^2/day)	1.04 ± 0.47	1.19 ± 0.33	0.91 ± 0.45	1.18 ± 0.48	ns	ns	ns
Tibial periosteal							
Perimeter (mm)	7.03 ± 0.56	7.17 ± 0.46	7.28 ± 0.63	7.27 ± 0.41	ns	ns	ns
MS (%)	0.22 ± 0.05	0.21 ± 0.04	0.27 ± 0.05	0.20 ± 0.08	ns	ns	ns
MAR (μm/day)	1.27 ± 0.29	1.37 ± 0.44	1.12 ± 0.36	1.59 ± 0.31	ns	ns	ns
BFR (μm^3/μm^2/day)	0.30 ± 0.09	0.29 ± 0.10	0.30 ± 0.12	0.37 ± 0.13	ns	ns	ns

Means were compared with two-way ANOVA. Similar superscripts indicate values that are not significantly different from each other at $p < 0.05$ by Tukey's post hoc test

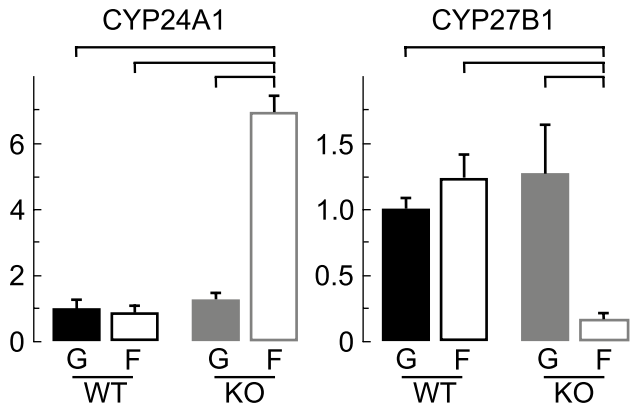

Fig. 5 Renal gene expression of Vitamin D-metabolizing enzymes from mice fed for 8 weeks on a 20% glucose (G, control) or 20% fructose (F) diet started after weaning at 4 weeks of age ($n=6$–8 mice per group; means ± SD). Significant differences between groups are determined by ANOVA with Tukey's post hoc test ($p<0.05$)

relationships between plasma fructose and bone parameters (Fig. 6, Table 4), including ultimate load ($R^2=0.48$, $p<0.02$), post-yield displacement ($R^2=0.49$, $p<0.02$), and post-yield and total work-to-failure ($R^2=0.55$, $R^2=0.57$, $p<0.01$). Sclerostin (SOST), an inhibitor of bone formation produced by osteocytes and involved in the FGF23/1,25VitD3 kidney to bone cytokine signaling axis, also demonstrated a strong positive relationship with plasma fructose levels ($R^2=0.58$, $p<0.01$). Significant, strong relationships between plasma fructose and trabecular bone μCT measurements were also observed (Tb.BMD: $R^2=0.71$, $p<0.001$, Distal Tb.BV/TV: $R^2=0.62$, $p<0.01$).

Discussion

Increased fructose in the KHK-KO impaired the attainment of peak bone length but increased mineral content of trabecular bone during growth. In humans, the attainment of these peaks contributes to osteoporosis prevention and a healthy lifespan [28, 29]. Importantly, our mouse study utilized a calorie-matched, 20% fructose-in-food diet, presenting a fructose level attained with some current western diets, and a lower level than often studied, probably due to a lack of association with obesity, early-onset metabolic syndrome, non-alcoholic fatty liver disease, or diabetes. Thus, as opposed to work with higher fructose concentration [12], we did not observe effects on plasma insulin, or the weights of the epididymal WAT pad, or liver (Tables S3 and S4).

The data (Figs. 1 and 3) also do not support our original hypothesis, that consumption of 20% dietary fructose by WT mice for 8 weeks would not only raise plasma levels, but affect normal bone growth. However, the data unveiled a previously unrecognized, possibly direct effect that may not require fructose metabolism in central (intestine and liver) or peripheral tissues to affect bone growth. The KHK-KO (both isoforms) does not possess the primary mechanisms for central metabolism and control of circulating fructose, and peripheral fructolytic activity is

Table 3 Fold differences (normalized to WT glucose) in renal and intestinal expression of genes involved in fructose and calcium metabolism ($n=6$–8 mice per group; means ± SD)

	WT		KHK-KO		*p* values		
	Glucose	Fructose	Glucose	Fructose	Diet	Genotype	Interaction
Kidney							
Khk	1.00 ± 0.34	1.34 ± 0.68	nd	nd	ns	–	–
Glut5	1.00 ± 0.28	1.19 ± 0.35	1.39 ± 0.49	1.20 ± 0.26	ns	ns	ns
CaBP9k	1.00 ± 0.23^{a}	1.24 ± 0.40^{a}	0.83 ± 0.51^{a,b}	0.49 ± 0.14^{b}	ns	0.003	0.043
Trpv5	1.00 ± 0.25^{a}	1.19 ± 0.46^{a}	1.67 ± 0.32^{a,b}	1.85 ± 0.78^{b}	ns	0.004	ns
Klotho	1.00 ± 0.29^{a}	1.20 ± 0.21^{a,b}	1.37 ± 0.13^{b}	1.17 ± 0.21^{a,b}	ns	ns	0.030
Napi-2a	1.00 ± 0.26^{a}	1.02 ± 0.38^{a}	1.89 ± 0.90^{b}	1.28 ± 0.24^{a,b}	ns	0.006	ns
G6pc	1.00 ± 0.12	1.01 ± 0.14	1.56 ± 0.50	1.29 ± 0.15	ns	ns	ns
Duodenum							
Glut5	1.00 ± 0.36^{a}	8.82 ± 5.75^{b}	1.28 ± 0.50^{a}	1.09 ± 0.35^{a}	0.003	0.003	0.002
CaBP9k	1.00 ± 1.32	1.93 ± 2.01	2.09 ± 2.16	1.09 ± 1.59	ns	ns	ns
Trpv6	1.00 ± 0.94	1.13 ± 0.93	2.14 ± 1.09	1.36 ± 0.87	ns	ns	ns
Jejunum							
Glut5	1.00 ± 0.38^{a}	3.90 ± 0.94^{b}	1.00 ± 0.53^{a}	1.00 ± 0.35^{a}	0.002	0.002	0.002
CaBP9k	1.00 ± 0.54	1.33 ± 1.52	1.57 ± 1.12	0.46 ± 0.24	ns	ns	0.025
Trpv6	1.00 ± 0.32	0.71 ± 0.26	1.83 ± 2.17	1.18 ± 1.02	ns	ns	ns
Tph1	1.00 ± 0.09	0.96 ± 0.08	0.99 ± 0.12	1.04 ± 0.07	ns	ns	ns

Means were compared with two-way ANOVA. Similar superscripts indicate values that are not significantly different from each other ($p>0.05$ by Tukey's post hoc test)

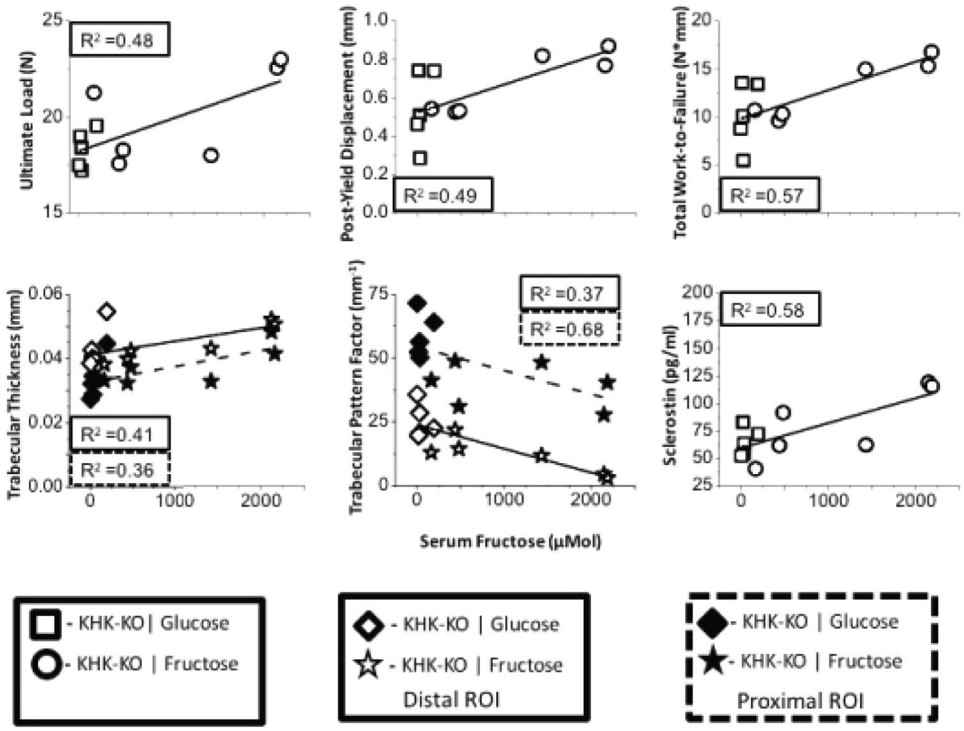

Fig. 6 Relationships ($p < 0.05$) between bone parameters and plasma fructose in KHK-KO mice. For trabecular thickness and pattern factor, open and closed shapes refer to the distal and proximal ROIs, respectively (see Fig. 4)

also substantially inhibited. Peripheral plasma fructose concentrations rose over 40-fold, to the mM range in this current study (Fig. 1a). These data confirm the recent demonstration that as intestinal capacity to cope with fructose is overwhelmed, fructose plasma concentration rises [14, 30]. Similar to our findings in KHK-KO mice, the plasma fructose concentrations are elevated in individuals diagnosed with EBF [31, 32]. Increased plasma fructose in the mice may be responsible for the significant bone growth effects, including decreased long bone length, and increased trabecular volume fraction with altered pattern factor after 8 weeks of feeding (Figs. 3 and 4).

Plasma fructose concentrations are rarely accurately measured in rodents or humans and, based on this study and one previous, may be highly variable despite utilization of controlled-content diets [33]. We utilized HPLC to isolate fructose from other sugars and metabolites found in plasma. Though more labor-intensive and costly than enzymatic assays that measure fructose by direct metabolism of fructokinase or indirect sugar conversion [24], the high sensitivity of HPLC is required to measure concentrations that do not normally exceed 100 µM. Additionally, in the current study, this high sensitivity allowed possible relationships between fructose and bone to emerge (Fig. 6 and Table 4). Feeding fructose at a supra-physiological level, 60% or greater to WT rats for 4–6 weeks, also elevates plasma fructose to the mM range [9, 34]. Supporting the hypothesis that an elevated plasma fructose level contributes to inhibiting long bone growth, our group reported that a high, chronic diet (63%, 4 weeks) reduced femur length by ~5% in rapidly growing male rats [35].

In the present study, there were no differences in gene expression for the calcium transport genes Trpv6 in the jejunum or CaBP9K and Trpv6 in the duodenum, confirming our previous findings [4, 9]. However, in the kidney, Trpv5 was greater and CaBP9K lower by half in the fructose-fed KO compared to the age-matched, WT fed glucose (Table 3). CaBP9K and Trpv5 are Vitamin D-dependent calcium transporters. Previously, we also found fructose-dependent reductions in Vitamin D receptor binding to the promoter of CaBP9K [34].

Fructose consumption in KHK-KO also resulted in reduced and increased renal expression of Cyp27b1 and Cyp24a1 genes, respectively, confirming the effects we observed previously and associated with reduced systemic levels of 1,25(OH)Vitamin D3 (1,25VitD3) [4, 9, 33-35]. There are strong relationships between these Cyps and the bone morphological parameters (Table S6). The Cyp27b1 gene encodes for a mitochondrial cytochrome P450 enzyme, 1α-hydroxylase, which hydroxylates 25-hydroxyvitamin D3 at the 1α position to its active form of 1,25VitD3. 1α-hydroxylase-KO mice have shortened long bones and increased trabecular BV/TV on high-calcium diets [36-38]. Osteogenic expansion of their marrow in the ob-CFU assay also leads to fewer number of ALP-stained colonies [36]. The poor growth associated with Cyp27b1 deficiency could account for decreased long bone length in our work, though the effect previously

Table 4 Significant ($p < 0.05$) relationships between plasma fructose and other measured study parameters

	r^2			*p* values		
	All	WT	KO	All	WT	KO
Mechanical testing						
Ultimate load	+**0.20**	+0.10	+**0.48**	0.037	0.354	0.018
Max. displacement	+**0.20**	+0.14	+**0.53**	0.043	0.279	0.012
PY displacement	+0.09	−0.06	+**0.49**	0.245	0.485	0.016
PY work	+**0.19**	−0.05	+**0.55**	0.040	0.509	0.009
Total work	+0.17	−0.06	+**0.57**	0.059	0.475	0.007
µ*CT*						
Ct.B.Ar	+**0.18**	+0.19	+0.16	0.046	0.162	0.217
Ct.Th	+**0.18**	−0.01	+0.12	0.042	0.709	0.290
Distal.Tb.BMD	+**0.60**	+**0.42**	+**0.71**	0.001	0.023	0.001
Distal.Tb.BV/TV	+**0.52**	+0.32	+**0.62**	0.001	0.053	0.004
Distal.Tb.Th	+**0.38**	+0.10	+**0.36**	0.002	0.316	0.050
Distal.Tb.Sp	+0.01	−**0.45**	−0.01	0.943	0.017	0.895
Distal.Tb.N	+**0.27**	+**0.38**	+0.31	0.011	0.034	0.073
Distal.Tb.Pf	−**0.47**	- 0.19	- **0.68**	0.001	0.159	0.002
Proximal.Tb.BMD	+**0.23**	+**0.64**	+0.27	0.021	0.002	0.101
Proximal.Tb.Th	+**0.32**	+0.16	+**0.41**	0.005	0.192	0.035
Proximal.Tb.N	+0.01	+**0.84**	+0.01	0.595	0.001	0.759
Proximal.Tb.Conn.Dn	−0.01	+**0.78**	−0.01	0.881	0.001	0.742
Proximal.Tb.Pf	−**0.26**	−0.29	−**0.37**	0.013	0.070	0.048
Histomorphometry						
Oc.Lc.Dn/Avg.BFR	+**0.35**	−0.01	+**0.60**	0.020	0.959	0.025
Bone markers						
SOST	+**0.54**	+0.19	+**0.58**	0.001	0.185	0.007
FGF23	+0.06	+**0.40**	+0.04	0.300	0.037	0.598
Weights						
Kidney	−0.01	+**0.45**	−0.03	0.882	0.023	0.607
Liver	−0.01	+**0.35**	−0.03	0.578	0.041	0.598
Caecum	+**0.65**	+0.19	+**0.58**	0.001	0.151	0.007
Gene expression						
Renal Cyp24a1	+**0.41**	+**0.67**	+0.25	0.002	0.004	0.145
Renal Trpv5	+**0.21**	+0.14	+0.08	0.041	0.279	0.419
Duodenal Trpv6	−0.04	+**0.41**	−**0.49**	0.349	0.024	0.017
Jejunal Khk	+**0.47**	+**0.47**	nd	0.014	0.014	–
Jejunal CaBP9k	−**0.17**	−0.12	−0.23	0.049	0.276	0.140

described in Cyp27b1 knockout mice is much greater [39, 40]. Cyp24a1 is the gene responsible for degrading active 1,25VitD3 to its inactive form 24,25VitD and a regulator is FGF23, an endocrine cytokine produced by osteocytes that binds with a renal co-factor Klotho produced in the kidney, to reduce systemic 1,25VitD3 and to increase phosphate clearing [41]. No differences in systemic FGF23 (Table S3) or Klotho (Table 3) expression were detected. Thus, overall, our data raise the important question of how circulating fructose with a limited ability for metabolism can mediate regulators of Vitamin D with the possible effect of lowering calcium reabsorption in the kidney. To help answer this question in future work will require accurate measurement of not only Vitamin D (25VitD and 1,25VitD3), but also parathyroid hormone, tissue non-specific ALP, calcium, and phosphate in plasma.

Generally, studies that found detrimental effects on growing bone in other rodent models included increased fat and calories by introducing sugars in ad libitum drinking water [7, 8, 10, 11, 42]. Bass et al. fed two-month-old rats for 12 weeks into skeletal maturity and found that a 40% fructose diet (with 10% glucose) increased distal femur BV/TV (+14%) and trabecular thickness (+16%) with no differences demonstrated by dynamic histomorphometry [12]. Our group found that this level of fructose would not be tolerated by the KHK-KO for more than a few days [20]. To our

knowledge, no other groups have specifically examined the effects of fructose alone on bone growth.

Fructose feeding reduced the number of potential osteogenic progenitor cells in an Ob-CFU assay of bone marrow cells (Table 1). Felice et al. made similar findings with a high-fructose diet that was not matched for calories [7, 8]. This potential reduction of in vitro primary osteoblasts did not manifest in any in vivo mineral apposition or bone formation rate differences in either study. While interesting, the differences may simply reflect a loss of progenitor cell plasticity after removal from their niche and introduction to an osteogenic media, or a lag in the ability of these progenitors to spread and attach based on their biophysical traits that should be quantified in future studies [43]. Though we did not detect overall significant differences in plasma sclerostin (SOST; Table S3), there is a relationship between elevated plasma fructose and elevated plasma sclerostin (Fig. 6). Sclerostin is an inhibitor of osteoblast activation and knocking out the gene in mice leads to increased cortical bone mass [44]. The elevation of systemic sclerostin in fructose-fed mice could reduce the potential of bone marrow cells to commit to the osteogenic lineage. These studies should be repeated with pooling of marrow from multiple bones of individual mice to examine whether the variability between individual mice in blood fructose levels might explain differences in progenitor cell populations in the bone marrow. While we found no differences in dynamic histology measurements, bones from these mice should also be examined in future studies for osteoblast and osteoclast numbers, and osteoid surface.

Several other limitations exist in this study. Plasma collection from each mouse was limited to 100–300 µL. Thus, we utilized the most economical assays of HPLC and magnetic enzyme-linked analysis. Our bone plasma parameters were limited in that we were unable to measure osteocalcin and RANKL, due to conflict arising from dilution and cross reactivity, and active 1,25VitD3 and tartrate-resistant acid phosphatase 5b, due to volume limitations. The possibility exists that KHK-KO did not adjust as quickly to the fructose diet as indicated by smaller body weights early on in feeding. All other measurements were completed at the end of the 8 weeks of feeding and after body weights had quickly equilibrated so that all groups had equivalent weights (Fig. 2). We recently found that, during the first 3 weeks after fructose-diet introduction, daily water intake is significantly greater in KHK-KO versus WT. Though we are not aware of studies directly demonstrating that increased water intake affects bone growth, this difference in drinking pattern may be indicative of hyperosmotic plasma. Hyperosmotic microenvironments alter biophysical cell properties by shrinkage [43]. Additional fructose also alters gut microbiota composition and metabolism [45]. The metabolic by-products of these alterations may be utilized to benefit the host. These issues deserve further study. Experiments in male mice thus far should be repeated in females and results should be verified at younger ages to ascertain growth dynamics at the growth plate and determine whether the effects on jejunum, kidney, and bone are cumulative as plasma fructose levels rise or could be reversed.

In conclusion, fructose affected bone independent of fructose metabolism in mice. In KHK-KO, dysregulated fructose metabolism raised the concentration of the sugar ~ 40-fold with fructose diet (Fig. 1a). Like observations after chronic fructose feeding of rats, our KHK-KO mice demonstrated reduced bone length and perturbation of Vitamin D metabolism, possibly indicating a direct interaction between fructose and the proximal tubule cells (Figs. 2a and 5; Table 3). The bones of KHK-KO mice with the highest levels of fructose were shorter in stature, but had increased trabecular bone volume, similar to mice with Cyp27b1 gene deletion. Because diets free of fructose are becoming very difficult to attain, those with inhibited fructose metabolism due to downstream enzyme defects that result in HFI may benefit from newly proposed treatment that blockades KHK [23]. Surprisingly, the long-term blockade appears to have only minor consequence for bone growth, and further study of the effects of temporarily altering the entry of fructose into its breakdown pathway or increasing plasma fructose on bone and other organs should be made.

Acknowledgements Publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health (NIH Award Numbers: AR063351, AR074670). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We thank Drs. Joseph Geissler, George Pellegrino, Stephen Flowers, Patricia Buckendahl, and Chirag Patel, as well as Brian Canter, Mayuri Kinkhabwala, Juby Roy, David Sadegh, Timothy Ngoge, Alexander Kheshvadjian, Joe Lumuti, and Luke Fritzky for technical assistance.

Author Contributions The manuscript was approved by all authors, whom are solely responsible for the decision to submit for publication. There are no other persons who satisfied the criteria for authorship but are not listed. EW, VD, RF, and JF contributed to study design; EW, KS, HI, FD, XZ, and KK were involved in data collection; EW, VD, RF, and JF performed data analysis and interpretation; EW wrote the first draft; and VD, RF, and JF were involved in critical editing of the final version of the manuscript.

Compliance with Ethical Standards

Conflict of interest Edek A. J. Williams, Veronique Douard, Keiichiro Sugimoto, Hiroshi Inui, Fabienne Devime, Xufei Zhang, Kunihiro Kishida, Ronaldo P. Ferraris, and J. Christopher Fritton declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent Animal care and experimental protocols were approved by the Rutgers Institutional Animal Care and Use Committee (IACUC) and were done in accordance with institutional guidelines and under the supervision of authorized investigators.

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