Abstract

Background: Type 2 diabetes (T2D) is the leading cause of nephropathy in the United States. Renal complications of T2D include proteinuria and suboptimal serum 25-hydroxycholecalciferol (25D) concentrations. 25D is the major circulating form of vitamin D and renal reabsorption of the 25D–vitamin D–binding protein (DBP) complex via megalin-mediated endocytosis is believed to determine whether 25D can be activated to 1,25-dihydroxycholecalciferol (1,25D) or returned to circulation. We previously demonstrated that excessive urinary excretion of 25D–DBP and albuminuria occurred in rats with type 1 diabetes (T1D) and T2D. Moreover, feeding rats with T1D high-amylose maize partially resistant to digestion [resistant starch (RS)] prevented excretion of 25D–DBP without significantly affecting hyperglycemia.

Objective: We used Zucker diabetic fatty (ZDF) rats, a model of obesity-related T2D, to determine whether feeding RS could similarly prevent loss of vitamin D and maintain serum 25D concentrations.

Methods: Lean control Zucker rats (n = 8) were fed a standard semi-purified diet (AIN-93G) and ZDF rats were fed either the AIN-93G diet (n = 8) or the AIN-93G diet in which cornstarch was replaced with RS (550 g/kg diet; 35% resistant to digestion) (n = 8) for 6 wk.

Results: RS attenuated hyperglycemia by 41% (P < 0.01) and prevented urinary DBP excretion and albuminuria, which were elevated 3.0- (P < 0.01) and 3.6-fold (P < 0.01), respectively, in control diet–fed ZDF rats. Additionally, urinary excretion of 25D (P = 0.01) and 1,25D (P = 0.03) was higher (89% and 97%, respectively), whereas serum 25D concentrations were 31% lower (P < 0.001) in ZDF rats fed the control diet compared with RS-fed ZDF rats. Histopathologic scoring of the kidney revealed that RS attenuated diabetes-mediated damage by 21% (P = 0.12) despite an ~50% decrease in megalin protein abundance.

Conclusions: Taken together, these data provide evidence that suggests vitamin D balance can be maintained by dietary RS through nephroprotective actions in T2D, which are independent of vitamin D supplementation and renal expression of megalin. J. Nutr. 144: 1667–1673, 2014.
via endocytosis in concert with the intracellular adaptor protein disabled-2 (DAB2) (3). 25D is either hydroxylated by cytochrome p450, family 27, subfamily B, polypeptide 1 (CYP27B1) to generate its active derivative, 1,25D, or returned to the circulation as 25D–DBP. Because the maintenance of circulating 25D and 1,25D concentrations is dependent on kidney function, it is not surprising that compromised vitamin D status is a concern in diabetic patients (6–9), particularly those who experience symptoms of nephropathy (10–12). Moreover, with a proclivity to exhibit suboptimal vitamin D status, diabetics may be at an increased risk of developing complications that are consistent with vitamin D deficiency, such as bone disease, autoimmune disorders, and multiple forms of cancer, as was comprehensively reviewed (13).

We recently reported that in both type 1 diabetes (T1D) and T2D animal models, urinary excretion of the 25D–DBP complex was markedly elevated (14,15). Specifically, we observed a marked increase in urinary 25D excretion. Furthermore, reduced expression of megalin and DAB2 was associated with a decline in serum 25D concentrations and loss of 25D–DBP in the urine (14,16,17). We also reported that these observations were prevented in dietary intervention studies with rats with T1D, in which feeding rats high-amylose maize, which is partially resistant to digestion, as a carbohydrate source prevented the loss of renal megalin and DAB2 expression, as well as the urinary excretion of 25D and DBP (15).

Resistant starch (RS) is a family of fermentable dietary fibers, some of which were shown to improve the classic symptoms of obesity-related diabetes (18–20). However, no studies have reported a benefit of RS with respect to renal vitamin D metabolism and systemic vitamin D balance in obesity-related diabetes. In the present study, our objective was to determine whether feeding an AIN-93G diet in which the cornstarch was replaced with RS could protect against the perturbation of vitamin D metabolism in Zucker diabetic fatty (ZDF) rats, a well-characterized animal model of obesity-induced T2D. Specifically, we determined whether feeding ZDF rats high-amylose maize, which was chosen because it is partially resistant (~35%) to digestion (21), would maintain serum vitamin D status through the prevention of excessive urinary excretion of 25D, 1,25D, and DBP.

### Materials and Methods

**Rats and diets.** All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. All diet ingredients, with the exception of high-amylose maize (Amylogel, Cargill), were purchased from Harlan Teklad. Male ZDF and lean Zucker control rats were purchased at 8 wk of age (Charles River Laboratories) and housed individually in plastic cages in a room with a 12-h light-dark cycle and consumed food ad libitum. Rats were randomly assigned to a diet (AIN-93G) (Supplemental Table 1) containing either cornstarch (550 g/kg diet, control diet) or high-amylose maize (550 g/kg diet) that was ~35% resistant to digestion. Thus, rats were divided into 3 groups (n = 8 per group): 1) Zucker lean control rats fed a standard semi-purified AIN-93G control diet (LCs); 2) ZDF rats fed a standard semi-purified AIN-93G control diet containing 55% cornstarch (DCs); and 3) ZDF rats fed an RS diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize (DRSs). All dietary starches were prepared as described previously (15). The RS content (percentage) was verified in all diets over the 1 wk usage timeframe after it was prepared by in vitro digestion analysis (Association of Official Analytical Chemists 991.43 method) to confirm the stability of resistant starch (22). All rats were provided free access to experimental diets and water for 6 wk. Prior to being killed at the end of wk 6, rats were placed in metabolic cages for a 12 h period in which food was deprived. Subsequent urine samples were then collected and stored at −20°C until analysis. At the time they were killed, rats were anesthetized with a ketamine: xylazine cocktail (90:10 mg/kg body weight) via i.p. injection. Whole blood was then collected via cardiac puncture and blood glucose concentrations were measured with a glucometer (Bayer Healthcare), after which tissues were removed and stored at −80°C prior to analysis.

**Assessment of urinary creatinine, total protein, albumin, and DBP.** Urinary creatinine was measured by using a commercial colorimetric kit (Cayman Chemical). Urinary total protein concentrations over 12 h were assessed by using a bicinchoninic acid assay (Thermo Scientific Pierce). Urinary albumin and DBP were measured by using commercial ELISA kits as described (15). Urinary excretion of DBP and albumin were expressed as mg excreted/12 h.

**Assessment of urinary and serum 25D and 1,25D.** Serum and urinary concentrations of 25D were measured by using a commercial enzyme immunoassay kit (Immunodiagnostic Systems) as previously described (15). Assessment of 1,25D in both serum and urine were measured with a commercially available ELISA kit (My BioSource). The urinary total excretion of 25D and 1,25D was calculated and normalized to urinary creatinine as reported previously (15).

![FIGURE 1](https://academic.oup.com/jn/article-abstract/144/11/1667/4590061)
RNA Isolation and Real-Time PCR. Kidney total RNA was isolated as previously described (15). Total RNA was then quantified by UV detection and single-strand cDNA synthesis was carried out with a Verso cDNA Synthesis kit (Thermo Scientific). Real-time PCR reactions were performed in duplicate by using iScript SYBR Green Detection reagents (Bio-Rad) at 200 ng/well for the detection of megalin, Dab2, Cyp27b1, and cytochrome p450, family 24, subfamily A, polypeptide 1 (Cyp24a1) with an Applied Biosystems Plus real-time PCR system (Life Technologies). The primers sets specific for megalin (forward primer: AAGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: GAGATGGTCAGTGTATTCCAGACAGGCGCTGTGAAC; reverse primer: TCCAACATCAACACTTCTTTACGCCATTCGGTA), Dab2 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: TTCAGACGCGGTGTTGAAAG; reverse primer: TGTCAGTCTACCTGTGTTGTC) were normalized against 18S (forward primer: ACATCCAAGGAAGGCAGCAG; reverse primer: TTGGCAGTCGTCATCTCCATCACA), Cyp27b1 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGCAGTCGTCATCTCCATCACA), and Cyp24a1 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGCAGTCGTCATCTCCATCACA). Expression of each target gene was determined as mean fold change in gene expression relative to the LCs.

Histology and Immunohistochemistry. Kidneys were fixed in formalin, embedded in paraffin, sectioned at 5 μM, and stained with hematoxylin and eosin Y for histologic assessment of kidney health. Histopathologic scoring of kidneys was performed as described (23). The lesions in the renal cortex that were evaluated included tubular degeneration, interstitial fibrosis, dilated glomerular space, hydropnephrosis, and dilated tubules in renal cortex. Immunohistochemistry for the detection of DAB2 and megalin was performed as described previously (14). Staining intensity was expressed as fold change relative to lean control rats.

Statistical Analysis. All data were analyzed by using SAS (SAS Institute). Repeated measures ANOVA was performed on the body weight changes and relative daily food intake. All other endpoint analyses were evaluated statistically for differences between groups by using 1-factor ANOVA followed by Tukey’s post hoc test. A nonparametric analysis was used when normality failed via Kruskal-Wallis 1-factor ANOVA by ranks followed by Tukey’s or Dunn’s multiple comparison test for unequal groups. Interactions between treatments and differences between means were considered significant at $P \leq 0.05$.

Results

RS normalized blood glucose, urinary volume, creatinine excretion, and proteinuria in ZDF rats. Blood glucose concentrations after food deprivation were 3-fold higher in DCs compared with LCs. Concentrations in DRSs were 1.4-fold higher compared with LCs and were 41% lower compared with DCs (Table 1). Likewise, the total volume of urine collected during the 12-h period of food deprivation was 1.9-fold higher in DCs compared with both LCs and DRSs (Table 1). Urinary creatinine concentrations were 91% and 86% higher in LCs and DRSs, respectively, with an Applied Biosystems Plus real-time PCR system (Life Technologies). The primers sets specific for megalin (forward primer: AAGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: GAGATGGTCAGTGTATTCCAGACAGGCGCTGTGAAC; reverse primer: TCCAACATCAACACTTCTTTACGCCATTCGGTA), Dab2 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: TTCAGACGCGGTGTTGAAAG; reverse primer: TGTCAGTCTACCTGTGTTGTC) were normalized against 18S (forward primer: ACATCCAAGGAAGGCAGCAG; reverse primer: TTGGCAGTCGTCATCTCCATCACA), Cyp27b1 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: GAGATGGTCAGTGTATTCCAGACAGGCGCTGTGAAC; reverse primer: TCCAACATCAACACTTCTTTACGCCATTCGGTA), and Cyp24a1 (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: TTGGATGAGCTGTGCGATGA; reverse primer: GAGATGGTCAGTGTATTCCAGACAGGCGCTGTGAAC; reverse primer: TCCAACATCAACACTTCTTTACGCCATTCGGTA) were normalized against 18S (forward primer: ACATCCAAGGAAGGCAGCAG; reverse primer: TTGGCAGTCGTCATCTCCATCACA). Expression of each target gene was determined as mean fold change in gene expression relative to the LCs.

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Results

RS normalized growth pattern despite reducing food intake in ZDF rats. By the beginning of the 3 wk treatment period, DRSs gained considerably more weight than LCs and DCs. By the end of the 6 wk study, DRSs gained 24% and 51% more weight than LCs and DCs, respectively (Fig. 1A). Moreover, LCs and DRSs continued to gain weight throughout the treatment period, whereas the growth of the DCs leveled off at week 3 and rats did not gain any additional weight. However, these data do not reflect food intake by ZDF rats. Despite the fact that the DRSs exhibited the greatest growth rate of all of the groups, as of day 35, they were consuming 35% less diet, and by the end of the treatment period, they were consuming 40% less, compared with DCs (Fig. 1B).

**FIGURE 2** Dietary resistant starch improved the renal histopathologic scoring in ZDF rats. Renal histopathologic scores of LCs, DCs, and DRSs (A). Kidney weight of LCs, DCs, and DRSs (B). Data are means ± SEMs; n = 4. Bars with different letters differ, $P < 0.05$. DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS, Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker lean control rat fed a standard semi-purified AIN-93G control diet.
urinary total protein did not differ between LCs and DRSs, but was 4.2- and 1.6-fold higher in DCs compared with LCs and DRSs, respectively (Table 1). Urinary albumin was 3.6-fold greater in DCs than in DRSs, and there were no differences in urinary albumin between LCs and DRSs (Table 1).

**Protection of the kidney by dietary RS rescued serum 25D concentrations and prevented urinary loss of 25D, 1,25D, and DBP.** The renal histopathologic score of DCs was highest among the treatments (48% higher than LCs) (Fig. 2A) and renal pathologic scores of DRSs did not differ from LCs or DCs. Similarly, kidney weights in DCs were greater than in LCs and DRSs (16% and 26%, respectively), and we did not detect a difference in kidney weight between LCs and DRSs (Fig 2B). The vitamin D status of DCs, as indicated by serum 25D concentrations, was 45% lower than in LCs and 31% lower than in DRSs (Fig. 3A). Serum 1,25D did not differ between groups regardless of serum 25D status (Fig. 3B). Consistent with the decline in vitamin D status in DCs, urinary excretion of DBP was markedly elevated in DCs compared with LCs, in which DBP was virtually undetectable, and was 75% greater in DCs than in DRSs (Fig. 3C). Moreover, urinary 25D concentrations were higher in DCs compared with LCs and DRSs (92% and 89% higher, respectively) as were urinary 1,25D concentrations (94% and 97% higher, respectively, Fig. 3D, E).

**mRNA expression of renal vitamin D transport and metabolism proteins did not change in ZDF rats regardless of diet.** We did not detect any statistical differences in megalin, Dab2 (Supplemental Fig. 1), Cyp27b1, or Cyp24a1 mRNA expression between any of the treatment groups (Supplemental Fig. 2).

Unlike mRNA expression, immunohistochemical staining of the kidney sections revealed that megalin protein abundance in renal proximal tubules was ~50% lower in DRSs compared with LCs or DCs (Fig. 4A). In contrast, expression of DAB2 protein in the renal proximal did not differ between DCs and DRSs, which was ~40% and 50% greater, respectively, than in LCs (Fig. 4B).

**Discussion**

We previously reported that vitamin D metabolism is disrupted in T1D and T2D and that dietary RS attenuated the urinary excretion of 25D in a model of T1D (14,15). Here, we demonstrate that the impact of dietary RS on maintaining vitamin D balance and the attenuation of symptoms were markedly more robust in T2D than we found in our T1D studies. Specifically, our data show that RS virtually prevented proteinuria and the urinary excretion of 25D and 1,25D, and maintained serum 25D concentrations. Furthermore, inclusion of dietary RS promoted the growth of DRSs despite the fact that RS-fed rats consumed the lowest volume of diet based on body weight, indicating better overall health of DRSs.

It is interesting that body weight gain in DRSs was significantly higher than in both LCs and DCs with no changes in food intake compared with LCs. A typical symptom of uncontrolled diabetes with renal complications is glucosuria, which can lead to substantial fluid and calorie loss (24). We suspect a reason for our observation is that, instead of excreting glucose in the urine, DRSs likely were able to retain and store or metabolize it because of their better overall renal health. Consistent with this
RS protected kidney health in the ZDF rats, we suspect that the decreased expression of megalin in the proximal tubules of DRSSs may have been a protective action. 25D–DBP is 1 of many known megalin ligands, which also include proinflammatory cytokines, chemokines, and nephrotoxins (25,26). Our data also suggest that absorption of the 25D–DBP complex by the kidney can be achieved by different means. Thus, we have not ruled out the possibility that there is more than 1 mechanism by which the 25D–DBP complex can be internalized by renal proximal tubules. Furthermore, we are currently investigating the possibility that the activity and/or expression of megalin can be regulated at the translational or post-translational levels. Our earlier T2D work also involved dietary restriction of vitamin D to ZDF rats, which may help explain why we did not detect differences in the expression of Cyp27b1 and Cyp24a1 in the present study during which vitamin D intake was not restricted. Because both genes are potently regulated by 1,25D (27–29), a likely reason for the fact that there was no change in their expression is that serum 1,25D concentrations were not different between any of the treatment groups, which was also observed in a recent clinical trial with subjects with T1D (30).

With the worldwide obesity-induced diabetes epidemic and the many questions that remain with respect to vitamin D requirements and vitamin D metabolism in diabetes, the present study is timely, because it is a highly translatable advancement in vitamin D research. Consistent with our rat studies, Thraillkill et al. (12) observed elevated urinary excretion of 25D, 1,25D, and DBP in a clinical study with subjects with T1D whom also exhibited compromised vitamin D status. Collectively, these are new insights into why suboptimal vitamin D status is common in diabetes and could prove to be valuable with respect to further interpretation of observational studies that reported compromised vitamin D status in both types of diabetes (31–34). However, there is still a lack of controlled clinical trials that show that vitamin D intervention can reduce the incidence and severity of diabetes. Yet, in part because of the complexity of coordinating long-term trials with supplemental vitamin D, especially in diabetic patients, the knowledge gap with respect to the role of vitamin D in T2D outcomes may be difficult to overcome. Moreover, vitamin D trials in diabetic patients, which have mostly been used in the context of glucose metabolism, have not yielded promising results (35–38) and resistance to vitamin D supplementation was reported in diabetic patients with suboptimal vitamin D status (39). Elevated vitamin D excretion in both types of diabetes, as we observed, could therefore be an important limitation for such studies and thus a vitamin D supplement and/or UV exposure may not be effective for the maintenance of circulating vitamin D. Hence, alternative dietary strategies that can reduce the excretion of vitamin D may be more viable with respect to improving vitamin D status in diabetes.

In this study, we demonstrated that, independent of vitamin D supplementation, vitamin D balance can be protected from the effects of obesity-induced diabetes through the inclusion of RS in the diet. Because the ZDF rat is an extreme model of T2D compared with what is typical of a human with T2D, it is reasonable to hypothesize that the beneficial effects of RS on vitamin D balance could be produced in a clinical setting by a diet containing less RS than we used for this study. In support of this concept, we recently found that reducing the RS content in the diet by half of what was used in the present study normalized growth of rats with T1D (G. Koh and M. Rowling, unpublished observations). Additionally, our future work will focus on concept, RS attenuated the rise in blood glucose concentrations after food deprivation in ZDF rats, which can explain at least in part the absence of osmotic diuresis due to hyperglycemia in RS-fed rats. However, these rats were still clearly diabetic, as indicated by a glucose concentration that was 2 times greater than in LCs. Although DCs fed the control diet consumed more overall diet than DRSSs and thus more vitamin D, serum 25D concentrations in DRSSs were markedly higher compared with DCs. These findings, combined with our histology data that demonstrated that kidneys from DRSSs did not exhibit the same degree of pathology as those from DCs, suggest that RS consumption did not prevent diabetes. Rather, it supports the possibility that dietary RS, because of the nature of its digestibility, attenuated a glycemic insult to the kidney of diabetic rats fed the cornstarch-based control diet. Thus, kidney function was protected, which in turn promoted vitamin D balance in hyperglycemic ZDF rats.

Surprisingly, we did not detect a decrease in mRNA expression of megalin and Dab2 as we consistently observed previously in diabetic rats (14,15). A possible explanation for this is that the macronutrient compositions are different between the AIN-93G diet we used for this study and the high-energy diabeticogenic diet we used previously, which may have had a stronger impact on renal health. Moreover, in our earlier studies, rats were fed a diabetogenic diet for 8 wk prior to being killed compared with a 6 wk period feeding the AIN-93G diet for the present study. We also observed decreased megalin protein expression in the renal proximal tubules of DRSSs. Because our study demonstrated that

![FIGURE 4](https://academic.oup.com/jn/article-abstract/144/11/1667/4590061) Renal megalin expression was reduced and DAB2 expression was enhanced by dietary resistant starch in the kidneys of ZDF rats. Renal expression of megalin in LCs, DCs, and DRSSs (A). Renal expression of DAB2 in LCs, DCs, and DRSSs (B). Data are means ± SEMs (n = 4). Bars with different letters differ, P < 0.05. LC, Zucker lean control rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; DRS, Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker lean control rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty rat.
whether other types of dietary fiber could provide similar benefits with respect to vitamin D balance and the prevention of vitamin D–related secondary complications.

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