

# Dietary Fructose Inhibits Intestinal Calcium Absorption and Induces Vitamin D Insufficiency in CKD

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## ABSTRACT

Renal disease leads to perturbations in calcium and phosphate homeostasis and vitamin D metabolism. Dietary fructose aggravates chronic kidney disease (CKD), but whether it also worsens CKD-induced derangements in calcium and phosphate homeostasis is unknown. Here, we fed rats diets containing 60% glucose or fructose for 1 mo beginning 6 wk after 5/6 nephrectomy or sham operation. Nephrectomized rats had markedly greater kidney weight, blood urea nitrogen, and serum levels of creatinine, phosphate, and calcium-phosphate product; dietary fructose significantly exacerbated all of these outcomes. Expression and activity of intestinal phosphate transporter, which did not change after nephrectomy or dietary fructose, did not correlate with hyperphosphatemia in 5/6-nephrectomized rats. Intestinal transport of calcium, however, decreased with dietary fructose, probably because of fructose-mediated downregulation of calbindin 9k. Serum calcium levels, however, were unaffected by nephrectomy and diet. Finally, only 5/6-nephrectomized rats that received dietary fructose demonstrated marked reductions in 25-hydroxyvitamin D<sub>3</sub> and 1,25-dihydroxyvitamin D<sub>3</sub> levels, despite upregulation of 1 $\alpha$ -hydroxylase. In summary, excess dietary fructose inhibits intestinal calcium absorption, induces marked vitamin D insufficiency in CKD, and exacerbates other classical symptoms of the disease. Future studies should evaluate the relevance of monitoring fructose consumption in patients with CKD.

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In the past 20 yr, the cases of end stage renal disease (ESRD) in the United States quadrupled to 382,000 in 2000, and its prevalence is expected to increase by 85% to 713,000 patients in 2015, so reducing the incidence of ESRD is widely recognized as a major public health goal.<sup>1</sup> When patients have been identified as having the major risk factors (diabetes, hypertension, and uricemia) for rapid progression from chronic kidney disease (CKD) to ESRD,<sup>2,3</sup> efforts are made to slow the rate of progression, and these efforts involve tight management of these risk factors. Dietary fructose may be a major contributor to the rapid development of all of these chronic diseases considered risk factors for ESRD. For example, diabetes *via* hyperinsulinemia, hypertriglyceridemia *via* hepatic lipogenesis,<sup>4,5</sup> and hypertension *via* uricemia<sup>6,7</sup> each are exacerbated by

excessive fructose consumption. Despite these documented linkages to symptoms of metabolic syndrome, fructose consumption instead has increased in the overall population and is not restricted or even monitored in patients with CKD. The average rate of fructose consumption is now approximately 80 g/d in the United States.<sup>4,8,9</sup> In addition, more recent work indicated a strong correlation between

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excessive fructose consumption and renal disease. Chronic fructose but not glucose or starch consumption in 5/6-nephrectomized (5/6Nx) rats leads to a marked decrease in creatinine clearance as well as to sharp increases in blood urea nitrogen (BUN), renal mass index, and histologic biomarkers of renal pathology.<sup>10</sup> Fructose-induced pathology seems to be caused by increases in levels of oxidative stress and inflammation, as demonstrated in immortalized proximal tubular cells (HK-2) incubated in fructose.<sup>11</sup>

Other complications of CKD, such as osteodystrophy, hyperphosphatemia, hyperparathyroidism, and deficiency in 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>] levels,<sup>12–14</sup> may also be affected by dietary fructose. For example, dietary fructose perturbs intestinal phosphate (Pi)<sup>15</sup> and calcium (Ca) absorption<sup>16</sup> as well as Ca and Pi balance.<sup>17,18</sup> Excessive consumption of carbonated beverages known to contain high-fructose corn syrups have been strongly associated with increased incidence of bone fractures in the adolescent population.<sup>19</sup> It seems clear from these studies that fructose could exacerbate the mineral imbalance usually associated with CKD; however, the studies investigating fructose effects on mineral metabolism have focused on young, actively growing individuals whereas ESRD and CKD are diseases that generally affect the aged population.

We first tested the hypothesis that dietary fructose consumption exacerbates the classical symptoms of CKD that can lead to ESRD. Using sham-operated rats as controls, we allowed 5/6Nx rats to develop clear symptoms of CKD for 6 wk, then subsequently fed either a high-fructose or -glucose diet for an additional 4 wk and determined the classical blood and bone markers of renal disease. No study has related fructose consumption to vitamin D metabolism, yet the liver and the kidney, the main sites of vitamin D synthesis, are organs adversely affected by both chronic fructose consumption<sup>20,21</sup> and renal failure<sup>10,11</sup>; therefore, we next tested the hypothesis that fructose consumption aggravates the already adverse effects of ESRD on the synthesis of the major active form of vitamin D, 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Because 1,25-(OH)<sub>2</sub>D<sub>3</sub> regulates intestinal Ca and Pi absorption,<sup>22,23</sup> we also examined rates of transepithelial transport of these minerals as well as determined expression levels of transporters and proteins involved in their uptake. Expression and activity levels of sugar transporters were

also monitored as positive control because these transporters are affected by dietary sugars and as negative control because they are not affected by nephrectomy.

## RESULTS

### Biomarkers of CKD

Rats (250 to 275 g) were randomly assigned to either the group that underwent 5/6 nephrectomy or the group that had the sham operation. Compared with sham-operated rats, 5/6Nx rats had a slower (approximately 10%) increase in body weight after surgery (Table 1); however, daily food intake relative to body weight was similar between the two groups. BUN and creatinine levels increased by approximately 50% in 5/6Nx compared with sham-operated rats 2 and 6 wk after surgery. Pi level in the blood was not significantly different between 5/6Nx and sham-operated rats at wk 2; however, after 6 wk, the 5/6Nx rats started to develop hyperphosphatemia ( $P = 0.08$ ). In contrast, serum Ca concentrations were similar between 5/6Nx and sham-operated rats at 2 or 6 wk after surgery.

Table 2 shows the body and blood parameters at week 10 after surgery, after the rats were fed glucose or fructose diets for 4 wk. The diets had no significant effect on body weight and food intake; however, 5/6Nx rats had significantly lower body weights compared with sham-operated rats. The kidney mass index increased with 5/6 nephrectomy, and within the 5/6Nx group, the rats fed fructose (5/6Nx-F) had a significantly higher kidney mass than rats fed glucose (5/6Nx-G). 5/6Nx rats had greater BUN and creatinine serum levels than sham-operated rats, and both biomarkers of CKD were significantly exacerbated by fructose feeding. After 10 wk, 5/6 nephrectomy also induced a significant hyperphosphatemia that likewise was significantly aggravated by dietary fructose. Even in the absence of significant effects of the diet and nephrectomy on serum Ca, the Pi-Ca product ( $[Pi] \times [Ca]$ ) was significantly higher in 5/6Nx than in sham-operated rats, with a tendency to increase further with fructose feeding ( $P = 0.09$ , one-way ANOVA least significant difference [LSD]). Serum fructose was detectable in our samples only in fructose-fed rats, and the concentration was highest in 5/6Nx-F.

**Table 1.** Clinical and biochemical parameters in 5/6Nx and sham-operated rats 2 and 6 weeks after surgery

Parameter	2 Wk		6 Wk		Significance		
	Sham	5/6Nx	Sham	5/6Nx	Surgery	Time after Surgery	Surgery × Time Interaction
Food type	Chow	Chow	Chow	Chow			
Body weight (g)	399 ± 9	361 ± 6	526 ± 12	459 ± 14	<0.001	<0.001	0.205
Food intake (g/d per kg body wt)	68 ± 9	66 ± 8	88 ± 12	90 ± 14	0.981	<0.001	0.723
BUN (mg/dl)	27.9 ± 3.7	54.3 ± 5.6	22.7 ± 1.8	51.2 ± 4.3	<0.001	0.261	0.775
Creatinine (mg/dl)	0.46 ± 0.07	0.71 ± 0.11	0.43 ± 0.15	0.86 ± 0.02	<0.001	0.358	0.958
Pi (mg/dl)	6.4 ± 0.1	6.7 ± 0.2	6.3 ± 0.2	7.0 ± 0.3	0.077	0.708	0.389
Ca (mg/dl)	10.4 ± 0.3	9.8 ± 0.1	10.7 ± 0.5	9.8 ± 0.1	0.120	0.442	0.959

Data are means ± SEM;  $n = 10$  per group. Statistical significance refers to two-way ANOVA.

**Table 2.** Clinical and biochemical parameters in 5/6Nx and sham-operated rats 10 wk after surgery and after 4 wk of special sugar diet

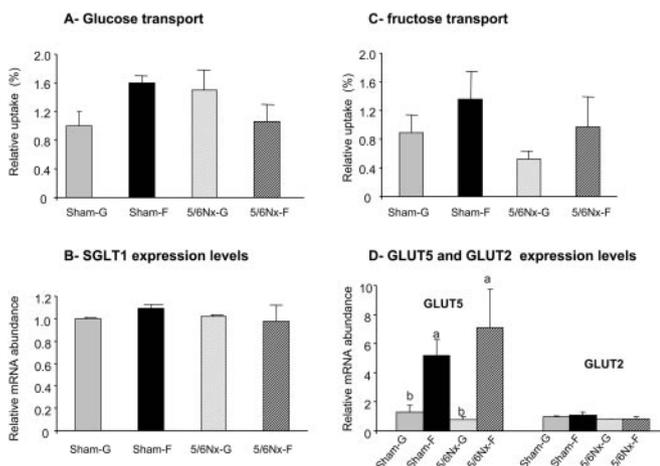
Parameter	Sham		5/6Nx		Significance			
	Glucose	Fructose	Glucose	Fructose	Two-Way			One-Way
					Diet	Surgery	Diet × Surgery	
Body weight (g)	644 ± 26	596 ± 16 <sup>a,b</sup>	505 ± 39 <sup>c</sup>	538 ± 29 <sup>b,c</sup>	0.959	0.005	0.242	0.034
Food intake (g/d/kg body wt)	86 ± 7	84 ± 8	80 ± 4	83 ± 7	0.951	0.450	0.548	0.311
Kidney mass index (g/kg body wt)	3.02 ± 0.20 <sup>a</sup>	3.05 ± 0.20 <sup>a</sup>	4.21 ± 0.20 <sup>b</sup>	5.39 ± 0.40 <sup>c</sup>	0.086	0.002	0.105	0.007
BUN (mg/dl)	13.9 ± 7.2 <sup>a</sup>	28.7 ± 8.7 <sup>a,b</sup>	40.1 ± 6.2 <sup>b</sup>	56.0 ± 3.1 <sup>c</sup>	0.022	0.0002	0.554	0.0004
Creatinine (mg/dl)	0.72 ± 0.11 <sup>a</sup>	0.75 ± 0.05 <sup>a</sup>	1.13 ± 0.04 <sup>b</sup>	1.45 ± 0.07 <sup>c</sup>	0.146	0.0004	0.197	0.0005
Pi (mg/dl)	6.3 ± 0.2 <sup>a</sup>	6.1 ± 0.2 <sup>a</sup>	7.1 ± 0.5 <sup>a</sup>	9.1 ± 0.6 <sup>b</sup>	0.208	0.016	0.143	0.017
Ca (mg/dl)	10.1 ± 0.6	8.8 ± 1.1	11.2 ± 0.3	11.5 ± 0.3	0.521	0.090	0.527	0.211
[Pi] × [Ca] (mg <sup>2</sup> /dl <sup>2</sup> )	64 ± 5 <sup>a</sup>	54 ± 7 <sup>a</sup>	81 ± 7 <sup>a,b</sup>	102 ± 10 <sup>b</sup>	0.520	0.005	0.117	0.007
Fructose (mM)	ND	1.4 ± 0.6	ND	3.9 ± 1.6	0.027	0.274	0.274	0.120

Data are means ± SEM; n = 4 to 5 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. ND, not detectable.

### Sugar, Pi, and Ca Transporter Activity and Expression after 4 Wk of Fructose Feeding

#### Sugars.

The intestinal uptake of glucose was independent of nephrectomy ( $P > 0.8$ ) and diet ( $P > 0.7$ ; Figure 1A). Fructose feeding induced



**Figure 1.** Dietary sugar effects on the regulation of intestinal glucose and fructose absorption in sham-operated and 5/6Nx rats. (A and C) Glucose (A) and fructose (C) uptakes were measured from the jejunum of sham-operated or 5/6Nx rats that had been fed a glucose or fructose diet for 4 wk. (B and D) The effects of dietary sugar and nephrectomy were measured on the mRNA levels of the intestinal apical glucose transporter, SGLT 1 (B), and the intestinal apical fructose transporter, GLUT5 and the basolateral glucose and fructose transporter GLUT2 (D), by real-time PCR using *EF1 $\alpha$*  as a reference gene. Data are expressed relative to the level seen in Sham-G rats (Sham-G = 1.0). Data are means ± SEM (n = 4 to 5 per group). For glucose uptakes and for SGLT1 and GLUT2 expression, a two-way ANOVA showed no significant diet or surgery effects. For fructose uptake and GLUT5 mRNA, there were significant diet but not nephrectomy effects by two-way ANOVA; <sup>a,b</sup>Significant differences ( $P < 0.05$ ).

a significant increase in fructose uptake ( $P < 0.05$ ; Figure 1C), resulting from a specific increase in GLUT5 but not GLUT2 expression ( $P < 0.01$ ; Figure 1D). The expression level of the Na<sup>+</sup>/glucose co-transporter (SGLT1) remained unchanged (Figure 1B). The 5/6 nephrectomy did not affect the rate of fructose uptake and the expression of GLUT5 and GLUT2 ( $P > 0.8$ ).

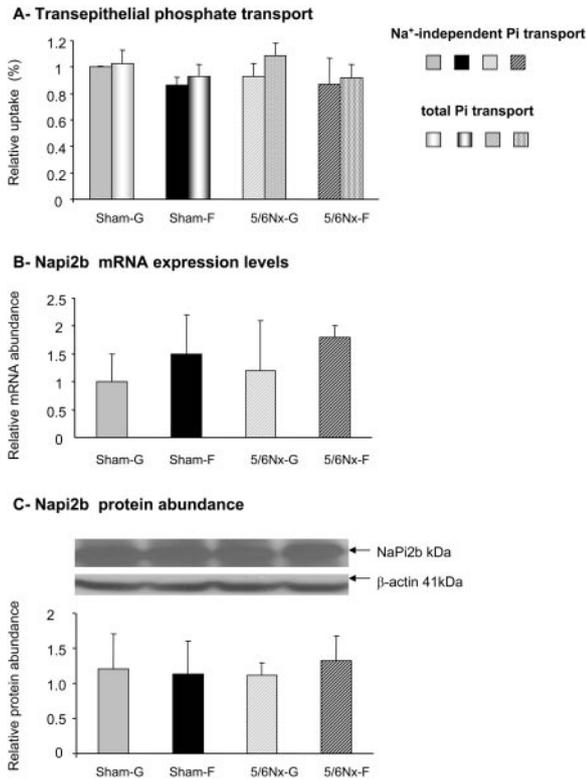
#### Phosphate.

Pi uptake in the jejunum is mainly Na<sup>+</sup> independent (Figure 2A). Thus, the active Na<sup>+</sup>-dependent Pi transport system mediated by NaPi2b contributes little to total Pi transport in adult rats, as would be expected from the dramatic decrease in NaPi2b expression with age that we<sup>15</sup> and others<sup>24</sup> found in rats. Total ( $P > 0.8$ ) and Na<sup>+</sup>-independent ( $P > 0.8$ ) Pi uptake did not change in sham-operated compared with 5/6Nx rats. Likewise, dietary sugar did not affect total ( $P > 0.3$ ) Na<sup>+</sup>-independent ( $P > 0.5$ ) Pi uptake. NaPi2b mRNA levels were not affected by 5/6 nephrectomy or dietary sugar ( $P > 0.8$  for both factors; Figure 2B), and so were NaPi2b protein levels ( $P > 0.7$  for both factors; Figure 2C). In this study, we used older rats exhibiting insignificant rates of active Na<sup>+</sup>-dependent Pi transport, and this minor transport pathway must not be regulated by fructose, as was observed in weaning pups.<sup>15,25</sup>

#### Calcium.

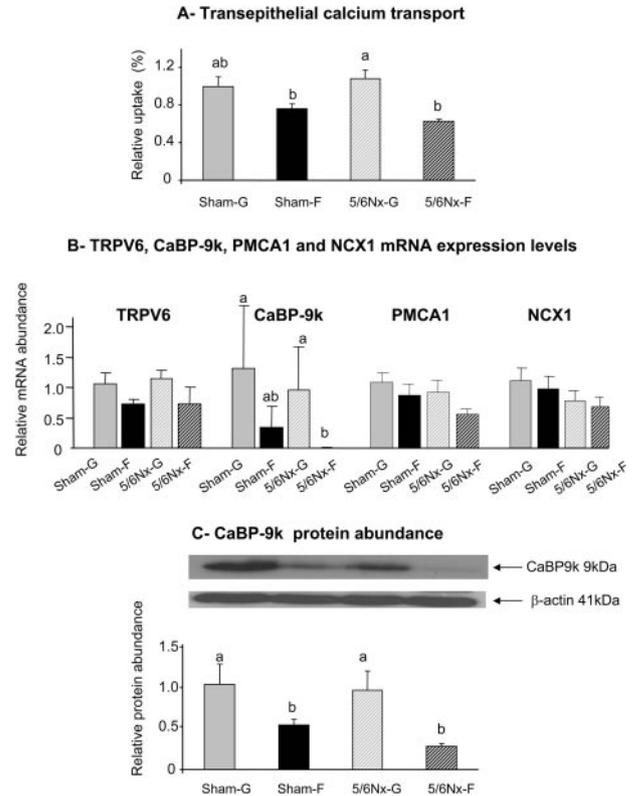
Fructose feeding induced a highly significant ( $P < 0.004$ ) decrease in intestinal Ca uptake (Figure 3A). Interestingly, there was no effect of nephrectomy on intestinal Ca uptake ( $P > 0.7$ ). In sham-operated ( $P = 0.08$  by one-way ANOVA LSD) and 5/6Nx ( $P < 0.05$  by one-way ANOVA LSD) rats, there was a 23 and 42% decrease, respectively, in Ca transport with fructose feeding.

Nephrectomy ( $P > 0.8$ ) and dietary fructose ( $P = 0.1$ ) had no effect on mRNA expression of TRPV6 (Ca transporter 1), which mediates the uptake of dietary Ca from the lumen across the apical membrane into the cytosol (Figure 3B). Dietary fructose ( $P < 0.05$ ) but not nephrectomy ( $P > 0.10$ ) significantly



**Figure 2.** Dietary sugar effects on the regulation of intestinal Pi absorption in sham-operated and 5/6Nx rats. Pi transport was measured using everted intestinal sacs formed from the jejunum of sham or 5/6Nx rats fed glucose or fructose diet for 4 wk. (A) The total and Na<sup>+</sup>-independent Pi transport from the apical to the basolateral compartments was expressed as a ratio of the final quantity of (<sup>33</sup>Pi inside/<sup>33</sup>Pi outside) of the everted gut sacs. Data are means ± SEM (n = 4 to 5 per group). (B) Napi2b mRNA expression was analyzed by real-time PCR using *EF1α* as a reference. (C) The expression level of Napi2b protein was analyzed by Western blot, and the protein abundance was expressed as the ratio of Napi2b to β-actin. Data are normalized relative to levels seen in Sham-G rats (Sham-G = 1.0). A two-way ANOVA did not show significant diet and surgery effects.

decreased the mRNA expression of the intestinal intracellular Ca binding protein 9k (CaBP-9k) that mediates the transfer of dietary Ca from the apical to the basolateral pole of the enterocyte. By one-way ANOVA LSD, CaBP-9k expression in 5/6Nx-F was less than that in sham-operated rats that were fed glucose (Sham-G) and 5/6Nx-G ( $P < 0.05$ ). The mRNA expression levels of the two basolateral Ca transporters, Ca-AT-Pase 1 (PMCA1) and the Na/Ca exchanger 1 (NCX1), each were not affected by nephrectomy ( $P > 0.6$  and  $P > 0.5$ , respectively) or by diet ( $P = 0.09$  and  $P > 0.2$ , respectively). Dietary fructose ( $P < 0.005$ ) but not nephrectomy ( $P > 0.4$ ) diminished the protein expression of CaBP-9k, which decreased by 50% in sham-F compared with sham-G ( $P < 0.05$ , one-way ANOVA LSD) and by 70% in 5/6Nx-F compared with 5/6Nx-G ( $P < 0.02$ , one-way ANOVA LSD; Figure 3C). No specific antibody for TRPV6 is available. Likewise, after several

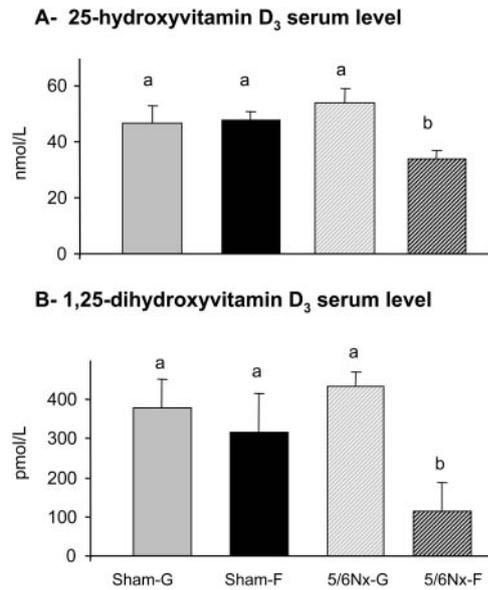


**Figure 3.** Dietary sugar effects on the regulation of intestinal Ca transport in sham-operated and 5/6Nx rats. Ca transport was measured using everted intestinal sacs formed from the duodenum of sham or 5/6Nx rats that had been fed glucose or fructose diet for 4 wk. (A) Trans epithelial Ca transport from the apical to the basolateral compartment was expressed as a ratio of the final quantity of (<sup>45</sup>Ca inside/<sup>45</sup>Ca outside) of the everted gut sacs. (B) mRNA expression of TRPV6, CaBP-9k, PMCA1, and NCX1 was analyzed by real-time PCR using *EF1α* as a reference. (C) The protein abundance of CaBP-9k was expressed as the ratio of calbindin to β-actin protein. All data were normalized relative to levels seen in Sham-G rats (Sham-G = 1.0). Data are means ± SEM (n = 4 to 5 per group). Differences ( $P < 0.05$ ) among means, indicated by differences in superscript letters, were analyzed by one-way ANOVA LSD after a two-way analysis showed significant diet or surgery effects.

attempts, we found no suitable antibody for PMCA1 and NCX1.

### Vitamin D

The serum concentration of 25-hydroxyvitamin D<sub>3</sub> [25-(OH)D<sub>3</sub>] was not significantly altered by dietary fructose ( $P = 0.07$  by two-way ANOVA) and by nephrectomy ( $P > 0.5$ ; Figure 4A); however, the significant interaction ( $P = 0.05$ ) indicated that the effect of diet depended on the type of surgery. Indeed, when analyzed by one-way ANOVA LSD, there clearly was a significantly lower 25-(OH)D<sub>3</sub> serum level (by 30 to 40%) in 5/6Nx-F rats compared with those in the other three groups ( $P < 0.05$ ). Diet ( $P < 0.02$ ) but not nephrectomy ( $P > 0.1$ ) affected the levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the serum. Subse-



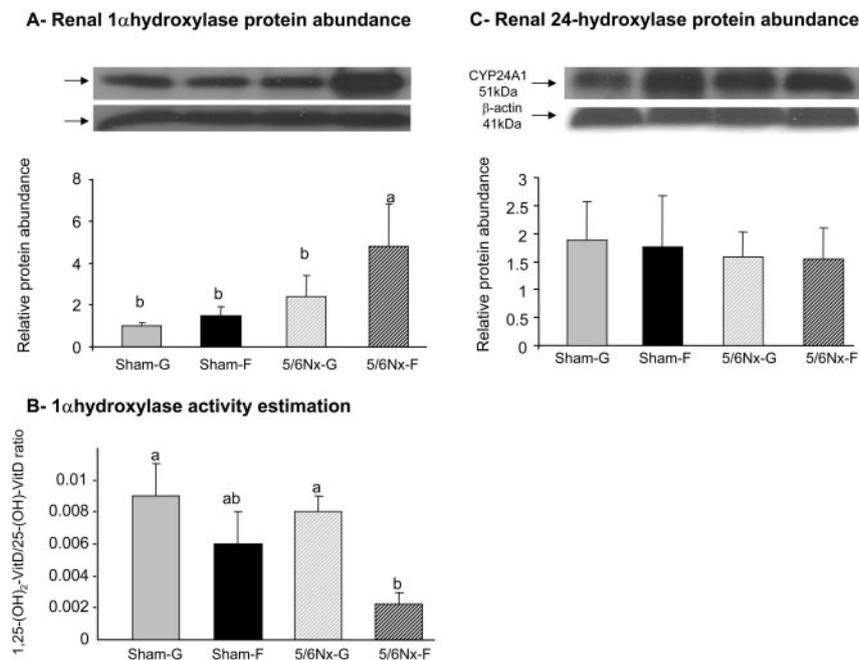
**Figure 4.** Dietary sugar effects on levels of vitamin D in the serum. (A) Levels of 25-(OH)D<sub>3</sub> and (B) 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the serum of sham and 5/6Nx rats that had been fed glucose or fructose diet for 4 wk. Data are means  $\pm$  SEM ( $n = 4$  to 5 per group). Differences among means, indicated by differences in letters, were analyzed by one-way ANOVA LSD after a two-way analysis showed significant diet and surgery effects ( $P < 0.05$ ).

quent one-way ANOVA LSD indicated that the 5/6Nx-F rats had 2.5 to four-fold lower 1,25-(OH)<sub>2</sub>D<sub>3</sub> serum levels than those of the other three groups ( $P \leq 0.05$ ; Figure 4B). Hence, dietary fructose decreased 25-(OH)D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub> only in 5/6Nx rats.

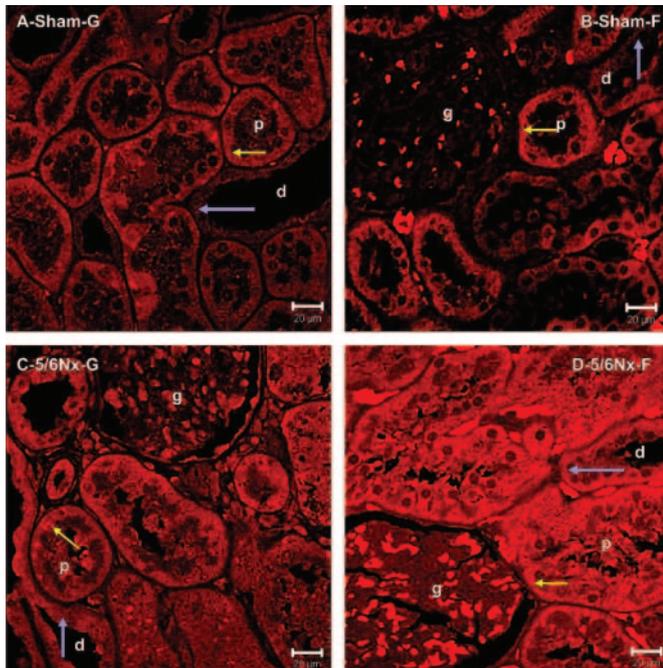
In the kidney, the protein expression level of 1 $\alpha$ -hydroxylase [CYP27B1, which mediates the 1 $\alpha$ -hydroxylation of 25-(OH)D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub>] is significantly affected by diet ( $P < 0.005$ ) but not by surgery ( $P > 0.3$ ; Figure 5A). By one-way ANOVA, 1 $\alpha$ -hydroxylase protein abundance increased (two- to five-fold) significantly in the 5/6Nx-F group compared with that in the other three groups ( $P \leq 0.05$ ; Figure 5A). The relative 1 $\alpha$ -hydroxylase activity, evaluated by the ratio [1,25-(OH)<sub>2</sub>D<sub>3</sub>/25-(OH)D<sub>3</sub>] shows the same pattern as that of serum levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub>: The ratio [1,25-(OH)<sub>2</sub>D<sub>3</sub>/25-(OH)D<sub>3</sub>] was significantly affected by diet ( $P < 0.04$ ) but not by surgery ( $P > 0.1$ ; Figure 5B). In contrast, the protein level of 24-hydroxylase [CYP24A1 involved in the degradative pathway of 25-(OH)D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub>] remained similar among the four groups (Figure 5C).

#### Renal Histology and 1 $\alpha$ -Hydroxylase Immunohistology

1 $\alpha$ -Hydroxylase is expressed at high levels only in the proximal tubule of normal kidneys (Figure 6, A and B, yellow arrow for proximal tubule, which is brighter red compared with the distal tubule [blue arrow]). In 5/6Nx rats, however, 1 $\alpha$ -hydroxy-



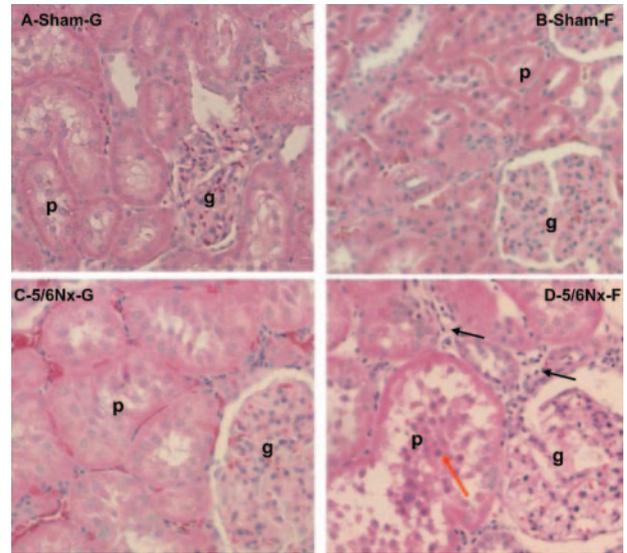
**Figure 5.** Dietary sugar effects on protein levels of hydroxylases involved in vitamin D metabolism. (A and C) Protein expression level of 1 $\alpha$ -hydroxylase (CYP27B1; A) and of 24-hydroxylase (CYP24A1; C) was analyzed on the whole kidney. (B) Relative 1 $\alpha$ -hydroxylase activities expressed as ratio of 1,25-(OH)<sub>2</sub>D<sub>3</sub>/25-(OH)D<sub>3</sub> in sham or 5/6Nx rats that had been fed glucose or fructose diet for 4 wk. The protein abundance values corresponded to a ratio of CYP27B1 (A) or CYP24A1 (B) to  $\beta$ -actin, and the data are normalized relative to levels seen in Sham-G rats (Sham-G = 1.0). Data are means  $\pm$  SEM ( $n = 4$  to 5 per group). Differences among means, indicated by differences in letters, were analyzed by one-way ANOVA LSD after a two-way analysis showed significant diet and surgery effects ( $P < 0.05$ ).



**Figure 6.**  $1\alpha$ -Hydroxylase (CYP27B1) is immunolocalized in kidney of sham or 5/6Nx rats fed glucose or fructose diets. (A through D) Analysis of  $1\alpha$ -hydroxylase expression in the distal tubules (d), proximal tubules (p), and glomerulus (g) in the Sham-G rats (A), Sham-F rats (B), 5/6Nx-G rats (C), and 5/6Nx-F rats (D). Yellow arrows indicate  $1\alpha$ -hydroxylase occupying the basolateral cytosolic pole of cells lining the proximal tubules (A through D). Blue arrows indicate the absence of  $1\alpha$ -hydroxylase in the cells of the distal tubules in sham rats (A and B) or its presence in the distal tubules of 5/6Nx rats (C and D).

lase becomes equally expressed in both proximal and distal tubules (Figure 6, C and D, yellow arrow *versus* blue arrow), suggesting that distal tubular cells were recruited to express  $1\alpha$ -hydroxylase. The intensity of the staining in both the proximal and distal tubules is higher in the 5/6Nx-F rats (Figure 6D, yellow and blue arrows, respectively) than in the other three groups.

In association with the increase in kidney mass index, hematoxylin and eosin staining showed significantly larger and seemingly more deformed glomeruli and proximal tubules in 5/6Nx (Figure 7, C and D) compared with sham-operated (Figure 7, A and B) rats. The increase in proximal tubule diameter has been shown to originate from hypertrophy and not hyperplasia of tubular cells.<sup>26</sup> There may be a greater incidence of proximal tubular damage as well as an increase in cellular debris in the tubular lumen (Figure 7, D *versus* C, red arrow) of 5/6Nx-F rats compared with glucose controls. The number of periglomerular interstitium that was infiltrated by mononuclear cells (inflammatory signals) was observed to be higher in 5/6Nx-F rats than that in the other three groups (Figure 7D, black arrows); however, the increase in levels of serum  $[Ca] \times [Pi]$ , as is often the case in long-term ESRD patients, did not lead to its accumulation in tissues, as shown by the absence of



**Figure 7.** The effect of diet on the progression of CKD damage in 5/6Nx rats is histologically assessed. Renal tissue was obtained from Sham-G (A), Sham-F (B), 5/6Nx-G (C), or 5/6Nx-F (D) rats. Hematoxylin and eosin staining shows marked glomeruli (g) and proximal tubular (p) hypertrophy in 5/6Nx rats; the tubular hypertrophy is much higher in the 5/6Nx-F rats (D) than in the 5/6Nx-G rats (C). (D) The red arrow shows the accumulation of cell debris in the lumen of the proximal tubules of 5/6Nx-F rats; the black arrows show increased infiltration of macrophages in 5/6Nx-F rats.

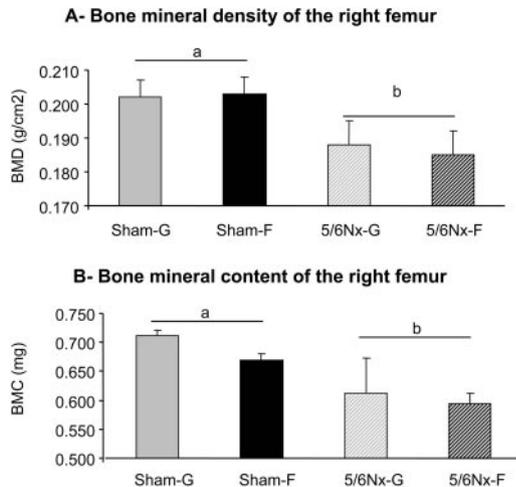
hydroxyapatite deposits in the kidney of hyperphosphatemic 5/6Nx-F and 5/6Nx-G rats (Supplemental Figure S1).

### Bone Mineral Density and Content after Nephrectomy

Bone mineral density (BMD) of the left femur decreased significantly with nephrectomy ( $P < 0.05$ ) but was independent of diet ( $P > 0.9$ ; Figure 8A). Bone mineral content (BMC) was also significantly affected by nephrectomy ( $P < 0.02$ ) but not by diet ( $P > 0.4$ ; Figure 8B). No significant interaction between surgery and diet were observed.

## DISCUSSION

This study has three major findings. First, chronic consumption of a high-fructose diet when renal function is already compromised drastically reduces serum levels of  $25\text{-(OH)}_2\text{D}_3$  and  $1,25\text{-(OH)}_2\text{D}_3$ . Second, chronic consumption of a high-fructose diet has a marked deleterious effect on intestinal Ca absorption mediated mainly by a decrease in CaBP-9k expression. Finally, dietary fructose worsens the progression of renal dysfunction, as indicated by increases in most of the blood parameters (BUN, creatinine, Pi,  $[Pi] \times [Ca]$ ), indicative of CKD. We also confirmed the augmentation of kidney mass and the changes in renal histologic structure in 5/6Nx-F rats first mentioned by Gersch *et al.*<sup>10</sup>



**Figure 8.** Dietary sugar effects on BMD (A) and BMC (B) of the right femur determined 10 wk after surgery from Sham-G, Sham-F, 5/6Nx-G or 5/6Nx-F rats. Data are means  $\pm$  SEM ( $n = 4$  to 5 per group). Differences between means, indicated by differences in letters, were analyzed by two-way ANOVA ( $P < 0.05$ ).

### Fructose Reduces Serum Vitamin D Levels in 5/6Nx Rats

The conversion of vitamin D to the active form 1,25-(OH)<sub>2</sub>D<sub>3</sub> involves hepatic 25-hydroxylation by cytochrome P450 enzymes of vitamin D<sub>3</sub> to 25-(OH)D<sub>3</sub>. There are still controversies about the specific enzymes mediating this step.<sup>27,28</sup> The second step involves the hydroxylation of 25-(OH)D<sub>3</sub> by 1 $\alpha$ -hydroxylase in renal proximal tubule cells, leading to the formation of 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

#### The 25-Hydroxylation Step.

Levels of dietary vitamin D and of light exposure (factors regulating the serum levels of 25-(OH)D<sub>3</sub>) were similar in all groups of rats, so dietary precursors or substrate levels cannot explain the low level of 25-(OH)D<sub>3</sub> in 5/6Nx-F rats. The six hepatic cytochrome P 450 having a 25-hydroxylase activity (CYP27A1, CYP2R1, CYP2C11, CYP2D25, CYP33A4, and CYP2J3)<sup>27,28</sup> all are NADPH dependent. It has been shown that fructose induces the NADPH-consuming synthesis of fatty acids in the liver, as well as of reactive oxygen species production by NADPH oxidase (unpublished observations), thereby potentially reducing levels of NADPH available for the 25-hydroxylation step. Moreover, hepatic oxidative stress increases with renal failure,<sup>29</sup> which may amplify the NADPH reduction in the 5/6Nx-F rats compared with the sham-F rats and explain why the decrease in 25-(OH)D<sub>3</sub> level is observed only in 5/6Nx-F rats.

#### The 1,25-Hydroxylation Step.

Although 1 $\alpha$ -hydroxylase is expressed in extrarenal tissues,<sup>30</sup> their contribution to the levels of serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> is modest.<sup>31</sup> Thus, there are likely three proximate factors regulating the 1,25-(OH)<sub>2</sub>D<sub>3</sub> serum level: The amount of precursor 25-

(OH)D<sub>3</sub> from the liver, the rate of degradation from 24-hydroxylase, and the rate of synthesis arising from the activity of renal 1 $\alpha$ -hydroxylase. First, the precursor 25-(OH)D<sub>3</sub> is indeed already quite low in 5/6Nx-F rats. Second, there is no significant change in the amount of 24-hydroxylase, suggesting there is no diet- and nephrectomy-induced change in degradation rate of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Finally, the reduction of 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels in 5/6Nx-F rats was significant despite marked increases in 1 $\alpha$ -hydroxylase protein abundance.<sup>32</sup> The renal abundance of 1 $\alpha$ -hydroxylase is inversely regulated by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, a potent inhibitor of CYP27B1 gene expression.<sup>33</sup> The paradoxical decrease in serum levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub> despite an increase in 1 $\alpha$ -hydroxylase protein may also be due to decreases in 1 $\alpha$ -hydroxylase activity. Indeed, the ratio [1,25-(OH)<sub>2</sub>D<sub>3</sub>/25-(OH)D<sub>3</sub>], commonly used as an index of the 1 $\alpha$ -hydroxylase activity,<sup>34</sup> is reduced in 5/6Nx-F rats. 1 $\alpha$ -Hydroxylase is also a mitochondrial cytochrome P450, which, like the other hepatic CYP proteins mentioned, is NADPH dependent. During CKD, renal gluconeogenesis is strongly repressed, which likely leads to decreases in NADPH production. Moreover, as in the liver, fructose consumption as well as renal failure results in increased levels of oxidative stress in the kidney.<sup>35</sup> Such increases would reduce NADPH levels, leading to marked decreases in 1 $\alpha$ -hydroxylase activity in 5/6Nx-F rats.

### Intestinal Ca Uptake and CaBP-9k Expression

The inhibitory effect of fructose on intestinal Ca transport is specific, because fructose had no effect on transepithelial Pi transport and transapical glucose uptake. Transepithelial Ca transport and CaBP-9k expression each are clearly reduced only by fructose in both 5/6Nx and sham-operated rats, suggesting the reduction is mainly a diet effect. Previous studies showed a strong correlation between Ca intestinal absorption and CaBP-9k expression levels<sup>36</sup>; however, this effect of fructose is a novel and unexpected observation. In accordance with our findings, previous studies on the interaction between dietary sugars and mineral absorption showed that lactose (a digestible disaccharide like fructose) decreased intestinal Ca absorption as well as CaBP-9k protein levels.<sup>37</sup> In contrast, indigestible oligosaccharides enhanced intestinal Ca absorption,<sup>38</sup> probably *via* an increase in the passive paracellular route.<sup>39</sup> It would be interesting to determine whether the fructose-induced decrease in transepithelial Ca absorption is also associated with a change in paracellular intestinal Ca transport.

The link between the fructose-induced decrease in 1,25-(OH)<sub>2</sub>D<sub>3</sub>, which normally regulates intestinal Ca transport, and the fructose-induced decrease in Ca transport is not clear because fructose affected 1,25-(OH)<sub>2</sub>D<sub>3</sub> only in 5/6Nx-F rats. The effect of fructose on Ca transport may be partly vitamin D independent. Indeed, other studies showed vitamin D receptor-independent regulation of intestinal Ca absorption and of CaBP-9k expression.<sup>40</sup> Moreover, fructose metabolism leads to rapid reductions in ATP and Pi levels in hepatocytes<sup>41</sup> and enterocytes,<sup>42</sup> thereby affecting all ATP- and Pi-dependent processes in the cells, such as PMCA1 and NCX1. Further stud-

ies are needed to evaluate the contributions of PMCA1 and NCX1 as well as TRPV6 to fructose-induced changes in intestinal Ca transport.

### Fructose Exacerbates Classical Symptoms of CKD

The mechanisms underlying the fructose-induced enlargement of the kidney and degeneration of renal function are unknown. The effect can be indirect because dietary fructose increases the incidence of hypertension, insulin resistance, and dyslipidemia,<sup>4</sup> which are metabolic diseases that often lead to kidney dysfunction. The effect can also be direct, because fructose promotes the production of proinflammatory factors such as reactive oxygen species and monocyte chemoattractant protein 1 in cultured proximal tubular cells HK2.<sup>11</sup> Because GLUT5 is expressed in the proximal tubular cells,<sup>43</sup> they must be able to absorb fructose, possibly promoting fructose-induced inflammation.

Enlargement of the kidney usually reflects a compensation for a decrease in kidney filtration rates, as would be expected after nephrectomy. Fructose-induced increases in renal mass index therefore indicate further decreases in renal filtration rate, which may lead to fructose causing a further elevation of nephrectomy-induced changes in serum biomarkers related to renal function (*e.g.*, Pi, fructose).

### Other Consequences of Fructose Feeding: Bone Integrity and Tissue Calcification

Most likely, an experimental duration longer than 1 mo is required before significant effects of fructose on bone integrity can be observed. In fact, bone remodeling takes at least 4 wk to happen in rodents.<sup>44</sup>

There was no deposition of hydroxyapatite in the kidney (Supplemental Figure S1) of 5/6Nx-F rats, the group with the highest blood levels of phosphate and of  $[Ca] \times [Pi]$ . In our study, in the 5/6Nx-F rats, the Pi was only 9 mg/dl (1 mM), well below the critical concentration required for calcification (*e.g.*, 11.5 mg/dl Ca).<sup>45</sup> Because serum Pi level increased by as much as 50% in the 5/6Nx-F rats after only 4 wk, we predict that the calcification of soft tissues and blood vessels would be another major complication of chronic fructose consumption in advanced renal failure.

## CONCLUSION

Until now, the recommended diet for the patient with CKD is traditionally based on protein and phosphorus restriction. Excessive fructose intake may also be carefully avoided by the patient with CKD to slow the progression of the disease. Besides renal degradation, we show here that not only mineral but also vitamin D homeostasis is markedly altered by excessive fructose consumption. The decrease in 25-(OH)D<sub>3</sub> level indicates that liver function during ESRD is also compromised by fructose feeding. Further studies need to be conducted to evaluate whether the impairment of vitamin D synthesis by

fructose is direct or is a secondary, indirect effect of the deleterious consequence of fructose on renal function. Dietary fructose constitutes approximately 60% of our rat diet, but the average fructose content of American diets is 15 to 17%; therefore, further studies need to be done using lower levels of dietary fructose and longer durations of feeding to evaluate properly the effect of this sugar on the progression and complications of CKD.

## CONCISE METHODS

### Animals and Treatment

All of the procedures conducted in this study were approved by the Institutional Animal Care and Use Committee, UMDNJ-New Jersey Medical School. Studies were conducted on 20 male Sprague-Dawley rats (approximately 3 mo old and 200 to 210 g at the beginning of the experiment). Male sham-operated and 5/6Nx rats were purchased from Charles River Laboratories US (Wilmington, MA). Five-sixths nephrectomy was performed by ligating two of three branches of the renal artery of the right kidney and then removing 1 wk later the left kidney. The rats were delivered 1 wk after the second surgery and at this time divided in two groups: The sham-operated rats (sham; *n* = 10 rats) and the 5/6Nx rats (*n* = 10). They all were housed in the research animal facility and kept under standard conditions, 12-h light/dark photoperiod and controlled temperatures (22 to 24°C), and fed for 5 wk a commercial rat chow (Purina Mills, Richmond, IN) and water *ad libitum*.

### Experimental Design

Body weights and food consumption of 5/6Nx and sham-operated rats were measured weekly. Serum biochemical parameters were measured at 2 and 6 wk. Blood was collected from the tail vein in anesthetized (0.2 to 0.4 ml/100g body wt, intraperitoneally, of ketamine 20 mg/ml and xylazine 2.5 mg/ml), unconscious rats. Six weeks after surgery, the sham and 5/6Nx groups were each randomly divided in two groups fed either a 60% glucose or a 60% fructose AIN-93M-based diet<sup>46</sup> (Research Diets; Supplemental Table S1). Rats were fed the high-sugar diets for 4 wk *ad libitum* because the weekly measurement of food intake and body weight showed similar food consumption rates (when normalized to body weight) among the four groups. After 4 wk of fructose or glucose diets (total 10 wk after surgery), rats were anesthetized and killed.

### Tissue Sampling

After opening the abdominal cavity, a small incision was made 5 cm distal to the stomach, and a catheter was inserted into the lumen of the small intestine. After its contents were flushed with ice-cold KRB, the small intestine was removed entirely and placed in ice-cold KRB and divided into five segments. Segment 1 was the proximal 5 cm of the duodenum for use in Ca uptakes. Segment 2 was the subsequent 3 cm, which was quickly frozen in liquid nitrogen and then stored at -80°C for subsequent protein and mRNA analyses. Segment 3 was the first 4 cm of the jejunum and was stored in ice-cold KRB for glucose and fructose uptakes. Segments 4 and 5 were the following 10 cm divided in two equal pieces and reserved in ice-cold KRB for measurements of

total and  $\text{Na}^+$ -independent Pi uptake, respectively. The entire right kidney was removed, weighed, and either fixed in 4% paraformaldehyde (for two rats in each group) or quickly frozen in liquid nitrogen and then stored at  $-80^\circ\text{C}$  for subsequent protein extractions (for three rats in each group). The blood was then sampled by cardiac puncture, and the serum was separated in a BD Vacutainer (BD Scientific) by centrifugation for 10 min at 5000 rpm and stored at  $-80^\circ\text{C}$ . The left femur was dissected, the muscle fibers were removed, and the bone was stored at  $-80^\circ\text{C}$  until later analyses.

### Intestinal In Vitro Uptake Measurements

All of the segments were everted quickly after isolation and prepared as everted sacs or sleeves to determine nutrient function as described next.

#### Ca Uptake.

The intestinal Ca transport determination was modified from the classical everted sac method described by Benn *et al.*<sup>47</sup> The everted gut sacs were made by using the first 5-cm segment of proximal duodenum, where active transport of Ca is localized. The everted intestinal segment was filled with 400  $\mu\text{l}$  of transport buffer (125 mM NaCl, 10 mM fructose, 1.3 mM HEPES, and 0.25 mM  $\text{CaCl}_2$  [pH 7.4]), and two ligations were tied at both extremities of the segment. The sacs were incubated in tubes containing 30 ml of transport buffer containing  $^{45}\text{CaCl}_2$  (40,000 cpm/ml) and kept in a water bath at  $37^\circ\text{C}$  for 1 h aerated continuously with 95%  $\text{O}_2/5\% \text{CO}_2$ . At the end of the incubation, sacs were removed from the flask, gently washed in cold KRB, blotted dry, and cut open. A total of 50  $\mu\text{l}$  of internal fluid from the sac and 50  $\mu\text{l}$  from the external incubation medium were assayed in duplicate for  $^{45}\text{Ca}$  using a scintillation counter. The active accumulation of  $^{45}\text{Ca}$  in the inside fluid was expressed as a ratio of the final quantity of  $^{45}\text{Ca}$  inside/outside. The everted gut sac assay selectively measures active transepithelial Ca transport, which occurs primarily at low concentrations of Ca and is saturable.<sup>47</sup>

#### Fructose and Glucose Uptake.

From the first 4 cm of everted jejunum, four 1-cm jejunal segments were prepared and preincubated at  $37^\circ\text{C}$  for 5 min in Ringer bubbled with 95%  $\text{O}_2/5\% \text{CO}_2$  as described previously.<sup>48</sup> The sleeves were then incubated in 50-mM sugar solutions containing either 10  $\mu\text{Ci}$  D- $^{14}\text{C}$ glucose (NEN, Boston, MA) for 1 min or 10  $\mu\text{Ci}$  of D- $^{14}\text{C}$ fructose for 2 min. We used 20  $\mu\text{Ci}$  of L- $^3\text{H}$ glucose to correct for adherent fluid and passive diffusion of glucose or fructose.

#### Pi Uptake.

Intestinal Pi transport was determined by a modification of the everted gut sac assay using two consecutive 5-cm segments of medial jejunum. The everted intestinal segment dedicated to the measurement of total Pi transport was filled with 400  $\mu\text{l}$  of  $\text{Na}^+$ -containing transport buffer (in mM: 128 NaCl, 4.7 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 1.2  $\text{KH}_2\text{PO}_4$ , and 19  $\text{NaHCO}_3$  [pH 7.3]). The everted intestinal segment dedicated to the measurement of  $\text{Na}^+$ -independent Pi transport was filled with 400  $\mu\text{l}$  of  $\text{Na}^+$ -free transport buffer (128 mM choline chloride replacing NaCl). Then two ligations were tied at both extremities, thereby trapping the buffer within the segment. The everted sac with  $\text{Na}^+$ -containing buffer was incubated in a tube with 30 ml of  $\text{Na}^+$ -containing buffer with  $^{33}\text{Pi}$  (40,000 cpm/ml) and kept in a water bath at  $37^\circ\text{C}$  for 1 h and aerated

continuously with 95%  $\text{O}_2/5\% \text{CO}_2$ . The everted sac with  $\text{Na}^+$ -free transport buffer was likewise incubated in a tube containing  $\text{Na}^+$ -free transport buffer. At the end of the incubation, sacs were removed from the flask, gently washed in cold KRB, blotted dry, and cut open. A total of 50  $\mu\text{l}$  of internal fluid from each sac and 50  $\mu\text{l}$  from the external incubation medium were assayed in duplicate for  $^{33}\text{Pi}$  using a scintillation counter. The active accumulation of  $^{33}\text{Pi}$  in the inside fluid was expressed as a ratio of the final concentration of  $^{33}\text{Pi}$  inside/outside.

### Measurements of Serum Clinical Parameters

Creatinine concentrations in serum samples were determined by the Jaffe method using QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA), serum urea nitrogen (BUN) concentrations were determined by the Jung method using QuantiChrom Urea Assay Kit (BioAssay Systems), and the total serum Pi concentrations were determined by the Malachite en method using QuantiChrom Pi Assay Kit (BioAssay Systems). The total serum Ca concentrations were determined by previously described techniques using flame atomic absorption spectrophotometry (Model 603; Perkin-Elmer, Norwalk, CT).

### Vitamin D Assays

1,25-(OH) $_2\text{D}_3$  levels were measured from serum by enzyme immunoassay according to the manufacturer's protocol (ImmunoDiagnostic Systems). Briefly, serum samples are delipidated and 1,25-(OH) $_2\text{D}_3$  immunoextracted before assaying. 25-(OH) $_2\text{D}_3$  levels was measured directly from serum by enzyme immunoassay according to the manufacturer's protocol (ImmunoDiagnostic Systems).

#### Western Blot Analysis.

For Western blot analysis, supernatants were prepared from 100 mg of tissue (liver, kidney, or intestine) using T-Per Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) and analyzed for protein concentration by the Bradford method. For calbindin-D9k (Swant Swiss Antibodies, Bellinzona, Switzerland), 50  $\mu\text{g}$  of intestinal protein was analyzed by Western blot using precast gradient gel 4 to 20% Tris-HCl (BioRad, Hercules, CA). For other proteins, CYP27B1 (CYP27B1 M-100, Santa Cruz Biotechnology, Santa Cruz, CA) CYP24A1 (CYP24 S-20; Santa Cruz Biotechnology), Napi2B (Chemicon International), and 50  $\mu\text{g}$  of renal or intestinal proteins were analyzed using a precast gel 12% Tris-HCl. All membranes were stripped and reprobed with  $\beta$ -actin antibody (Chemicon International). The relative OD obtained using the test antibody was divided by the relative OD obtained after probing with  $\beta$ -actin to normalize for variations in sample loading and transfer.

#### Extraction of mRNA and Reverse Transcription Reaction.

Total RNA was isolated from 100 mg of frozen small intestine tissue ( $-70^\circ\text{C}$ ) using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 5  $\mu\text{g}$  of total RNA and 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) at  $37^\circ\text{C}$  for 1 h as described by the manufacturer.

#### Real-Time PCR.

Primers were designed using primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/>) and were purchased from Integrated DNA Technologies

(Coralville, IA). Real-time PCR was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA) as follows: The reactions were performed in 12.5  $\mu$ l with 300 nM of each primer and 5  $\mu$ l of a 1:30 dilution of the real-time reaction and the SYBR-Green PCR Master Mix (Stratagene) according to the manufacturer's instructions. The thermal cycling protocol was as follows: 95°C for 10 min, followed by 40 cycles of PCR at 95°C for 30 s, then annealing step at adequate temperature in function of the primers (Supplemental Table S2) and 72°C for 1 min for extension. After the final cycle, melting curves were monitored from 55 to 95°C (0.05°C/s). The relative expression ratio of target genes was calculated as described previously.<sup>49</sup> In all of the experiments, the control group was the Sham-G rats. The target gene expression was also normalized by a reference gene,  $\alpha$ -elongation factor 1 (*EF1 $\alpha$* ), which we already validated as having a stable expression level in our previous experiments.

#### Immunohistochemistry.

After overnight fixation in fresh 4% paraformaldehyde in PBS (pH 7.35), rat kidneys were then embedded in paraffin. Some slides were then processed for routine hematoxylin-eosin staining. For immunohistochemistry, heat-induced antigen retrieval was performed in a programmable pressure cooker (Retriever, Electron Microscopy Services) on 5- $\mu$ m sections in 0.01 M Na citrate buffer (pH 6.0). The sections were rinsed in PBS and blocked in 1% normal goat serum for 1 h at room temperature. The kidney sections were incubated in rabbit anti-mouse CYP27B1 (Santa Cruz Biotechnology; 1:200 in 1% BSA in PBS) overnight at 4°C, then washed in PBS. The secondary antibody was goat anti-rabbit IgG labeled with Cy3 (1:100; Chemicon International), diluted in 1% BSA in PBS and applied for 1 h at room temperature. After PBS wash, the stained sections were mounted in Vectashield (Vector Laboratories) and examined at  $\times 20$  or  $\times 40$  with a laser scanning confocal microscope (Radiance 2100; BioRad). All images of sections being compared were obtained with the same settings of the microscope. Nonspecific staining with secondary antibodies was consistently negligible.

#### Determinations of BMC and BMD.

BMD and BMC were evaluated using dual-energy x-ray absorptiometry (GE-Lunar PIXImus; GE Healthcare). Excised bone measurements were obtained by placing the tibia or femur on a Delrin block. The total tibia or femur was scanned. The PIXImus densitometer uses a 14° stationary anode x-ray tube generator with a 0.25  $\times$  0.25-mm focal spot that generates a cone beam x-ray (55/80 kVp at 400  $\mu$ A). The right femurs were analyzed for the whole bone "total" BMD and BMC. The age coefficient of variation (CV) for three repeated BMD and BMC measurements for four samples was calculated. The CV for the tibia and femur BMD was 2.2 and 1.4%, respectively. The BMC values for CV for tibia and femur were 3.5 and 1.5%, respectively.

#### Statistical analyses.

Data are presented as means  $\pm$  SEM. A two-way ANOVA was first used to determine the significance of differences as a function of surgery and of diet. When there was a significant difference, a one-way ANOVA was used followed by a Fisher paired LSD test to determine

the particular effect that caused that difference (STATVIEW; Abacus Concepts). Differences were considered significant at  $P < 0.05$ .

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#### DISCLOSURES

Y. Sabbagh is an employee of Genzyme Corporation and owns stock in the company.

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