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Dietary Resistant Starch Prevents Urinary Excretion of 25-Hydroxycholecalciferol and Vitamin D-Binding Protein in Type 1 Diabetic Rats[1,2]

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Abstract

Diabetes is a rapidly growing epidemic affecting millions of Americans and has been implicated in a number of devastating secondary complications. We previously demonstrated that type 2 diabetic rats exhibit vitamin D deficiency due to aberrant megalin-mediated endocytosis and excessive urinary excretion of 25-hydroxycholecalciferol (25D3) and vitamin D-binding protein (DBP). Here, we examined whether a model of type 1 diabetes [T1D; streptozotocin (STZ)-treated Sprague-Dawley rats] would similarly excrete abnormally high concentrations of 25D3 and DBP due to renal damage and compromised expression of megalin and its endocytic partner, disabled-2 (Dab2). Moreover, we tested whether feeding diabetic rats starch that is resistant to digestion could alleviate these abnormalities. Control (n = 12) rats were fed a standard, semipurified diet (AIN-93G) containing 55% total dietary starch and STZ-treated rats were fed the AIN-93G diet (n = 12) or a diet containing 55% high-amylose maize that is partially resistant to digestion [20% total dietary resistant starch (RS); n = 12] for 2 and 5 wk. The RS diet attenuated weight loss and polyuria in STZ-treated rats. Histology and immunohistochemistry revealed that dietary RS also attenuated the loss of Dab2 expression in renal proximal tubules. Moreover, urinary concentrations of both 25D3 and DBP were elevated ~10-fold in STZ-treated rats (5 wk post STZ injection), which was virtually prevented by the RS. We also observed a ~1.5-fold increase in *megalin* mRNA expression in STZ-treated rats, which was attenuated by feeding rats the RS diet for 2 wk. Taken together, these studies indicate that consumption of low-glycemic carbohydrates can attenuate disruption of vitamin D homeostasis in T1D through the rescue of megalin-mediated endocytosis in the kidney. J. Nutr. 143: 1123–1128, 2013.

Introduction

In the United States, the percentage of individuals with diagnosed diabetes mellitus (DM)[7] has grown by 200% in the past 30 y (1). The disease is characterized by impaired insulin secretion and/or signaling. If uncontrolled, diabetes has a high mortality rate, which is dependent on the severity of secondary complications, such as cardiovascular disease, cancer, and renal failure (2). Such DM-related diseases are typically the end result of prolonged hyperglycemia and the formation of advanced glycation end-products in the vascular network of tissues, such as the eye and kidney (3–5). With respect to the kidney, advanced glycation end-product formation can lead to damage of the renal proximal tubule and microalbuminuria (6–8), the latter of which can also result from the shedding of endocytic proteins within the renal tubule (9).

Renal reabsorption of nutrients and hormones bound to proteins of low molecular weight, such as vitamin D-binding protein (DBP; 50 kD), is dependent on endocytic proteins to keep them in circulation (10–16). Hence, maintenance of adequate nutritional status for the prevention of secondary complications is a major challenge during prolonged DM (17). Among the nutrients that are affected during the progression of DM is vitamin D. Both insulin-dependent [type 1 diabetes (T1D)] and noninsulin dependent [type 2 diabetes (T2D)] diabetes have been associated with increased urinary loss of the major circulating form of vitamin D, 25-hydroxycholecalciferol (25D3), and DBP (18–20). Because 25D3 circulates bound to DBP, the 25D3-DBP complex must be filtered by the

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[6] These authors contributed equally to this work.
[7] Abbreviations used: C, AIN-93G control diet; D, diabetic; Dab2, disabled-2; DBP, vitamin D-binding protein; DM, diabetes mellitus; D+RS, streptozotocin-injected rats fed a resistant starch diet; RS, resistant starch; STZ, streptozotocin; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus; 25D3, 25-hydroxycholecalciferol; 1,25D3, 1,25-dihydroxycholecalciferol.
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nephron and internalized by the kidney for both reabsorption and activation to 1,25-dihydroxycholecalciferol (1,25D3) (21). Hence, in the diabetic kidney, increased filtration of 25D3-DBP due to damage of the glomerular basement membrane of the nephron combined with compromised reabsorption by the renal proximal tubule may lead to excessive urinary loss of 25D3. The specific mechanism by which the 25D3-DBP complex is internalized by the renal proximal tubule is dependent on a large transmembrane receptor, megalin, and its adaptor protein, disabled-2 (Dab2), for endocytosis within the renal proximal tubule (14). Once this complex is internalized, 25D3 is released from DBP and can be hydroxylated by 1α-hydroxylase (cytochrome P450, family 27, subfamily B, polypeptide 1) for activation to 1,25D3 or release back into circulation. This process has clear implications with respect to diabetic nephropathy. We as well as others have observed reduced megalin expression on the apical surface of the renal proximal tubule and/or shedding of megalin in T1D and T2D (9,18).

Perturbation of vitamin D homeostasis has been postulated to contribute to the comorbidities associated with microvascular complications in DM (20,22). However, dietary intervention strategies to optimize vitamin D metabolism in DM have not been clearly defined. Dietary starches resistant to digestion (RS) and other fibers have been shown to attenuate hyperglycemia and complications from DM (23). The objective of the current study was to determine whether inclusion of starch that is partially resistant to digestion in the diet could prevent nephropathy-related aberrant vitamin D homeostasis in a rat model of T1D. Specifically, we determined whether feeding high-amylose maize as a RS source to streptozotocin (STZ)-treated rats would prevent the loss of megalin and Dab2 function as well as diminish urinary excretion of 25D3 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 Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-Dawley (Harlan) rats (100–130 g) were individually housed in plastic cages in a 12-h-light/-dark cycle and consumed feed ad libitum. After a 10-d acclimation period, rats were randomly assigned to 1 of 3 groups: control rats fed a standard, semipurified control diet [AIN-93G (24)] containing commercial corn starch (C; n = 12), STZ-injected [60 mg/kg body weight; diabetic (D)] rats (to induce a T1D condition) fed the C diet (n = 12), and STZ-injected rats fed an RS diet (D+RS; n = 12) in which the cornstarch was replaced by an equivalent amount of high-amylose maize (Cargill) that is 37% resistant to digestion based on in vitro digestion analysis. For both diets, starch was provided at 55% (wt:wt) of the diet; thus, the total RS in the treatment diet was ~20%. For diet preparation, both starches were cooked in water for 20 min and allowed to completely cool prior to their addition to the remaining ingredients (Harlan-Teklad). Raw cornstarch and diets were analyzed throughout the preparation and experimental period, verifying that the level of resistance remained stable (25). In addition to cornstarch, the diets contained (g/kg): vitamin-free casein, 200; glucose monohydrate, 150; corn oil, 50; AIN93 mineral mix, 40; AIN93 vitamin mix, 10; L-methionine, 3; and choline bitartrate, 2 All rats were acclimated to the C diet for 1 wk, followed by 2 wk of being fed either the control or RS diet. On d 21, diabetic rats received a single intraperitoneal injection of STZ, whereas control rats were vehicle (10 mmol/L citrate buffer, pH 4.5) injected. One-half of the rats from each of the 3 treatment groups were killed at 2 and 5 wk post-STZ injection. At 2 or 5 wk, 6 rats from each of the 3 treatment groups were feed-deprived overnight (12 h) and placed in metabolic cages for collection of urine, then anesthetized (single intraperitoneal injection) with a cocktail of ketamine:xylazine (90:10 mg/kg body weight). Whole blood was obtained by cardiac puncture and whole kidneys were either snap-frozen in liquid nitrogen for mRNA analysis or preserved in 100 mL/L formalin for routine histological assessment and immunohistochemistry procedures.

Assessment of renal function. Serum and urinary creatinine concentrations were measured via a commercial colorimetric kit (18). Urinary albumin was measured via a commercial ELISA kit (18).

Assessment of urinary 25D3 excretion. Total urinary 25D3 concentrations were measured using a commercial enzyme immunoassay kit appropriate for analysis of 25D3 in urine that has cross reactivity of <0.3% for other vitamin D metabolites not hydroxylated at the 25 position (Immunodiagnostic Systems). Urinary DBP concentration was measured using a commercial ELISA kit appropriate for analysis of DBP in urine (Life Diagnostics). The urinary excretions of 25D3 and DBP were calculated and expressed relative to urinary creatinine concentrations as previously described (18).

Real-time PCR. RNA was extracted using a Quickgene 810 with a Quickgene RNA Tissue kit (Autogen). Single-strand cDNA was prepared using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Three stocks of cDNA were generated per kidney sample and independently analyzed for *megalin* and *Dab2* by real-time PCR as previously described (18). Gene expression for the 2- and 5-wk time points was determined as fold-induction relative to their respective controls at each time point.

Histology and immunohistochemistry. Formalin-fixed kidneys were embedded in paraffin, sectioned at 5 μM, and stained with Hematoxylin and Eosin Y for routine histological assessment. Immunohistochemistry for the detection of megalin and Dab2 was performed as previously described (18).

Statistical analysis. Data were analyzed by 1-way ANOVA and Fisher's Least Significant Difference post-test using SigmaStat software (version 3.5, Systat) for either the 2- or 5-wk time point. When the normality and/or equal variance test failed, data were analyzed by Kruskal-Wallis 1-way ANOVA on ranks followed by either the Student Neuman-Keuls multiple comparison test or Dunn's multiple comparison test when the groups were unequal. Interactions between treatments and differences between means were considered significant at $P \leq 0.05$.

Results

Dietary RS attenuated weight loss and renal diabetes symptoms in STZ-diabetic rats. Rats fed the D diet gained 60% less weight than control rats during the 5-wk study period following the STZ treatment (**Fig. 1**). Though the RS diet did not completely prevent weight loss in diabetic rats, weight gain in the D+RS rats was ~50% greater at the end of the study period compared with diabetic rats fed the C diet. Furthermore, the RS diet effectively attenuated polyuria at 2 and 5 wk (**Table 1**). However, blood glucose concentrations were lower in D+RS rats than in D rats at 2 wk but did not differ at 5 wk (Table 1).

Dietary RS attenuated diabetic nephropathy and prevented urinary loss of 25D3 and DBP. Histological analysis revealed a mild deterioration in renal proximal tubule integrity in D rats compared with C and D+RS rats (**Fig. 2***A*). Moreover, immunohistochemical staining showed that Dab2 (Fig. 2*B*) and megalin (Fig. 2*C*) expression in the renal proximal tubules of D rats were a much lower intensity compared with C rats and, though similar in intensity, less abundant than in D+RS rats. Consistent with this observation, loss of 25D3 and DBP in the urine of D rats was attenuated by feeding rats the RS diet at both 2- and 5 wk (**Fig. 3**). Urinary excretion of 25D3 by D rats was

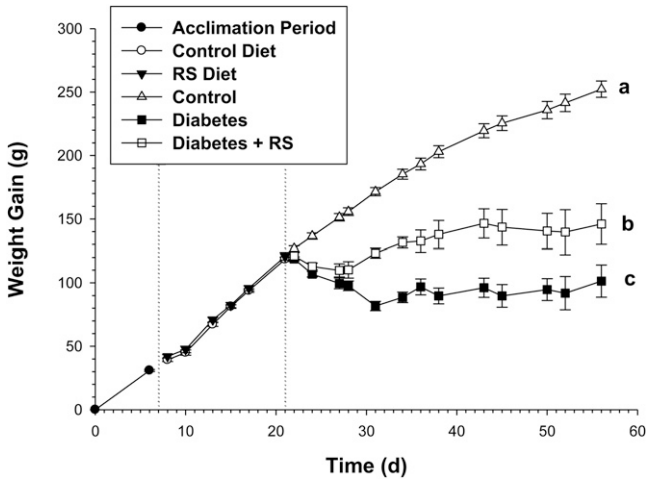

FIGURE 1 Dietary RS attenuated weight loss in STZ-treated T1D rats. Cumulative weight gain for male Sprague-Dawley rats fed a standard semipurified diet (AIN-93G) containing commercial cornstarch (n = 12), STZ-treated rats fed the AIN-93G diet (n = 12), or STZ-treated rats fed a RS diet (n = 12) in which the cornstarch was replaced by an equivalent amount of high-amylose maize (RS). Values are means ± SEs. Final mean weight gain values without a common letter differ, $P < 0.05$. RS, resistant starch; STZ, streptozotocin; T1D, type 1 diabetes mellitus.

roughly 9-fold greater than that by C rats (Fig. 3*A*), whereas urinary concentrations of 25D in D+RS rats did not differ from those of C rats. Likewise, the urinary excretion of DBP in D rats was ~3- and 9-fold greater after 2 and 5 wk, respectively, compared with C rats (Fig. 3*B*). In D+RS rats, loss of DBP was attenuated by ~50% at 2 wk. At 5 wk, though urinary DBP excretion by D+RS rats did not differ from D rats, it also did not differ from the control rats, indicating that the RS diet attenuated DBP loss in diabetic rats.

RS normalized megalin and Dab2 mRNA overexpression in diabetic rats. Expression of renal megalin was 1.5-fold higher in D rats at 2 wk following STZ treatment compared with C rats (**Fig. 4***A*). *Megalin* expression in D+RS rats was not greater than C rats at 2 wk; however, at 5 wk, *megalin* mRNA levels did not differ between the D+RS and D rats. *Dab2* mRNA expression did not statistically differ at 5 wk between the D and D+RS rats; at 2 wk, expression was 3.5-fold greater in D than in C and D+RS rats, which did not differ from one another (Fig. 4*B*).

Discussion

In the present study, we demonstrated that the inclusion of high-amylose maize that is partially resistant to digestion in the diet of T1D rats normalized the expression of megalin and Dab2, as well as prevented urinary excretion of 25D3 and DBP. Moreover, the RS diet prevented diabetic nephropathy as indicated by the normalization of serum creatinine (data not shown), urinary volume, and urinary albumin concentrations (data not shown) in D+RS rats as well as attenuated growth stunting of D rats. Because the rodent diet we utilized for these experiments contained

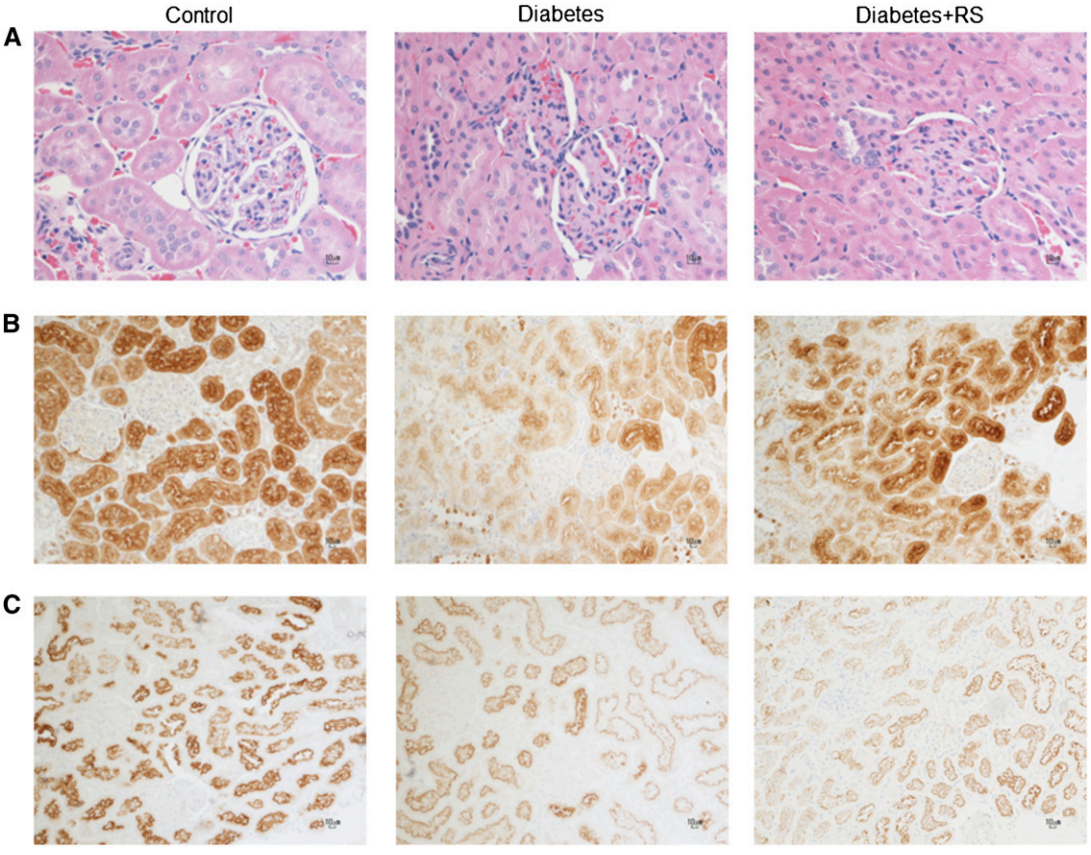

FIGURE 2 Histology and immunohistochemical analysis of megalin and Dab2 abundance in kidney of C and STZ-treated type 1 diabetic rats. Renal tissue stained with Hematoxylin and Eosin Y for routine histological assessment (*A*) or subjected to immunohistochemical staining for megalin and Dab2 as described in ''Materials and Methods.'' Dab2 (*B*) and megalin (C) appear dark brown against the blue Hematoxylin counterstain. Dab2, disabled-2; RS, resistant starch; STZ, streptozotocin.

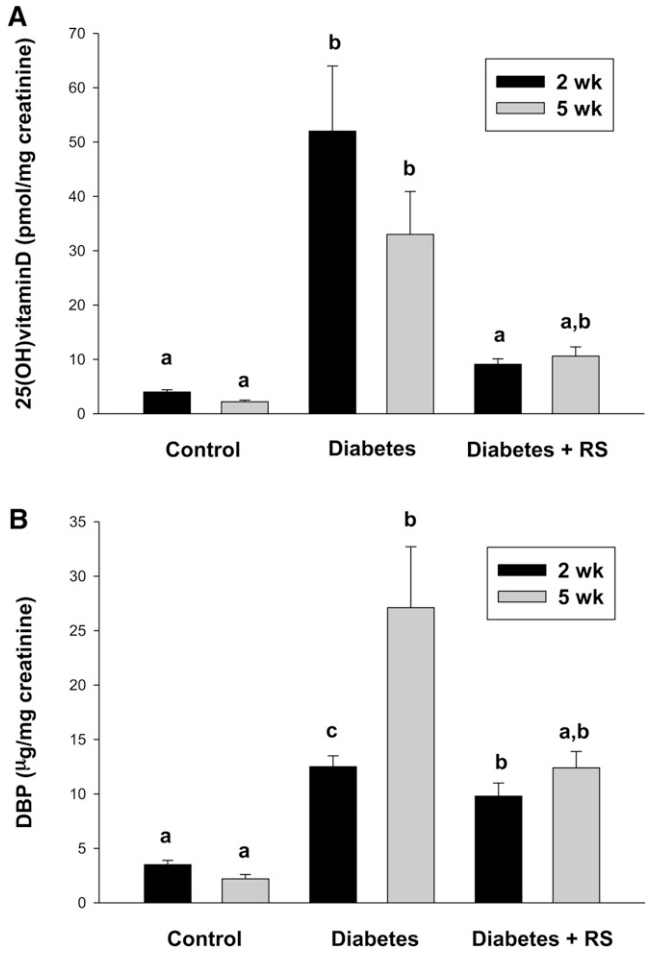

FIGURE 3 Dietary RS prevented urinary excretion of 25D3 and vitamin DBP in STZ-treated type 1 diabetic rats. Urinary 25D3 and DBP concentrations were determined as described in ''Materials and Methods.'' (*A*) Urinary 25D3 concentrations from control and diabetic rats. (*B*) Urinary DBP concentrations from control and diabetic rats. Values are means ± SEs, $n = 4$–6. Labeled means at a time without a common letter differ, $P < 0.05$. DBP, D-binding protein; RS, resistant starch; STZ, streptozotocin; 25D3, 25-hydroxycholecalciferol.

20% RS, we hypothesized that a subsequent reduction in blood glucose concentrations in D+RS rats would protect the renal proximal tubules from damage and subsequently prevent urinary excretion of 25D. Our hypothesis appeared to be only partially correct, as the glucose concentrations of the D rats were minimally affected by the RS diet after 2 wk of the diet. After 5 wk, the blood glucose concentrations of the D+RS rats did not differ from those of the D rats. Hence, though we have not ruled out the possibility, the protection of the kidney by RS was not the result of reducing blood glucose concentrations. An intriguing observation from our studies is that that hyperglycemia in D rats was associated with elevated mRNA levels of *megalin* and *Dab2*, indicating that as blood glucose rose, renal damage may have affected megalin expression at the surface of the proximal tubule and a compensatory increase in *megalin* mRNA was triggered.

Suboptimal vitamin D status (25D3 concentrations <60 nmol/L) has been associated with several chronic diseases that are known secondary complications of DM, such as osteoporosis, cancer, and cardiovascular disease (2). Low vitamin D status has been observed in both T1D and T2D (26–31); however, whether disruption of vitamin D metabolism during progression of DM exacerbates secondary complications of DM is unclear. A major factor associated with low vitamin D status reported in T1D and T2D appears to be the development of nephropathy, characterized by the shedding and excretion of the endocytic protein megalin, which is required for vitamin D absorption by the renal proximal tubule (21). Additionally, we recently reported that T2D rats excreted markedly elevated concentrations of 25D3 and DBP in the urine and exhibited reduced megalin and Dab2 expression in the renal proximal tubule (18). Because megalin and Dab2 are essential for reabsorption of 25D3 for reentry into the circulation and activation to 1,25D3, these findings may have important implications with respect to designing intervention strategies to prevent secondary complications related to aberrant vitamin D metabolism during the onset and progression of diabetes. Moreover, our observations suggest that protection of the kidney, independent of supplementation, should be a primary objective when optimizing dietary recommendations for diabetics, as damage to the nephron can not only affect vitamin D homeostasis but also the metabolism of additional nutrients (12,13,32,33).

Outside of a slight reduction in the blood glucose concentration, we have not fully characterized the mechanism by which the RS diet prevented the urinary loss of 25D3 and DBP. We have

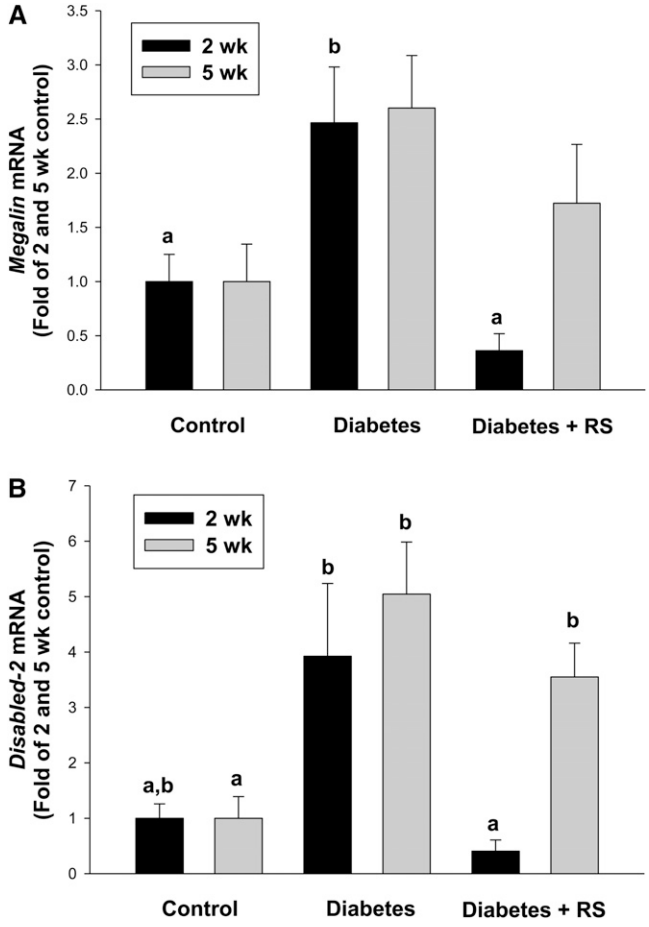

FIGURE 4 Dietary RS attenuates changes in *megalin* and *Dab2* mRNA expression in STZ-treated type 1 diabetic rats. Renal tissue was collected from the same rats as described for Figure 1. (A) *Megalin* mRNA abundance in control and diabetic rats. (B) *Dab2* mRNA abundance in control and diabetic rats. Values are means ± SEs, $n = 4$–6. Labeled means at a time without a common letter differ, $P < 0.05$. RS, resistant starch; STZ, streptozotocin.

TABLE 1 Blood glucose concentrations and 12-h urinary volume in C, D, and D+RS-fed rats[1]

	C	D	D+RS
Blood glucose, *mg/dL*			
2 wk	209 ± 26[a]	613 ± 23[c]	438 ± 39[b]
5 wk	249 ± 13[a]	591 ± 93[b]	574 ± 59[b]
Urinary output, *mL*			
2 wk	5 ± 1[a]	20 ± 4[b]	8 ± 2[a]
5 wk	4 ± 1[a]	24 ± 4[c]	13 ± 3[b]

[1] Values are means ± SEMs, $n = 5$–6. Mean values within a row without a common letter differ, $P < 0.05$. C, AIN-93G control diet; D, diabetic; D+RS, streptozotocin-injected rats fed a resistant starch diet; RS, resistant starch.

not eliminated the possibility that the RS reduced urinary excretion of 25D by lowering the glomerular filtration rate. Another possibility is the production and absorption of microbial fermentation products, such as SCFAs, from the RS diet could have prevented damage to the kidney. It has been shown that microbial fermentation products of fiber, such as butyrate, can be absorbed by enterocytes and mediate a pronounced antiinflammatory response (34) as well as prevent aortic endothelial dysfunction (35). Additionally, treatment of diet-induced obese mice with tributryn, a molecule containing 3 butyl esters, reduced the production of the proinflammatory cytokines, TNFα and IL-1β, by macrophages (36). With respect to the kidney, butyrate has been reported to repress a number of genes related to cell cycle control and apoptosis in kidney epithelial cells (37).

Though we and others have found that vitamin D metabolism can be dramatically altered under diabetic conditions (18,38,39), little is known about whether vitamin D supplementation can help prevent secondary complications from DM. The negative impact of DM on vitamin D homeostasis may have important implications with respect to evaluating the outcomes of diabetic patients. It is well documented that diabetics are at a disproportionately high risk for the development of various forms of cancer, osteoporosis, and cardiovascular disease, diseases that have been shown to be prevalent in individuals with suboptimal vitamin D status (40–44). Additionally, the degree to which dietary interventions that may protect the kidney during diabetes progression can attenuate the perturbation of vitamin D metabolism is not known. We think that by focusing our future work on characterizing vitamin D metabolism at the onset through the progression of diabetic nephropathy and expanding on our work with RS or other dietary fibers to improve vitamin D homeostasis, we will be better able to assess strategies to improve vitamin D status in diabetes for the reduction of preventable diabetic complications.

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