Excessive fructose intake causes 1,25-(OH)_2D_3-dependent inhibition of intestinal and renal calcium transport in growing rats

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Douard V, Sabbagh Y, Lee J, Patel C, Kemp FW, Bogden JD, Lin S, Ferraris RP. Excessive fructose intake causes 1,25-(OH)_2D_3-dependent inhibition of intestinal and renal calcium transport in growing rats. Am J Physiol Endocrinol Metab 304: E1303–E1313, 2013. First published April 9, 2013; doi:10.1152/ajpendo.00582.2012.—We recently discovered that chronic high fructose intake by lactating rats prevented adaptive increases in rates of active intestinal Ca^{2+} transport and in levels of 1,25-(OH)_2D_3, the active form of vitamin D. Since sufficient Ca^{2+} absorption is essential for skeletal growth, our discovery may explain findings that excessive consumption of sweeteners compromises bone integrity in children. We tested the hypothesis that 1,25-(OH)_2D_3 mediates the inhibitory effect of excessive fructose intake on active Ca^{2+} transport. First, compared with those fed glucose or starch, growing rats fed fructose for 4 wk had a marked reduction in intestinal Ca^{2+} transport rate as well as in expression of intestinal and renal Ca^{2+} transporters that was tightly associated with decreases in circulating levels of 1,25-(OH)_2D_3, bone length, and total bone ash weight but not with serum parathyroid hormone (PTH). Dietary fructose increased the expression of 24-hydroxylase (CYP24A1) and decreased that of 1α-hydroxylase (CYP27B1), suggesting that fructose might enhance the renal catabolism and impair the synthesis, respectively, of 1,25-(OH)_2D_3. Serum FGF23, which is secreted by osteocytes and inhibits CYP27B1 expression, was upregulated, suggesting a potential role of bone in mediating the fructose effects on 1,25-(OH)_2D_3 synthesis. Second, 1,25-(OH)_2D_3 treatment rescued the fructose effect and normalized intestinal and renal Ca^{2+} transporter expression. The mechanism underlying the deleterious effect of excessive fructose intake on intestinal and renal Ca^{2+} transporters is a reduction in serum levels of 1,25-(OH)_2D_3. This finding is significant because of the large amounts of fructose now consumed by Americans increasingly vulnerable to Ca^{2+} and vitamin D deficiency.

bone; growth; intestine; kidney; parathyroid hormone; vitamin D; FGF23

IN CHILDREN AND YOUNG ADULTS, excessive consumption of sugar-sweetened beverages and of sweets may increase the incidence of bone fracture, decrease bone mineral density (BMD), and reduce the rate of bone mineral accrual (35, 50). Moreover, chronic consumption of these sweeteners, including fructose, by young rats reduces bone mechanical strength and mineral content (11, 16, 48, 54). These studies suggest that sweetness, including fructose, compromise bone integrity, but the mechanism remains unclear (49). Sufficient intake, intestinal absorption, and renal reabsorption of Ca^{2+} as well as increased levels of 1,25-(OH)_2D_3, or calcitriol, the active form of vitamin D_3, are essential in maintaining Ca^{2+} homeostasis and bone quality. Total intestinal Ca^{2+} absorption consists of a passive paracellular and a 1,25-(OH)_2D_3-dependent active transcellular pathway (21). Active Ca^{2+} transport involves Ca^{2+} entry through the apical transient receptor potential vanilloid Ca^{2+} channels (TRPV6 for the intestine and TRPV5 for the kidney), its intracellular diffusion via Ca^{2+}-binding proteins (CaBP9k for the intestine and CaBP28k for the kidney), and its extrusion across the intestinal or renal basolateral membrane through the Na^+/Ca^{2+} exchanger (NCX1) along with the plasma membrane Ca^{2+}-ATPase (PMCA1) (6). Since the major source for Ca^{2+} acquisition is intestinal absorption, vertebrates display adaptive increases in active Ca^{2+} transcellular transport, achieved by augmenting levels of 1,25-(OH)_2D_3, whenever physiological demands for Ca^{2+} increase. The precursor of vitamin D, cholecalciferol, is hydroxylated in the liver by 25-hydroxylases, producing calcidiol or 25-(OH)-D_3, which is further hydroxylated in the kidney by 1α-hydroxylase (encoded by CYP27B1) to 1,25-(OH)_2D_3. Degradation of 25-(OH)-D_3 and 1,25-(OH)_2D_3 is mediated by 24-hydroxylase (CYP24A1). Serum levels of 1,25-(OH)_2D_3 are tightly regulated by feedback loops controlling renal CYP27B1 and CYP24A1 expression (39).

We recently discovered in adult rat models of chronic kidney disease, as well as in lactating rats, that dietary fructose reduced rates of intestinal Ca^{2+} transport and circulating levels of 1,25-(OH)_2D_3, in association with a decrease in the binding of the vitamin D receptor (VDR) on the promoter of TRPV6 and CaBP9k genes (11, 16). This indicated that high fructose intake, by altering 1,25-(OH)_2D_3 homeostasis, may inhibit the adaptive increases in Ca^{2+} transport during lactation.

U.S. dietary intake data pooled by Popkin and Nielsen (40) show an 83 kcal/day increase of caloric sweetener consumption in the U.S. between 1962 and 2000. This upsurge represents a 22% increase in the proportion of daily energy requirements obtained from caloric sweeteners, including fructose. Fructose is transported passively across the apical membrane of the intestinal cell by the facilitative glucose transporter GLUT5 (Slc2A5), while the sodium-dependent glucose transporter SGLT1 (Slc5A1) is responsible for most glucose transport (14).

Both sugars are then transported across the basolateral mem-

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brane by GLUT2 (Slc2A2) into the portal vein and the liver, where fructose is rapidly metabolized. Many recent epidemiological studies correlate the rise in fructose consumption with various modern-day health concerns, including obesity, metabolic syndrome, and osteoporosis (5, 49). Since fructose is a natural component of human diets, its association with metabolic diseases may arise from recently documented, chronically excessive intake. Particular emphasis is placed on health concerns of actively growing children and adolescents whose intake per unit body weight of sweeteners, particularly fructose, is much greater than that of adults (34).

Few of the large number of studies on the metabolic effects of dietary fructose in humans and rodent models have utilized neonatal, weaning, or postweaning age groups (9, 10, 22, 25, 32), which consume the greatest quantity of fructose per kilogram of body weight. Since rapid growth in young mammals requires marked increases in Ca2+ intake and absorption driven by a higher level of 1,25-(OH)2D3 than older adults (17), we investigated in weaning rats the effect of a chronically high fructose intake on intestinal Ca2+ transport, 1,25-(OH)2D3 homeostasis, and bone quality. To test our hypothesis that the fructose-induced decrease in serum level of 1,25-(OH)2D3 is the mechanism by which dietary fructose impairs intestinal Ca2+ absorption, we then treated fructose-fed rats with 1,25-(OH)2D3 and determined whether the treatment prevented the deleterious effects of fructose.

MATERIALS AND METHODS

Animals. All the procedures in this study were approved by the Institutional Animal Care and Use Committee, UMDNJ-New Jersey Medical School. Studies were conducted on postweaning Sprague Dawley (Charles Rivers) male, virus/antibody-free rats (21 days old). Rats were kept under standard conditions: 12:12-h light-dark cycle and 22–24°C in dust-free cellulose bedding.

Experimental design. In the first study, postweaning rats (~55 g) were randomly divided into three groups (n = 7–9 rats) and then fed 63% glucose, fructose, or starch diets based on a standard American Institute of Nutrition (AIN)-93G formula containing normal Ca2+ and phosphate (Pi) levels designed to meet growth requirements of young rodents (11, 16). Animals were fed the diets ad libitum for 4 wk, from 21 to 50 days old. The AIN recommends either 73% (AIN-93M diet) or 63% (AIN-93G) carbohydrate levels, slightly higher than the average for humans (54%) consuming a high-carbohydrate diet (27). Since this is among the initial studies evaluating the effects of dietary fructose on 1,25-(OH)2D3 and Ca2+ homeostasis, we used an animal model with a high demand for these nutrients and then challenged this model with an experimental diet containing a high concentration of fructose to allow us to detect any potential effect it might have on selected outcomes. Although humans consume lower concentrations of fructose that are often mixed with other carbohydrates, studies have shown that mixing with glucose may accelerate fructose effects and that lower fructose concentrations cause similar deleterious effects to higher fructose concentrations if consumed for a longer time period (15, 24).

In a second study, same-age postweaning rats from a different batch (~45 g) were randomly divided into two groups fed 63% glucose or fructose diet, and then each group was further subdivided into two subgroups (n = 6 rats), each receiving daily intraperitoneal injections at noon of either 2.4% (vol/vol) ethanol in saline (vehicle) or 1 g/kg body wt 1,25-(OH)2D3 for 12 days. The animals were euthanized 18–20 h after the last 1,25-(OH)2D3 or saline injection.

In vitro intestinal nutrient transport measurements. Intestinal segments were everted quickly after isolation and prepared as everted sacs or sleeves to determine nutrient transport rates at 37°C with 95% O2-5% CO2 as described previously (11).

Ca2+ uptake. The everted gut sacs were made by using the first 4 cm of proximal duodenum, where active transcellular transport of Ca2+ is localized (4), and then incubated in Ca2+ transport buffer as previously described (11). The outer luminal and inner serosal compartments had equal initial concentrations (0.25 mM) of nonradioactive Ca2+; then, 45Ca2+ was added to the outer mucosal compartment. After 1 h, the active accumulation of 45Ca2+ in the inner serosal compartment was calculated as a ratio of the final concentration of (45Ca2+ serosal/45Ca2+ mucosal compartments) and then normalized to that of rats fed starch.

Fructose and glucose uptake. Four 1-cm jejunal segments were made into everted sleeves, mounted on rods, and preincubated for 5 min in Krebs ringer bicarbonate (KRB) as described previously (12). Two segments each were then incubated in 50 mM glucose- or fructose-KRB solutions containing tracer concentrations of [14C]glucose or [14C]fructose, respectively.

Phosphate uptake. Intestinal Pi transport was determined in two consecutive 4-cm segments of jejunal jejunum using the previously described everted gut assay (11). Briefly, the everted intestinal segment for determining total Pi transport was incubated for 1 h in Na+-containing Pi transport buffer (1.2 mM Pi), while the adjoining segment for determining Na+-independent Pi transport was incubated in Na+-free transport buffer. The total and Na+-independent transport of 33Pi was expressed as a ratio of the final concentration of (33Pi serosal/33Pi mucosal compartments) and normalized to the ratio obtained from sacs of rats fed starch.

Measurements of serum clinical parameters. Following earlier work (11), serum uric acid concentrations were determined using a QuantChrom Uric Acid Kit (BioAssay Systems, Hayward, CA), and Pi concentrations were determined using a QuantChrom Pi Assay Kit. The total serum Ca2+ concentrations were determined by previously described techniques using flame atomic absorption spectrophotometry (model 603; PerkinElmer, Norwalk, CT) (11).

1,25-(OH)2D3 fibroblast growth factor 23, and PTH assays. Following earlier work (11, 53), serum 1,25-(OH)2D3 levels were measured by enzyme immunoassay [ImmunoDiagnosticSystems (IDS)]. Briefly, serum samples were delipidated and 1,25-(OH)2D3 immunoextracted before the assay. Serum 25-(OH)D3 levels were measured directly by IDS. Intact rat PTH (Immutopics, San Clemente, CA) and intact fibroblast growth factor 23 (FGF23; Kainos, Tokyo, Japan) ELISAs were performed according to the manufacturers’ instructions.

Real-time PCR. Total RNA from homogenized intestinal mucosa or kidney was isolated and reverse transcribed, and real-time PCR was performed using MX3000P (Stratagene, La Jolla, CA) as previously described (11). 1,25-(OH)2D3 fibroblast growth factor 23, and PTH assays. Following earlier work (11, 53), serum 1,25-(OH)2D3 levels were measured by enzyme immunoassay [ImmunoDiagnosticSystems (IDS)]. Briefly, serum samples were delipidated and 1,25-(OH)2D3 immunoextracted before the assay. Serum 25-(OH)D3 levels were measured directly by IDS. Intact rat PTH (Immutopics, San Clemente, CA) and intact fibroblast growth factor 23 (FGF23; Kainos, Tokyo, Japan) ELISAs were performed according to the manufacturers’ instructions.

Bone analyses. Briefly, harvested right femora were cleaned of soft tissues and stored at −20°C wrapped in saline-soaked gauze (0.9% NaCl) until testing. Prior to testing, the longitudinal length and the diameter at the diaphysis (the midsection of the shaft) were measured with a sliding caliper and recorded. The diameters were measured at the midshaft of the diaphysis. The maximum (ao) and minimum (bo) outer diameters were measured before breaking the bone, whereas the maximum (ao) and minimum (bo) inner diameters were measured after breaking. The cortical thickness was calculated by taking the difference between the maximum and minimum outer and inner diameters [max = (ao − ai)/2; min = (bo − bi)/2].

Determination of bone calcium, magnesium, and phosphorus. Harvested humera were dried to constant weight, and then organic materials in the dried bone were removed using a methanol-chloroform mixture (1:1 vol/vol). After extraction, bones were ashed overnight in a muffle furnace at 482°C. Ashed bones were dissolved in a hot nitric-perchloric acid mixture (3:1) and diluted to 25 ml with 1%
RESULTS

Fructose feeding inhibits Ca\(^{2+}\) transport. Chronic consumption of a high-fructose diet containing normal Ca\(^{2+}\) levels more than doubled intestinal fructose uptake but reduced active transepithelial Ca\(^{2+}\) transport by almost one-half compared with starch and glucose diets (Fig. 1A). The dietary fructose effect on Ca\(^{2+}\) and fructose transport is specific, because active glucose uptake, and total Pi transport, as well as Na\(^{+}\)-independent passive Pi transport, remained similar among the three diet groups.

To determine whether changes in transport rates paralleled changes in mRNA levels, we next analyzed expression of the various transporters that play significant roles in fructose and Ca\(^{2+}\) transport (Fig. 1B). mRNA levels of TRPV6 and CaBP9k each decreased more than threefold in the duodenum of fructose-fed rats, paralleling marked reductions in active Ca\(^{2+}\) transport. In contrast, the mRNA expression of GLUT5 was upregulated more than tenfold by dietary fructose. In the distal jejunum (data not shown), fructose also markedly downregulated TRPV6 and CaBP9k, except that mRNA expression levels were already orders of magnitude less than in the duodenum. These fructose effects on TRPV6, CaBP9k, and GLUT5 levels were specific, as expression of the basolateral Ca\(^{2+}\) transporters PMCA1 and NCX1 were not affected by the diet. Likewise, the mRNA expression of the Na\(^{+}\)/glucose cotransporter SGLT1 and the intestinal Na\(^{+}\)-dependent phosphate cotransporter NaPi2b remained independent of diet. Changes in protein levels of CaBP9k followed the changes observed for mRNA and showed a lower level of proteins in the intestine of fructose-fed rats (Fig. 1C).

Blood chemistry and hormone levels. We then investigated the plasma level of 1,25-(OH)\(_2\)D\(_3\), since it is one of the key hormones controlling intestinal active Ca\(^{2+}\) transport, mainly by regulating TRPV6 and CaBP9k expression (Table 1). We observed a significant 30–40% decrease in 1,25-(OH)\(_2\)D\(_3\) plasma concentration in rats fed fructose. It is well established...
that a low plasma level of Ca\(^{2+}\) promotes synthesis of PTH, which in turn increases 1,25-(OH)\(_2\)D\(_3\) levels. Despite the marked decrease in rates of intestinal Ca\(^{2+}\) transport in the fructose group, plasma levels of Ca\(^{2+}\), Pi, and PTH remained similar among the three diet groups. Since fructose may cause hyperuricemia arising from fructose-induced renal damage (37) and hepatic uric acid production (30), concentrations of uric acid were determined but were also found to be independent of diet.

**Fructose alters CYP27B1 and CYP24A1 expression.** We examined the effects of fructose on the kidney, since it is the major organ system regulating metabolism of 1,25-(OH)\(_2\)D\(_3\), which synthesizes 1,25-(OH)\(_2\)D\(_3\), and catabolizes 1,25-(OH)\(_2\)D\(_3\), was decreased in the kidney of rats fed fructose compared with those fed glucose (Fig. 2A). In contrast, expression of CYP24A1, which catabolizes 1,25-(OH)\(_2\)D\(_3\), was two- to threefold higher in fructose-fed than in both glucose- and starch-fed rats (Fig. 2B). The protein levels of CYP27B1 decreased in the kidney of fructose-fed rats (Fig. 2C). Thus, the decrease in circulating levels of 1,25-(OH)\(_2\)D\(_3\) might have resulted from reduced synthesis and increased degradation.

We also performed gene expression analyses of 1,25-(OH)\(_2\)D\(_3\) target genes in the kidney involved in Ca\(^{2+}\) reabsorption from glomerular filtrates (Fig. 2D). Expression levels of TRPV5 and CaBP28k decreased in the fructose-fed compared with the glucose- and starch-fed rats, an expression pattern similar to that observed for Ca\(^{2+}\)-transporting proteins in the small intestine. The mRNA expression of CaBP9k (the sole CaBP in the small intestine but also expressed in the kidney, where it serves a significant but supportive role to CaBP28k), PMCA1 and NCX1 remained unchanged; thus, the inhibitory effect of fructose on TRPV5 and CaBP28k is specific. As in the small intestine, renal GLUT5 mRNA levels increased markedly, sixfold.

**1,25-(OH)\(_2\)D\(_3\) treatment rescues rats from the harmful effects of fructose.** To prove that the deleterious effect of dietary fructose on intestinal Ca\(^{2+}\) transport was directly mediated by fructose-induced reductions in 1,25-(OH)\(_2\)D\(_3\), fructose- and glucose (control)-fed rats were treated with 1,25-(OH)\(_2\)D\(_3\). After 2 wk, fructose feeding caused a twofold decrease in duodenal Ca\(^{2+}\) transport in vehicle-treated rats (Fig. 3A). However, 1,25-(OH)\(_2\)D\(_3\) treatment clearly prevented this deleterious effect of fructose. A similar, modest rescue effect of 1,25-(OH)\(_2\)D\(_3\) was also observed on the mRNA expression level of TRPV6 and CaBP9k (Fig. 3B). In vehicle-treated rats, dietary fructose clearly reduced TRPV6 and CaBP9k mRNA expression more than twofold. This reduction was not observed in fructose-fed 1,25-(OH)\(_2\)D\(_3\)-treated rats. In glucose-fed rats, treatment with 1,25-(OH)\(_2\)D\(_3\) did not induce any change in Ca\(^{2+}\) transport rate or in TRPV6 as well as CaBP9k mRNA expression but markedly increased the protein levels of CaBP9k (Fig. 3C). The protein level of CaBP9k also responded robustly to 1,25-(OH)\(_2\)D\(_3\) treatment in fructose-fed rats.

Exogenous 1,25-(OH)\(_2\)D\(_3\) also produced no toxic effects, since PMCA1, NCX1, and SGLT1 expression, which were not affected by diet, were also not affected by treatment with 1,25-(OH)\(_2\)D\(_3\). In fact, 1,25-(OH)\(_2\)D\(_3\) treatment did not inter-

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**Table 1. Serum biochemistry in 50-day-old rats fed glucose, fructose, or starch diet for 4 wk**

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Fructose</th>
<th>Starch</th>
<th>Significance</th>
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<tbody>
<tr>
<td>1,25-(OH)(_2)D(_3), pmol/l</td>
<td>949 ± 28(^a)</td>
<td>638 ± 50(^b)</td>
<td>885 ± 22(^c)</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>257 ± 62</td>
<td>200 ± 25</td>
<td>164 ± 32</td>
</tr>
<tr>
<td>Calcium, mg/dl</td>
<td>11.5 ± 0.3</td>
<td>10.2 ± 0.8</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td>Phosphate, mg/dl</td>
<td>18.9 ± 1.4</td>
<td>17.7 ± 1.1</td>
<td>18.5 ± 1.4</td>
</tr>
<tr>
<td>Uric acid, mg/dl</td>
<td>3.8 ± 0.19</td>
<td>3.5 ± 0.04</td>
<td>3.2 ± 0.23</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 7–9 per group. Superscript letters refer to results of least significant difference (LSD) post hoc tests after one-way ANOVA (P < 0.05). Means with different superscript letters are significantly different.

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Fig. 2. Expression of 1,25-(OH)\(_2\)D\(_3\) metabolic enzymes and Ca\(^{2+}\) transporters in kidney of 50-day-old rats fed glucose, fructose, or starch after weaning. mRNA expression of CYP27B1 (A) and CYP24A1 (B) analyzed by real-time PCR using EF1α as a reference. C: protein abundance of CYP27B1 using β-actin as a reference. D: mRNA expression of TRPV5, CaBP28k, CaBP9k, PMCA1, NCX1, and GLUT5. All data were normalized relative to levels seen in rats fed starch. Data are means ± SE (n = 8 per group). Means with different superscript letters are significantly different at P < 0.05. CYP27B1, TRPV5, and CaBP28k mRNA expression each decreased with dietary fructose; CYP24A1 and GLUT5 expression increased with fructose.
fere with regulation of vitamin D-independent genes, as clearly shown by the dramatic fructose-induced increase in GLUT5 expression in the presence or absence of 1,25-(OH)2D3.

What is the effect of exogenous 1,25-(OH)2D3 treatment on expression of CYP27B1 and CYP24A1? After 2 wk of feeding, the mRNA levels of CYP27B1 decreased threefold in fructose vehicle rats (Fig. 4A). Treatment with exogenous 1,25-(OH)2D3 inhibited expression of renal CYP27B1 in both glucose- and fructose-fed rats, as would be expected since CYP27B1 is inhibited by its product (39). 1,25-(OH)2D3 treatment also decreased the protein levels of CYP27B1 in both glucose- and fructose-fed rats (Fig. 4C). Since CYP27B1 protein levels were already very low in fructose-fed rats, 1,25-(OH)2D3 treatment resulted in no further decreases.

In contrast, 2 wk of dietary fructose was not sufficient to affect CYP24A1 expression (Fig. 4B). However, levels of CYP24A1 were already very low in this cohort of rats fed normal levels of Ca2+, and further decreases would likely not have been detectable. Treatment with exogenous 1,25-(OH)2D3 stimulated expression of renal CYP24A1 in both glucose- and fructose-fed rats, as would be expected since CYP24A1 is stimulated by excess Ca2+.

In the fructose vehicle rats, serum 1,25-(OH)2D3 levels decreased by ~40% compared with those in the glucose vehicle.
rats (Table 2), a trend similar to that observed in Table 1. It is not
clear why there are differences in levels of hormones between
experiments as indicated in Tables 1 and 2, but the second feeding
experiment was conducted over a year after the first, so different
batches of assay kits were used for analysis. Treatment with
exogenous 1,25-(OH)2D3 further decreased serum 1,25-(OH)2D3,
and fructose-fed rats compared with the glucose+vehicle group, for reasons mentioned in DISCUSSION. As expected, 1,25-(OH)2D3 treatment increased serum levels of CaBP9k in both glucose- and fructose-fed rats. Thus, as in the previous experiment, dietary fructose had no significant effect on Ca2+ levels.

The magnitude of the rescue effect of 1,25-(OH)2D3 was less
marked in the kidney (Fig. 4D). TRPV5 expression decreased
by over 50% in fructose+vehicle rats compared with that in
vehicle+vehicle rats. This inhibitory effect of fructose was
clearly albeit modestly rescued by 1,25-(OH)2D3 treatment, as
indicated by comparing the fructose
1,25-(OH)2D3 group to
1,25-(OH)2D3 rats. Fructose tended to reduce CaBP28k mRNA expression, and
1,25-(OH)2D3 treatment did not prevent this fructose-induced reduction in expression of CaBP28k in the kidney. Renal CaBP9k expression was similar between the glucose+vehicle and fructose+vehicle groups. Thus CaBP9k expression was similar between the glucose and fructose vehicle groups. 1,25-(OH)2D3 vehicle and fructose vehicle were not significantly different.

Fructose increased FGF23 serum levels. Among the key
regulators of 1,25-(OH)2D3 synthesis, FGF23 is known to
reduce 1,25-(OH)2D3 levels by inhibiting CYP27B1 expression.
We found that 4 wk of fructose feeding significantly increased circulating levels of FGF23 by more than 60% (Fig. 5A) and that there was a significant negative correlation between the serum levels of 1,25-(OH)2D3 and of FGF23 (Fig. 5B). PTH is another key factor regulating the circulating levels of 1,25-(OH)2D3. However, there was no significant correlation between the plasma levels of 1,25-(OH)2D3 and those of PTH (Fig. 5C), suggesting that the fructose-induced reduction in 1,25-(OH)2D3 levels may not involve PTH.

To support this new finding and demonstrate that fructose-
induced increases in FGF23 affected the kidney, where 1,25-
(OH)2D3 is synthesized, we investigated whether fructose feeding
was associated with the well-established inhibitory effects of FGF23 on renal Pi reabsorption, as FGF23 inhibits the translocation of NaPi2a to the proximal tubular membranes (18, 42, 44). Fructose-fed rats (Fig. 6) clearly displayed a lower level of NaPi2a in the apical membrane of cells of the proximal tubules (white arrow) than those of rats fed glucose or starch.

Fructose feeding compromised growth rate. Despite similar
rates of food intake among diets (data not shown) and similar
rates of initial growth in the first week, rats fed fructose had
slightly lower increases in body weight about 2 wk after the
beginning of feeding compared with littermates fed glucose or
starch (Fig. 7). After 2 wk more of feeding, at the time of death,
the fructose group weighed ~7% less than the glucose or the
starch group. This modest effect on body weight has been
observed previously in other studies involving fructose feeding of postweaning rodents (25, 32).

Table 2. Serum biochemistry in 50-day-old rats fed glucose or fructose and then treated with 1,25-(OH)2D3 or vehicle

<table>
<thead>
<tr>
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<th>Glucose</th>
<th>Fructose</th>
<th>1,25-(OH)2D3</th>
<th>Two-Way Significance</th>
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<tbody>
<tr>
<td>Calcium, mg/dl</td>
<td>10.1 ± 0.2a</td>
<td>10.2 ± 0.2b</td>
<td>11.4 ± 0.2a</td>
<td>11.7 ± 0.3b</td>
</tr>
<tr>
<td>PTH, pg/mL</td>
<td>0.3a 0.523</td>
<td>0.4b 0.004</td>
<td>0.7a 0.004</td>
<td>0.863</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 6 per group. Superscript letters refer to results of LSD post hoc tests after one-way ANOVA (P < 0.05). Means with different superscript letters are significantly different.
The modest effect of fructose on body weight was also observed in experiment 2. Feeding rates and body weights were likewise similar at the start of the experiment when rats were 22 days of age, but at the midway point of the experiment after 7 days of feeding, fructose-vehicle rats exhibited a significantly ($P < 0.03$) lower body weight (67.6 ± 3.8 g) than glucose-vehicle rats (78.2 ± 2.5 g). Treatment with 1,25-\((\text{OH})_2\)D$_3$ prevented the fructose-induced, significant decrease in body weight (glucose-fed, 78.6 ± 2.8; fructose-fed, 73.2 ± 3.4 g; $P > 0.20$). At the end of the rescue experiment, ~12 days after initiation of feeding, fructose+vehicle rats (100 ± 5 g) had lower body weights than glucose+vehicle rats (124 ± 4 g). However, the mean body weight of fructose+ 1,25-\((\text{OH})_2\)D$_3$ rats (110 ± 4 g) was not significantly different from that of glucose+1,25-\((\text{OH})_2\)D$_3$ rats (121 ± 4). Thus, 1,25-\((\text{OH})_2\)D$_3$ treatments partially rescued the rats from the deleterious effects of fructose on body weight.

Consequences of chronic fructose intake on bone. Fructose-fed rats had femur lengths significantly shorter than those of glucose- and starch-fed ones (Table 3), suggesting that the fructose-induced decrease in total body weight may have been due, in part, to retardation of linear skeletal growth. Although diet had no effect on outer bone diameters, glucose-fed rats tended to have greater inner bone diameters than fructose-fed and starch-fed rats. Thus, the net effect is a trend for glucose-fed rats to have less cortical thickness than fructose-fed and starch-fed rats.

Fructose-fed rats also displayed a lower (~10%) dry weight of the humeri than glucose and starch groups (Table 4). Subsequently, their bone ash weight was also significantly lower by ~12%: the percentage of bone ash to dry weight, however, was similar among the treatment groups. Bone Ca$^{2+}$ was also higher in the glucose- and starch-fed ones, although the difference from that of the fructose-fed rats was not statistically significant. Glucose-fed rats contained a significantly higher amount of P than those fed fructose. The [\(\text{Ca}^{2+} \times P\)] product accounts for much of the hydroxyapatite that makes up the bone matrix. [\(\text{Ca}^{2+} \times P\)] tends to be lower in humeri of fructose-fed rats than in those fed glucose or starch, explaining their reduced length and lower weight.

**DISCUSSION**

The present study demonstrates that a decrease in circulating levels of 1,25-\((\text{OH})_2\)D$_3$ is the key mechanism by which fructose inhibits active transport of Ca$^{2+}$ in the small intestine and likely in the kidney as well. This finding is highly significant,
because total fructose intake now constitutes almost 10% of total energy intake of average Americans, and ~20% of total energy intake of the highest 5% of fructose consumers (34).

1,25-(OH)2D3 treatment prevents the fructose-induced decrease in intestinal Ca2+ transport. When Ca2+ status is deficient because dietary supply is limiting or physiological demand is high, levels of the biologically active 1,25-(OH)2D3 hormone increase dramatically to restore Ca2+ sufficiency by inducing active intestinal absorption and renal reabsorption of Ca2+. We recently discovered that dietary fructose prevents these compensatory increases in Ca2+ transport in rats whose Ca2+ requirements are increased because of lactation (16). But the signal mediating the inhibitory fructose effect had not been clearly identified until the present study.

The fructose-induced decrease in intestinal Ca2+ transport was linked to reductions in duodenal expression of TRPV6 and CaBP9k, both known to be transcriptionally regulated by 1,25-(OH)2D3 (52). We focused on the duodenum because contributions of the more distal regions to adaptive, 1,25-(OH)2D3-mediated increases in active transcellular Ca2+ transport are likely to be small. Moreover, since fructose also decreases TRPV6 and CaBP9k expression levels in the distal jejunum, there likely can be no compensation of Ca2+ transport in these distal regions. CaBP9k-null mice are still able to transport Ca2+; thus, CaBP9k deletion alone will not stop active transport (4). In TRPV6 knockout mice, Ca2+ absorption can still be stimulated by a low-Ca2+ diet and 1,25-(OH)2D3 injections (4, 28); hence, TRPV6 deletion alone is insufficient to fully inhibit transport activity. However, simultaneous deletion of TRPV6 and CaBP9k impairs the ability to respond to Ca2+ insufficiency or 1,25-(OH)2D3 (4) treatment, indicating that both CaBP9k and TRPV6 are required for adaptive increases in Ca2+ transport. In our postweaning rat model requiring Ca2+ levels sufficient to support rapid growth, TRPV6 and CaBP9k expression was compromised by dietary fructose, providing a clue that 1,25-(OH)2D3 was involved.

Since serum levels of 1,25-(OH)2D3 decrease when it is injected (Table 2), how can 1,25-(OH)2D3 treatments rescue intestinal and renal Ca2+ transporters? The low serum 1,25-(OH)2D3 concentration is likely due to reductions in CYP27B1 expression and increases in CYP24A1 expression, potentially preventing the synthesis of endogenous 1,25-(OH)2D3 and ensuring the rapid clearance of injected 1,25-(OH)2D3. Similar findings had been previously observed in hypocalcemic CYP27B1-deficient mice treated daily with 1,25-(OH)2D3 for 5 wk that eventually rescued the hypocalcemia despite a dramatic increase in CYP24A1 expression and the undetectable amounts of 1,25-(OH)2D3 in the plasma (8).

Although 1,25-(OH)2D3 treatment clearly rescued intestinal Ca2+ transport, its acute effects on mRNA expression of intestinal target genes were modest. The acute effects of 1,25-(OH)2D3 may be difficult to demonstrate due to the short half-life of intestinal CaBP9k (~16 h (2)) and TRPV6 (~6 h (46)) relative to the time gap between the last 1,25-(OH)2D3 injection and death (~20 h). Thus, 1,25-(OH)2D3-induced changes in levels of intestinal CaBP9k mRNA were small and contrasted with those of CaBP9k protein, which has a longer half-life and thus would be more reflective of acute 1,25-(OH)2D3 effects. In vitamin D-deficient young rats, 1,25-(OH)2D3 treatment also caused gradual increases in CaBP9k mRNA and protein, but 24 h after injection, mRNA levels had returned to preinjection levels as protein levels remained high (2).

Although 1,25-(OH)2D3 treatment affects the paracellular component of Ca2+ transport, its effects are primarily on the active component, as indicated by the strikingly similar changes between active transepithelial transport and levels of intestinal TRPV6 and of CaBP9k expression (Figs. 1 and 3). Nonetheless, measurements of total and paracellular Ca2+ transport and of Ca2+ digestibility are very interesting and can be the subject of future work.

What is the mechanism underlying the fructose-induced decrease in 1,25-(OH)2D3? The proximate mechanism underlying the regulation of 1,25-(OH)2D3 by fructose may be the dramatic fructose-induced decreases in CYP27B1 expression and the increases in CYP24A1 expression as shown in the first experiment involving 5 wk of fructose feeding. In the second experiment, the renal expression of CYP27B1 was also reduced in fructose-fed compared with glucose-fed rats; however, the stimulatory fructose effect on CYP24A1 mRNA expression was not yet apparent, likely because fructose feeding lasted only 2 wk. When serum levels of Ca2+, Pi, and

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**Table 3. Femora length and diameters**

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Fructose</th>
<th>Starch</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Length, mm</td>
<td>34.4 ± 0.6a</td>
<td>32.4 ± 0.3b</td>
<td>34.0 ± 0.6a</td>
<td>0.031</td>
</tr>
<tr>
<td>Maximum outer diameter, a1</td>
<td>4.33 ± 0.07</td>
<td>4.30 ± 0.12</td>
<td>4.46 ± 0.08</td>
<td>0.367</td>
</tr>
<tr>
<td>Minimum outer diameter, b1</td>
<td>3.07 ± 0.09</td>
<td>2.95 ± 0.08</td>
<td>3.00 ± 0.05</td>
<td>0.469</td>
</tr>
<tr>
<td>Maximum inner diameter, a1</td>
<td>3.23 ± 0.06</td>
<td>2.82 ± 0.14</td>
<td>2.70 ± 0.22</td>
<td>0.076</td>
</tr>
<tr>
<td>Minimum inner diameter, b1</td>
<td>2.17 ± 0.07a</td>
<td>1.85 ± 0.11b</td>
<td>1.91 ± 0.12b</td>
<td>0.028</td>
</tr>
<tr>
<td>Maximum cortical thickness, a1</td>
<td>1.11 ± 0.05a</td>
<td>1.48 ± 0.12b</td>
<td>1.76 ± 0.17b</td>
<td>0.018</td>
</tr>
<tr>
<td>Minimum cortical thickness, b1</td>
<td>0.91 ± 0.09</td>
<td>1.10 ± 0.12</td>
<td>1.10 ± 0.10</td>
<td>0.241</td>
</tr>
</tbody>
</table>

Data are means ± SE in mm; n = 6–8 per group. Superscript letters refer to results of LSD post hoc tests after one-way ANOVA (P < 0.05). Means with different superscript letters are significantly different.

**Table 4. Humerus weight and mineral composition measurements**

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Fructose</th>
<th>Starch</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight</td>
<td>206 ± 11a</td>
<td>183 ± 8b</td>
<td>201 ± 13a</td>
<td>0.031</td>
</tr>
<tr>
<td>Total ash weight</td>
<td>126 ± 10a</td>
<td>108 ± 6b</td>
<td>120 ± 14a</td>
<td>0.029</td>
</tr>
<tr>
<td>% Ash bone</td>
<td>60.7 ± 2.0</td>
<td>59.2 ± 1.8</td>
<td>58.9 ± 2.8</td>
<td>0.874</td>
</tr>
<tr>
<td>Total weight of Ca2+</td>
<td>45.0 ± 2.3</td>
<td>43.1 ± 1.7</td>
<td>47.9 ± 3.4</td>
<td>0.341</td>
</tr>
<tr>
<td>% Ca2+/ash weight</td>
<td>34.6 ± 2.1</td>
<td>38.4 ± 0.9</td>
<td>37.0 ± 1.0</td>
<td>0.158</td>
</tr>
<tr>
<td>Total weight of P</td>
<td>25.7 ± 2.6a</td>
<td>19.3 ± 0.4b</td>
<td>22.2 ± 1.4ab</td>
<td>0.023</td>
</tr>
<tr>
<td>% Phosphat weight</td>
<td>19.8 ± 2.4</td>
<td>17.3 ± 0.4</td>
<td>17.2 ± 0.4</td>
<td>0.264</td>
</tr>
<tr>
<td>[Ca2+] × [P]</td>
<td>1153 ± 114</td>
<td>837 ± 38</td>
<td>1089 ± 146</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Data are means ± SE, weights in mg; n = 6–8 per group. Superscript letters refer to results of LSD post hoc tests after one-way ANOVA (P < 0.05). Means with different superscript letters are significantly different.
1,25-(OH)₂D₃ are normal, 1,25-(OH)₂D₃ inhibits its own synthesis by reducing CYP27B1 expression so that low levels of 1,25-(OH)₂D₃ are often associated with an increase in CYP27B1 expression to restore homeostasis. Since 1,25-(OH)₂D₃ levels and CYP27B1 expression are both low, the 1,25-(OH)₂D₃-CYP27B1 feedback loop is not responding appropriately in fructose-fed rats. This feedback loop was also shown by us to be disrupted in fructose-fed lactating rats (16), challenging our understanding of the regulation of 1,25-(OH)₂D₃ synthesis under conditions of chronically high fructose intake.

If this feedback loop is disrupted during chronic fructose feeding, what else may be mediating the fructose-induced reduction in 1,25-(OH)₂D₃? The two other main hormones potentially regulating 1,25-(OH)₂D₃ are PTH, which increases, and FGF23, which decreases, serum 1,25-(OH)₂D₃ concentrations (43). If the fructose-induced reduction in 1,25-(OH)₂D₃ concentrations is via PTH, fructose should reduce PTH levels; if via FGF23, fructose should increase FGF23 levels. In this study, PTH did not vary with diet, and there was no correlation between the circulating levels of PTH and 1,25-(OH)₂D₃, a finding similar to that in our previous work (16). In contrast, fructose increased FGF23 circulating levels, so that FGF23 and 1,25-(OH)₂D₃ were significantly negatively correlated. These findings suggest a potential role of FGF23 in the downregulation of 1,25-(OH)₂D₃ by fructose.

FGF23, which is highly expressed in bone tissue matrix-forming cells such as osteoblasts and osteocytes, is a phosphaturic hormone that decreases renal Pi reabsorption (43). We confirmed that the fructose-induced increase in FGF23 circulating levels is physiological, as it led to reduced translocation of NaPi2a protein expression to the apical membrane of proximal tubular cells, as previously shown (44). In fact, the marked effect of fructose via FGF23 on NaPi2a levels in the proximal tubular apical membrane (Fig. 6) may result in reduced Pi reabsorption as well as increased Pi excretion and may explain the increased loss of Pi from the bone (Table 4) to keep blood Pi levels from decreasing. In addition, FGF23 is known to regulate the renal synthesis of 1,25-(OH)₂D₃ by inhibiting CYP27B1 and stimulating CYP24A1 mRNA expression (43), findings replicated in this study. Future work using FGF23⁻/⁻ and klotho⁻/⁻ (the obligate renal coreceptor of FGF23) mice should confirm whether the effect of fructose is truly mediated through FGF23, and enable us to determine the mechanism underlying the fructose-induced increase in FGF23 concentrations.

It is not clear how fructose increases FGF23 levels, but 6-deoxy-6-[¹⁸F]fluoro-D-fructose did accumulate in the bone more than in any other organ system (51). Interestingly, we have immunocytochemical evidence that GLUT5 may be expressed in bone (V. Douard and P. Ferraris, unpublished observations), suggesting that some bone cells may be able to transport and metabolize fructose. FGF23 is an early-onset biomarker of renal insufficiency (23), but it is also not clear whether this increase in FGF23 is a cause or a consequence of kidney damage (29, 41, 45). Interestingly, excessive fructose consumption has been shown to damage the kidney, as evidenced by marked renal hypertrophy, accelerated progression of chronic kidney disease, and apparent aggravation of end-stage renal disease symptoms (7, 11, 16, 19). It also increases plasma concentration of uric acid (31), shown to be positively associated with increased FGF23 in adults and children with normal kidney function (3, 20). However, in this study, the rats fed fructose did not yet exhibit any increase in plasma levels of uric acid, although fructose did induce renal hypertrophy.

A recent study showed that FGF23 may directly alter 1,25-(OH)₂D₃-mediated intestinal Ca²⁺ transport in mice (26), suggesting that FGF23 may not act indirectly via the kidney.

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**Fig. 8.** Schematic diagram of fructose-induced disruptions in 1,25-(OH)₂D₃ homeostasis. Under normal conditions such as in rats fed starch or glucose, postweaning growth stimulates increases in circulating levels of 1,25-(OH)₂D₃, which in turn enhance rates of intestinal Ca²⁺ transport, thereby addressing the increased Ca²⁺ demand for bone growth. Under fructose feeding, serum fructose increases (solid line with arrowhead, step 1). Fructose intake is associated with decrease in levels of 1,25-(OH)₂D₃ (2), resulting in reduced intestinal Ca²⁺ transport (3), decreasing the supply of Ca²⁺ for appropriate growth (4; solid line with end point). Reduction in 1,25-(OH)₂D₃ levels results from increases in CYP24A1 and decreases in CYP27B1 renal expression, suggesting that fructose may directly enhance renal breakdown and impair synthesis of 1,25-(OH)₂D₃ (5, dashed arrow). These fructose effects were independent of any change in PTH (6) and the negative feedback loop by which 1,25-(OH)₂D₃ controls its own metabolism (7). Alternatively, fructose feeding is associated with an increase in circulating levels of FGF23, which may result from a direct effect of fructose on bone cells (8a). This fructose-induced increase in FGF23 (8b) may be the mechanism by which fructose indirectly affects renal CYP27B1 and CYP24A1 expression (8c).
However, klotho is an obligate coreceptor of FGF receptors for FGF23 that is expressed at high levels only in the kidney and is poorly expressed in the small intestine (38); thus, it is not clear how FGF23 would exert its direct effects on the gut.

Fructose and growth. Although serum Ca\(^{2+}\) concentrations were not perturbed, preserving its essential electrochemical gradients and cell signaling functions, fructose-induced reductions in Ca\(^{2+}\) transport may ultimately have affected growth rate, as indicated by reductions in bone length, bone dry weight, and body weight. The cause can only be ascribed to excessive fructose intake, as the diets were isocaloric and isonitrogenous. The effect of chronic fructose feeding on body weight observed in both experiments may be specific for this rapidly growing age group, as we did not observe such fructose effects on body weight in our previous studies involving adult male and female rats (11, 16). Two other studies focusing on fructose effects in postweaning rats also observed reductions in body weight (25, 32) between 4 and 8 wk of age. Continued fructose feeding in one study beyond 8 wk of age eventually led to the frequently observed fructose-induced increases in body weight and obesity in older age groups.

Modest differences in food intake were observed only after body weight was reduced, and even then, food consumption per kilogram of body weight remained the same. Thus, both Ca\(^{2+}\) and 1,25-(OH)\(_2\)D\(_3\) are critical for bone development, and herein we confirm, using a fructose model, previous work demonstrating that the action of 1,25-(OH)\(_2\)D\(_3\) on the contributions of Ca\(^{2+}\) to bone growth and remodeling is mediated by regulating intestinal Ca\(^{2+}\) absorption (1, 33). In contrast, fructose seems to influence Pi levels and metabolism in bone without affecting total and Na\(^+\)-independent Pi transport in the small intestine. However, fructose, via an increase in serum FGF23 level, decreased expression of NaPi2a in the apical membrane of the proximal tubule, which likely impaired renal reabsorption of Pi. Interestingly, consumption by humans of a high-fructose diet (20% of total calories) for 5 wk markedly increased renal Pi excretion, modestly increased renal Ca\(^{2+}\) excretion, and compromised intestinal Ca\(^{2+}\) absorption, thereby decreasing Ca\(^{2+}\) and Pi balance (36). Our findings provide a mechanism underlying these interesting observations.

Perspectives. In conclusion, our studies demonstrate that a chronically high fructose intake during postweaning has adverse impacts on the intestine, kidney, and bone (Fig. 8). More studies are required in order to better understand the exact signaling pathways involved in the different organs impacted by excess fructose intake and to demonstrate that a mixed diet with lower fructose content will have similar effects as was demonstrated previously (15, 24). Here, we used the same high fructose concentration as in an earlier work (16) to demonstrate that our previous observation in the rat lactation model could be repeated in growing rats that were not hyperphagic, to demonstrate a robust effect of fructose so that we could unequivocally show whether or not 1,25-(OH)\(_2\)D\(_3\) mediated those effects, and to induce the fructose effect quickly and demonstrate 1,25-(OH)\(_2\)D\(_3\) mediation while growth rate was still rapid.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.D. and R.P.F. conception and design of research; V.D., Y.S., J.L., C.P., and F.W.K. performed experiments; V.D., Y.S., J.L., C.P., F.W.K., J.D.B., S.L., and R.P.F. analyzed data; V.D., Y.S., C.P., J.D.B., S.L., and R.P.F. interpreted results of experiments; V.D., J.L., and R.P.F. prepared figures; V.D. drafted manuscript; V.D., Y.S., J.D.B., and R.P.F. edited and revised manuscript; R.P.F. approved final version of manuscript.

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