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Vitamin D homeostasis is compromised due to increased urinary excretion of the 25-hydroxycholecalciferol-vitamin D-binding protein complex in the Zucker diabetic fatty rat

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**Vitamin D homeostasis is compromised due to increased urinary excretion
of the 25-hydroxycholecalciferol-vitamin D-binding protein complex in the
Zucker diabetic fatty rat**

by

Rachel Lynn Anderson

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Nutritional Sciences (Molecular and Cellular Nutrition)

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2011

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LIST OF ABBREVIATIONS

- 1,25D3 – 1,25-dihydroxycholecalciferol
- 25D3 – 25-hydroxycholecalciferol
- BMD – bone mineral density
- CAMP - cathelicidin antimicrobial peptide
- CKD – chronic kidney disease
- CVD – cardiovascular disease
- Dab2 – disabled 2 gene product
- DBP – vitamin D binding protein
- FFA – free fatty acid
- FGF23 – fibroblast growth factor
- IL-6 – interleukin 6
- IRS-1 – insulin receptor substrate 1
- NIDDM – non-insulin dependent diabetes mellitus
- PTC – proximal tubule cell
- PTH – parathyroid hormone
- RAS – renin-angiotensin system
- Th—T helper
- TLR – toll-like receptor
- VDR – vitamin D receptor
- VDRE – vitamin D response element
- ZDF – Zucker diabetic fatty (rat)

CHAPTER 1: GENERAL INTRODUCTION

Introduction

Suboptimal vitamin D status has been linked to poor outcomes in cardiovascular disease and cancer, complications that occur frequently in individuals with noninsulin-dependent diabetes mellitus (NIDDM). Simultaneously, the North American population has seen increasing incidence of NIDDM, and a growing awareness of widespread vitamin D insufficiency. Therefore, understanding vitamin D metabolism in NIDDM becomes important in seeking to improve outcomes of this disease as it becomes more prevalent. Poorly controlled NIDDM can lead to impaired renal function, and NIDDM cases account for a large portion of advanced kidney disease in this country. Additionally, epidemiological studies have linked NIDDM and chronic kidney disease with low or inadequate vitamin D status, although a concrete explanation for this association remains elusive. In this thesis, we use a rat model of NIDDM to test the hypothesis that impaired renal reabsorption of circulating vitamin D contributes to the disruption of vitamin D homeostasis in NIDDM.

Thesis Organization

This thesis will examine recent literature pertaining to vitamin D metabolism, its mechanism of action, classical and non-classical roles of the vitamin, and diseases associated with poor vitamin D status, particularly cancer and cardiovascular disease. The review will also discuss the NIDDM disease model used in our study, outlining the associated pathologies, the likely etiology, and the specific rodent model used in our study. Secondly,

the thesis will contain the author's published article (10) reporting the results of work undertaken to test our hypothesis that vitamin D metabolism is compromised in NIDDM. The article will contain an abstract, an introduction to the problem along with the hypothesis to be tested, the experimental materials and methods used, the results of our experiments, and a general discussion of these results. Thirdly, the thesis will further discuss the ramifications of our findings in a general discussion section, and indicate areas for further research. References will be cited at the end of each chapter. Finally, the author's acknowledgements will end the thesis.

Literature Review

Introduction to Vitamin D Metabolism

Vitamin D is unique among the vitamins in that it can be produced photochemically in the skin from 7-dehydrocholesterol. Energy from ultraviolet radiation is used to break the B ring of 7-dehydrocholesterol and produce pre-D3. Pre-D3 then spontaneously isomerizes to cholecalciferol (vitamin D3) at a temperature-dependent rate (18, 73, 74). Alternatively, vitamin D can be obtained in the diet, either as vitamin D3 or ergocalciferol (vitamin D2), a plant- or fungal-derived form. Both of these vitamin D forms are metabolized in the same way and have equal physiological activity (75, 187), although vitamin D2 appears to be cleared from circulation more quickly than vitamin D3 (11). Since skin production of vitamin D3 is the primary source for most human populations (76) vitamin D3 becomes the most important form in determining vitamin D status in normal human conditions and will therefore be the focus of the following discussion. From the skin, vitamin D3 is transported to the liver in the blood bound to vitamin D binding protein (DBP) where it undergoes

hydroxylation by one of the many enzymes in the cytochrome p450 superfamily to produce 25-hydroxyvitamin D (25D3), the most stable metabolite of vitamin D and the major form circulating in the body. Both CYP27A1, a mitochondrial p450, and CYP2R1, a microsomal p450, have been identified as capable of this conversion in humans. However, the substrate affinity and distribution pattern of CYP2R1 seem to suggest that this enzyme also has primary 25-hydroxylase activity (40, 160, 181, 202), but this has not been definitively demonstrated (160, 181). Once produced, 25D3, again bound to DBP, is transported in the serum to the proximal tubule cells of the kidney where it is hydroxylated a second time by another p450-related enzyme, CYP27B1 (1- α -hydroxylase), to produce the active hormone form, 1,25-dihydroxyvitamin D (1,25D3). This active form 1,25D3 is responsible for the classical actions associated with the vitamin, namely the maintenance of calcium and phosphate homeostasis. When present in excess, both 25D3 and 1,25D3 can be inactivated by subsequent 24-hydroxylation. This inactivation is carried out by renal or hepatic CYP24A1 (24-hydroxylase), another mitochondrial cytochrome p450. The 24-hydroxylated metabolites are then catabolized further into water-soluble calcitroic acid and excreted in the urine.

Mechanism of Action

Once the active hormone 1,25D3 is released into circulation, it acts similarly to other steroid hormones by binding a nuclear receptor, the vitamin D nuclear receptor (VDR), to induce and regulate the expression of proteins involved in calcium absorption, reabsorption, and resorption in the intestine, kidney, and bone, respectively. The presence of 1,25D3 activates VDR, initiating recruitment of retinoid X receptor and binding to genomic DNA at the vitamin D response element (VDRE). Along with additional co-regulatory proteins, these complexes can act as activators or repressors of transcription.

Classical Roles for Vitamin D

The effects of vitamin D, in conjunction with parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), on calcium homeostasis and bone health have been widely documented, and its mechanism of action in this endocrine capacity is fairly well described (27, 161, 174). In this function, 1,25D₃ directly increases calcium absorption in intestinal epithelial cells by inducing the expression of the calcium channel transporters TRPV5 and TRPV6 on the apical membrane of the enterocyte while simultaneously inducing transcription calcium binding protein calbindin-D_{9k} and basolateral membrane Ca pump PMCA1b (42, 101), which are required for transcellular transport of calcium (100). Under conditions of low calcium intake, transcellular transport of calcium surpasses the passive paracellular transport as the primary mechanism for calcium absorption, therefore increasing the importance of vitamin D in calcium transport in these conditions (88, 98). Under conditions of adequate or abundant calcium availability paracellular transport appears to dominate and the regulation of absorption becomes less dependent on the presence of the vitamin, and more dependent of the levels of calcium itself (176). In hypocalcemic conditions, the calcium sensing receptor of the parathyroid gland detects the lack of calcium and induces PTH secretion. PTH in turn, induces renal CYP27B1 expression and production of 1,25D₃, increasing intestinal absorption and renal reabsorption of calcium and phosphorus. Additionally PTH, possibly in conjunction with 1,25D₃, activates osteoclasts and osteoclastogenesis in bone to increase bone resorption and release calcium and phosphate and restore blood calcium homeostasis. In response to 1,25D₃ and restored serum phosphate levels, osteocytes secrete FGF23, which then suppresses renal CYP27B1 expression and renal phosphate reabsorption (171). In a negative feedback loop, 1,25D₃ suppresses PTH

secretion via VDR binding to a repressor VDRE upstream of the PTH gene sequence (174). Additionally, 1,25OH₂D negatively regulates its own production by directly suppressing the renal expression of CYP27B1 and inducing its degradation by increasing CYP24A1 expression, and by increasing the production of FGF23 by osteoblasts (118, 161). These factors work together to regulate renal production and systemic release of 1,25D₃, and maintain proper blood calcium and phosphorus in normal conditions.

Through its powerful regulatory effects on calcium and phosphorus homeostasis, vitamin D necessarily impacts the growth and development of bone structure (151, 152, 178). Indeed, the low concentrations of vitamin D result in severe bone malformations known as Ricketts and osteomalacia. Less well understood, however, is the role of vitamin D in the development of osteoporosis. The numerous endocrine factors influencing disease development and progression are still being uncovered and characterized, however, clinical studies indicate that vitamin D supplementation is a critical player in the treatment and prevention of the disease through its regulation of PTH release (165). For example, in a large double-blind placebo controlled study, supplementation with vitamin D and calcium increased lumbar spine bone mineral density (BMD), decreased the relative risk of hip fractures, and reversed secondary hyperparathyroidism in elderly women (38). Additionally, vitamin D analogues were found to prevent bone mineral loss and spinal fractures in patients with primary osteoporosis or with glucocorticoid-induced osteoporosis (162), while supplementation with vitamin D itself plus calcium appears to do the same in patients treated with low-dose glucocorticoids (29, 162), although perhaps with slightly less efficacy. The primary mechanism appears to be the suppression of PTH expression and reversal of hyperparathyroidism (38), a condition that causes increased bone turnover and loss of BMD.

However, vitamin D may also decrease the risk of fracture by strengthening muscle tone and responsiveness, leading to reduced risk of falls in elderly populations (20).

Additionally, there is also evidence that 1,25D₃ may act more directly in bone formation by influencing osteoblast and chondrocyte differentiation and function (178). Both cell types express VDR and show responsiveness to 1,25D₃ treatment. However, the actions of 1,25D₃ appear to be sensitive to stage of cellular growth. In early, pre-osteoblastic cell cultures, 1,25D₃ treatment suppressed expression of type I collagen and alkaline phosphatase mRNA, markers of bone mineralization activity, while treatment of later stage cultures had the opposite effect (149). The effect of timing on the differential response to 1,25D₃ could explain contrasting results of in vivo animal studies. Both osteoblast-specific VDR null mice and mature VDR over-expressing transgenic mice showed increased BMD compared to controls (61, 184). Together, these studies indicate that the pleiotropic effects of vitamin D on bone formation and maintenance extend beyond systemic mineral homeostasis and depend on the developmental stage of the cells involved.

Non-classical Roles for Vitamin D

A more widespread role for vitamin D in many non-classical tissues has been postulated based on the observation of VDR and CYP27B1 expression in many extra-renal tissues (1, 17, 19, 58, 188). Thus, it is now widely acknowledged that 1,25D₃ also has significant paracrine or autocrine activity mediated by the local or intracellular activity of CYP27B1 in cells of the immune system (1), epithelium (17, 96), and those involved with insulin signaling, such as the β cells of the pancreas (22). The regulation of these non-classical functions of the vitamin appear to depend primarily on the availability of circulating 25D₃, and require delivery of the DBP-25D₃ complex to the target cells (96).

Vitamin D and Immune function

Since the first evidence of VDR in peripheral mononuclear leukocytes brought about the speculation of vitamin D activity in immune function, many cell types of both the innate and adaptive arms of the immune system have shown responsiveness to 1,25D₃, as well as VDR and CYP27B1 expression (18, 39, 173).

As part of the non-specific and non-memory-conferring cellular responses to pathogens, referred to as the innate immune response, 1,25D₃ directly induces the expression and production of cathelicidin antimicrobial peptide (CAMP) in isolated human keratinocytes, monocytes and neutrophils and myeloid cells (63). CAMP has direct activity in the destruction of pathogenic bacteria, suggesting the importance of active vitamin D in the first-line protection of epithelial cell surfaces from invasion and infection. CAMP levels, along with 1,25D₃ levels are often reduced in patients with cystic fibrosis (207), indicating vitamin D may also mediate healthy lung epithelial function. CAMP production also appears to be induced through activation of the toll like receptors (TLRs) upon recognition of specific microbial components. Moreover, TLR activation by microbial peptides induced VDR and CYP27B1 expression in human macrophages, and required the presence of 25D₃ in the sera to induce CAMP mRNA production. However, low serum 25D₃ levels or specific inhibition of CYP27B1 attenuates CAMP production, suggesting that local autocrine production of 1,25D₃ is crucial to the innate TLR-mediated CAMP response and is dependent on the adequate amounts of 25D₃ substrate (117).

Additionally, vitamin D appears to modulate the adaptive immune response that is responsible for the long-term protection mediated in part by the production of antigen-specific immunoglobulins. In this role, 1,25D₃ directly inhibits the differentiation,

proliferation, activation and survival of human dendritic cells. This inhibition limits the maturation of cells involved in antigen-recognition to only highly activated cells, possibly attenuating the first response to pathogenic recognition and preventing activation of autoreactive cells (157). Furthermore, treatment with 1,25D3 inhibited proliferation of activated B cells and inhibited their differentiation into immunoglobulin-secreting plasma cells. These cells also expressed CYP27B1, and the treatment effects seen with 1,25D3 administration were repeated with the administration of 25D3 at a 25-fold higher dose (39), suggesting intracellular 1,25D3 production could also be important in this function. Additionally, 1,25D3 regulates T cell development by inhibiting the proliferation of activated T cells (163). This regulation is accomplished in part by inhibiting the antigen presenting capability of dendritic cells, as mentioned previously (157), thus reducing recognition and subsequent T cell activation, and also by influencing the development of immature T helper (Th) cells. 1,25D3 treatment directs immature Th cell differentiation toward Th2 development by increasing the release of cytokines IL-4, IL-5, and IL-10 (26), and inhibits the development of Th1/Th17 cells, thus limiting their ability to activate macrophages and produce proliferative (IL-2) and pro-inflammatory (IFN- γ) cytokines (50, 109). Collectively, these actions effectively modulate the inflammatory response of the adaptive immune system, a response that is overactive in autoimmune diseases. Therefore it is not surprising that 1,25D3 administration has been shown to reverse or halt the progression of multiple animal models of autoimmune disease (35, 36, 64, 154). The fact that many of these immunomodulatory effects can be mediated via autocrine 1,25D3 production is consistent with the observed correlation between low serum levels of 25D3 and increased risk of

development of autoimmune diseases such as multiple sclerosis, type 1 diabetes, and systemic lupus erythematosus in humans (15, 92, 133).

Vitamin D and cancer

There is well-documented evidence that vitamin D has profound activity on cell development and cell cycle, specifically in regulating the proliferation, differentiation, and apoptotic signals in cell culture systems. This fact may explain the observed link between low serum 25D3 and increased risk of cancer mortality, especially among those involving growth-sensitive epithelial tissues such as the breast, colon, and prostate. Detection of VDR expression in these tissues further supports this idea (91, 96, 139, 155). Administration of 1,25D3 or its precursor 25D3 has been demonstrated to inhibit proliferation of cultured multipotent mesenchymal cells (12), colonic epithelial cells (91), prostate adenoma cells (155), and multiple breast cancer cell lines (43). It is thought to mediate this antiproliferative action by arresting cell cycle in the gap 0 or gap 1 phase, preventing DNA synthesis and cell division via increasing expression of cell cycle inhibitors p21 and p27 (12, 139). In hyperplastic prostate cells, this arrest was permanent since the cells did not resume growth after the 1,25D3 was removed (155). These growth inhibitory effects appear to be dependent on VDR expression. In fact, Hedlund et al. (71) was able to restore the antiproliferative effects of 1,25D3 in JCA-1 prostatic carcinoma cells, which do not express VDR are not normally responsive to 1,25D3 treatment, by reestablishing VDR expression via stable transfection.

Additionally, 1,25D3 actively promotes cell differentiation, a process that is commonly disrupted in malignant tumors. In vitro, Palmer et al. (150) demonstrated that 1,25D3 induced the expression of cell-adhesion protein E-cadherin in colon carcinoma cells.

Loss of E-cadherin, a tumor suppressor gene, is predictive of a transition of normal epithelial cell phenotype to an invasive carcinoma (66, 150). Normal hematopoietic cells differentiate to active macrophages after exposure to 1,25D3, but cells derived from VDR knockout mice did not (145), indicating the necessity of genomic actions of vitamin D in this process. Furthermore, a CYP27B1 null mouse model developed poorly differentiated epidermal cells compared to controls, indicating that local 1,25D3 production may also be an important factor in regulating cell differentiation (84).

Finally, 1,25D3 influences apoptosis signaling in cancer cell models. For example, MCF-7 breast cancer cells showed increased apoptotic signal induction after 1,25D3 treatment (175), but multipotent mesenchymal cells decrease these factors after 1,25D3 treatment (12). The difference may be related to the degree of differentiation in these cells and could be mediated in part by steroid hormones, with cells responsive to steroid signaling becoming more susceptible to apoptosis in the presence of 1,25D3 (16, 139). For instance, prostate cancer cells showed reduced responsiveness to 1,25D3-mediated growth inhibition when androgen receptors are down regulated (213). However, breast cancer cells lacking estrogen receptors do not show this same effect (56), and therefore the mechanism may be very specific to cancer cell type.

Vitamin D and Cardiovascular Disease

Vitamin D is also recognized as a factor influencing cardiovascular disease (CVD), with serum 25D3 levels being inversely and independently associated with reported CVD cases, hypertension and incidence of myocardial infarction (23, 97, 167, 168). The role of vitamin D in the development of the disease appears to be two-fold. First, 1,25D3 plays a role in the prevention of hypertension by actively regulating the renin-angiotensin system

(RAS) (114). Secondly, it appears to protect the integrity of vascular endothelial cells by reducing the inflammatory signals and macrophage recruitment, and may also reduce the thrombocytic response. Evidence shows that cultured human coronary artery endothelial cells express VDR and respond to treatment with 1,25D3 by decreasing the release of tumor necrosis factor α (TNF- α), a pro-inflammatory cytokine linked to the development of vasculitis and coronary artery lesions (182). In vivo, VDR knockout mice have decreased gene expression of antithrombin and thrombomodulin, proteins with anti-coagulation activity (6). Additionally, platelet aggregation was significantly increased in these animals under normal calcium conditions. Animal models also demonstrate the direct effect of 1,25D3 on blood pressure regulation, and this effect appears to be independent of its endocrine effect on calcium status. For example, VDR knockout mice had higher circulating renin and angiotensin II levels than controls, and developed hypertension prior to the detection of overt hypocalcemia (113). In addition, in a renal-targeted CYP27B1 knockout mouse model, 1,25D3 treatment normalized elevated circulating renin levels and blood pressure, but treatment with a calcium rescue diet restoring normocalcemia failed to show the same impact blood pressure or renin production (214). The direct inhibition of VDR-mediated renin production is further illustrated by the fact that anti-hypertensive agents, such as angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists, also normalize blood pressure in VDR null mice. However, their administration results in an additional increase in circulating renin levels due to the loss of feedback inhibition by angiotensin II, indicating that the effects of vitamin D are not mediated via angiotensin II signaling (102).

Vitamin D and NIDDM

Epidemiological evidence appears to implicate vitamin D in development of non-insulin dependent diabetes mellitus (NIDDM), with numerous large studies inversely linking low serum 25D3 with many indicators of metabolic syndrome, such as insulin resistance and insulin secretion, and as well as the incidence of NIDDM itself (8, 57, 87, 99, 103, 167). Intervention with daily dietary vitamin D supplementation significantly improved serum 25D3 levels and significantly decreased measures of insulin resistance and fasting blood insulin concentration in a large randomized placebo-controlled human trial (137). Significantly, serum 25D3 measurements correlate inversely with insulin secretion indices, suggesting another non-classical target, namely the pancreatic β cells responsible for insulin secretion (41). Isolated murine pancreatic β cells, along with a cultured β cell-derived line, have been shown to express CYP27B1 mRNA transcript similar to renal CYP27B1 transcript (22). This finding is consistent with evidence that rats deprived of sufficient dietary vitamin D have reduced insulin production and secretion and impaired glucose clearance compared to those fed a sufficient diet (31). Furthermore, 1,25D3 administration normalizes glucose clearance rate and insulin secretion in the vitamin D-deficient rats by increasing the biosynthesis of insulin (31). The combined evidence suggests that a major mechanism by which 1,25D3 may prevent the development of full-blown diabetes is by helping to maintain insulin production and secretion. In addition, 1,25D3 may also have effects on modulating insulin sensitivity through modulating insulin receptor expression and signaling (32, 215). Recently, a VDRE was identified in the human insulin receptor gene promoter, suggesting responsiveness to vitamin D in transcriptional regulation (124). Furthermore, cultured immature monocytes administered 1,25D3 have been shown to increase insulin receptor

expression in response to glucose (123), although this response was not elicited in a β cell-derived insulinoma model (107). When administered 1,25D₃, cultured muscle cells are protected against free fatty acid-induced reductions in glucose uptake in a dose-dependent manner (215). These protective effects appear to be mediated by increasing tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and Akt in response to insulin receptor activation.

Vitamin D and the Kidney

The kidney plays the primary role in the overall metabolism and regulation of vitamin D homeostasis. In particular, the cells of the renal proximal tubule are responsible for the systemic production of active 1,25D₃ from its circulating precursor 25D₃, and therefore the systemic endocrine activity of vitamin D on calcium and phosphorus homeostasis depends on the action of these cells. Since the metabolites of vitamin D circulate in the blood bound to DBP, the conversion of 25D₃ in the tubule is preceded by glomerular filtering of the 25D-DBP complex followed by its re-uptake at the apical membrane of these cells. Recently, it has been shown that megalin (a member of the LDL receptor family) is the receptor primarily responsible for endocytosis of filtered albumin, DBP, retinol binding protein, and many other proteins (44, 108, 140, 177). Mice lacking megalin exhibit low molecular weight proteinuria, which includes significant amounts of DBP (along with albumin and other low molecular weight proteins), vitamin D deficiency, impaired bone growth, and decreased bone density (142). When the normal activity of megalin is blocked with receptor activated protein, radiolabeled DBP is found almost entirely in urine with only a fraction remaining in circulation after one hour of perfusion. However, when this blockage is removed, nearly all radiolabeled DBP remains in circulation after the same time period, indicating the necessity

of megalin for the reabsorption of the DBP complex (142). Along with megalin, the adaptor proteins cubilin and Dab2 also appear to be necessary to the reuptake of vitamin D (143). Cubilin is known to co-localize with and bind megalin in polarized epithelial membranes, and is dependant on megalin expression for its stability (5). Cubilin also binds DBP, and dogs with genetic cubilin deficiency excrete high amounts of DBP in urine while none is detectable in those without the genetic deficiency. However, the cubilin deficient dogs do not show the same wide urinary protein excretion profile seen with megalin deficiency (143), suggesting that cubilin may confer some specificity in the recognition of the DBP-25D3 complex. In addition to these proteins, Dab2 has also been shown to have activity in regulating the endocytic process of DBP-25D3 reabsorption. Dab2 is a cytosolic ligand of megalin that co-localizes to the clathrin-coated pits of the tubule epithelium (146). Dab2 conditionally null mice show reduced numbers of clathrin pits, along with concurrent excretion of urinary DBP (132). Furthermore, cell culture models using anti-Dab2 antibodies and siRNA knockdown techniques show suboptimal internalization of megalin and diffusion of megalin localization outside the clathrin pits (127, 136), suggesting Dab2 might be important for proper trafficking and sorting of megalin during the endocytic process. Therefore the activity of megalin, cubilin, and Dab2 appear to be critical to proper renal 25D3-DBP reuptake and subsequent conversion of 25D to the active hormone after glomerular filtration, and making them important regulators of vitamin D homeostasis.

In addition to the proximal tubule, these proteins have been shown to be expressed in other polarized epithelial tissues, such as intestinal and mammary epithelial cells (164, 204), suggesting their importance may also extend to the local autocrine actions of vitamin D in these extra-renal tissues by facilitating the delivery of circulating 25D3-DBP to these cells.

The prevalence of vitamin D deficiency in populations with renal pathology also emphasizes the pivotal role that the kidney plays in vitamin D homeostasis (9, 52, 128). In the development of diabetic nephropathy, it is thought that hyperglycemia, glycosylated proteins, and proteinuria induce intra-renal activation of RAS and angiotensin II signaling (198). There is evidence from cell culture models of proximal tubule cells that angiotensin II type 1 receptor signaling causes decreased megalin expression. This down regulation of megalin appears to be inhibited by competitive insulin receptor-induced PI3K activation (79), suggesting that defective insulin signaling could contribute to reduced megalin levels and therefore to increased proteinuria.

Studies using the NHANES data have shown that diabetics with active nephropathy have significantly greater proportions of vitamin D deficiency (serum 25D3 <20 ng/mL) or insufficiency (serum 25D3 20-30 ng/mL) independent of all other factors (although the association was greater in minorities), and that the prevalence of albuminuria increased with decreasing rank of serum vitamin D concentration (52). Patients with chronic kidney disease (CKD) had a 30% increase in adjusted odds ratio for vitamin D deficiency which could not be explained by variations in vitamin D intake (128), and there was a significant trend toward deteriorating vitamin D status in children with CKD over a decade of follow-up (9). Additionally, vitamin D deficiency is an independent predictor of all-cause mortality in CKD patients, with CVD being the primary cause (14). The vitamin D deficiency associated with CKD also frequently involves a decreased renal CYP27B1 activity and thus decreased production and circulation of 1,25D3, resulting in hypocalcemia, secondary hyperparathyroidism, and eventually development of osteodystrophy (47). This association becomes even more unfortunate when considering the evidence reviewed earlier that 1,25D3

inhibits the hypertensive effects of the RAS, a primary target for CKD patients who are often given angiotensin converting enzyme inhibitors to suppress this system and prevent further renal endothelial injury (212). Beyond renin suppression, 1,25D3 may provide additional protection by modulating the inflammatory and growth factor responses that underlie degeneration of the glomerular structure. 1,25D3 has been shown to have anti-proliferative and protective effects on podocytes under increased glomerular pressure in partially nephrectomized rats (104), and to inhibit the production of signals inducing collagen matrix formation and fibrosis in cultured rat renal fibroblasts (115). Both of these actions are thought to be important in the development of glomerulosclerosis (144) and the progression towards renal failure in patients with CKD (2). Treatment with various active vitamin D analogues have shown effectiveness in slowing the progression of renal disease in various animal models (126, 211, 212), and are associated with decreased risk of overall vascular mortality in humans with CKD (172, 186). These studies highlight the necessity of maintaining proper vitamin D metabolism in protecting and improving renal outcomes.

NIDDM comorbidities

There is considerable epidemiological evidence that NIDDM creates increased risk for many of the vitamin D-related chronic diseases, most notably CVD and cancers of the breast, colon, pancreas and possibly prostate. For example, NIDDM positively correlates with increased incidence of breast cancer in large multiple observational studies, with the correlation being strongest in post-menopausal women (116, 130, 199). Insulin resistance was also independently associated with breast cancer incidence in post-menopausal Chilean women, although this effect was not always seen in all populations (62). A recent meta-

analysis found a 20% increase in relative risk for breast cancer among diabetic (primarily NIDDM) women (105).

Colon cancer incidence also appears to be positively associated with NIDDM. Increased risk for colon cancer was found in both male and females with NIDDM compared to non-diabetics in multiple populations (106, 169), while NIDDM also independently predicted greater rates of advanced colon adenomas in women (53). Although these correlations with NIDDM appear to be strong, they were not always found when other parameters of glucose metabolism were used, such as Hb_{A1C} measurements (158). In addition to colon cancer, two large meta-analyses have found significant increased risk of pancreatic cancer among NIDDM subjects compared to non-diabetics (54, 85). However, since the causal factor in this association cannot be conclusively determined, it may be that the presence of pancreatic cancer is causing the increased NIDDM incidence.

Interestingly, prostate cancer risk appears to be inversely associated with NIDDM in multiple observational studies and meta-analyses associate diabetes with decreased risk for prostate cancer incidence (25, 33, 95, 194). This association is thought to be due to a tendency for decreased androgen production in diabetics (65). However, while a recent cohort study showed no association with overall prostate cancer incidence, it found an association with NIDDM and increased risk of developing advanced prostate cancer in an Asian population (112), perhaps suggesting the difference in the pathogenesis of androgen-sensitive and androgen-insensitive prostate cancers and their relationship to diabetes. Overall, impaired glucose tolerance was associated with increased all cancer mortality rates in the NHANES II data set (166), suggesting a strong link between the development of both diabetes and cancer.

Lastly, it is widely known that NIDDM patients also have significantly increased cardiovascular morbidity and mortality, and this relationship also extends to the various pathologies of the metabolic syndrome, including obesity, hypertension, dyslipidemia, insulin resistance and glucose intolerance (191).

As previously stated, the diseases associated with NIDDM have also often been shown to correlate with low vitamin D status, but the mechanism behind the development of the deficiency remains unclear. However, as microalbuminuria and nephropathy are common comorbidities associated with NIDDM (67, 120) along with altered vitamin D status (8, 87, 103), we hypothesize that renal dysfunction, possibly through decreased activity of megalin-cubilin-Dab2 mediated endocytosis, may contribute to decreased retention of serum 25D3 and increased urinary excretion of the DBP-bound complex. The accumulated effects of this alteration over time could contribute to lowered serum 25D3 status and availability for conversion to the active 1,25D3 in peripheral tissues and increase the risk of the many previously mentioned chronic diseases, such as cancer and cardiovascular disease.

NIDDM disease etiology

NIDDM is a metabolic disease characterized by chronic insulin resistance, and progressive β cell failure leading to impaired insulin action, disrupted glucose handling, and hyperglycemia. While genetic factors play a part in the development of the disease, it is understood that inflammatory processes are associated with the development of both insulin resistance and obesity (196), which is present in a majority of NIDDM cases (192). Indeed, insulin and inflammatory signals appear to intersect in adipose tissue, thus proving it central in the regulation of both metabolic and immunologic signaling (196).

Adipose tissue has endocrine function and secretes adipokines such as leptin, adiponectin, and pro-inflammatory cytokines TNF- α and interleukin 6 (IL-6), among others (59). Leptin was first identified as an adipocyte-derived factor influencing the development of obesity through its regulation of food intake and energy expenditure (59). Genetically obese mice lacking the leptin gene product demonstrated hyperphagia, diabetes, reduced physical activity, and reduced thermoregulation. These same mice drastically reduced food intake, body mass, percent body fat, increased energy expenditure, and restored euglycemia when administered leptin injections daily (34, 68, 156), substantiating the profound importance of this adipokine in metabolic signaling and regulation of feeding behavior. In support of its role in appetite suppression, high levels of the leptin receptor were found expressed in the feeding regulation areas in the brain (129). In addition, leptin also acts in skeletal muscle to increase fatty acid oxidation (134), indicating a regulatory role in energy expenditure as well. However, in most human cases of obesity leptin levels are elevated even when normalized for body mass, indicating high levels of hormone secretion with no compensatory effect on appetite or energy expenditure, thus suggesting a state of resistance to leptin activity (59). Furthermore, at high levels of leptin administration (20x normal) in human obese populations, weight loss was induced in variable amounts by decreasing food intake, but energy expenditure did not change, indicating that the skeletal muscle might be the primary site of leptin resistance (72, 197). In rodents, leptin resistance can be induced after high-fat feeding (180) and appears to be mediated by reducing AMPK signaling and increasing SOCS3 signaling (21, 179).

The adipokine adiponectin appears to be protective in the development of obesity and NIDDM, as there is a strong negative correlation between adiponectin levels in humans

and measures of adiposity (83). Additionally, adiponectin increases fat oxidation and increases insulin sensitivity through its modulation of inflammatory signals (119, 203). For example, adiponectin directly suppressed the production of TNF- α and IL-6 (200, 208) in macrophages. Weight reduction itself increased adiponectin in obese humans (125, 148), suggesting a normalization of adipocyte secretion with reductions in obesity.

The link to inflammatory processes in obesity, insulin resistance and NIDDM was first suspected when the pro-inflammatory TNF- α was found to be overexpressed in adipose tissue of obese rodents (82, 170) and confirmed in humans (80). The presence of increased numbers of macrophages, also capable of secreting TNF- α , in white adipose tissue of obese individuals suggests an immune component contributes to this signaling (195, 201). Besides its immunologic functions, TNF- α induces serine phosphorylation of IRS-1, inhibiting the ability of IRS-1 to associate with the insulin receptor and mediate downstream intracellular insulin signaling (3, 4, 81), resulting in impaired insulin receptor activity. Furthermore, rodent studies using obese TNF- α null mice demonstrated improved insulin sensitivity compared to wild-type mice with diet-induced obesity (190), indicating TNF- α is a significant mediator of insulin resistance in obesity.

It has been suggested that a contributing mechanism behind the development of insulin resistance is adipose tissue hypoxia (205, 210). Indeed, a primary regulator of insulin-mediated glucose uptake, the foremost determinant of peripheral insulin resistance, is the degree of muscle perfusion (13, 24). In vitro, hypoxia induces secretion of vascular epithelial growth factor, an angiogenic factor, and leptin from adipocytes (121, 193), and reduces adiponectin expression (78, 206). Moreover, hypoxia induces gene expression of inflammatory cytokines TNF- α and IL-6 in adipocytes and macrophages (206). Indeed

adipose tissue hypoxia may explain increased accumulation of macrophages in adipose tissue of obese individuals (135), although the exact mechanism remains unknown (205). Since macrophages are known to secrete many factors involved in angiogenesis, their recruitment may be important response to hypoxia and subsequent adipose tissue remodeling (135, 153, 205). However, it is known that increased macrophage accumulation and decreased adiponectin expression in obesity are associated with proinflammatory TNF- α release (195, 200, 206), a response that directly inhibited insulin-mediated capillary recruitment and glucose uptake in a rat model (209). In contrast, leptin release, which is also induced in adipocytes in hypoxic conditions, increases vascular permeability when administered intradermally in mice (37), and directly induces vasodilation in humans (138). These actions indicate that leptin also functions in regulating the vascular response to hypoxia. In summary, it is possible that with excess fat storage as in obesity, adipose tissue blood perfusion may become inadequate inducing hypoxia. Hypoxia may then induce macrophage recruitment, angiogenic signaling and vessel dilation, with the simultaneous production of inflammatory signals, further leading to insulin resistance (205). Additionally, free fatty acids (FFAs) can mimic the negative effect of TNF- α on capillary recruitment (90) and insulin signaling via IRS-1 (60, 141), indicating dysfunctional lipid metabolism could play an important role in induction of insulin resistance as well.

Along with insulin resistance, the development of NIDDM requires impaired compensatory insulin secretion, which has been attributed to increased apoptosis of β cells found in NIDDM patients (30). In cultured human β cells, genes inducing apoptosis are overexpressed in hyperglycemic conditions (55). Additionally, in rodent models of NIDDM, controlling hyperglycemia pharmacologically preserved β cell function, insulin gene

expression, and glycemic control (70). However, slight decreases in insulin secretion are seen before impairment of glucose tolerance is observed in lean healthy subjects with a predisposing family history of NIDDM (93), indicating that toxic effects of hyperglycemia itself is not sufficient to explain the β cell failure in NIDDM. Insulin resistance in adipose tissue increases plasma FFAs due to decreased insulin-mediated suppression of lipolysis (48). Therefore, increased lipid accumulation in β cells may cause lipotoxicity (189). In fact, Kashyap et al. (93) found that sustained infusion of FFA impaired insulin secretion in patients genetically predisposed to NIDDM. Furthermore, reducing plasma FFAs by blocking lipolysis improved indicators of insulin secretion in the same population (49). Therefore, it appears that the inflammatory process involved in adipocyte insulin resistance, in conjunction with the known effects of glucotoxicity, may contribute to pancreatic β cell failure via β cell lipotoxicity and apoptosis (159).

Zucker diabetic fatty rat model of NIDDM

The Zucker diabetic fatty (ZDF) rat is a widely used rodent model of NIDDM that was developed by inbreeding the obese Zucker rat. The obese Zucker (fa/fa) rat is homozygous for a missense mutation in the leptin receptor gene (7, 86, 94, 183). As a result, the obese Zucker rat develops severe obesity early in life (3-5 weeks) due to defective leptin signaling, which is characterized by hyperphagia, defective thermoregulation, preferential adipose deposition, and elevated levels of circulating leptin. (7, 69, 89, 216). The male ZDF rat develops the same characteristic endocrine abnormalities of the obese Zucker rat that accompany defective leptin signaling, such as obesity, insulin resistance, dyslipidemia, hyperinsulinemia, and impaired glucose tolerance. Overt hyperglycemia, which is not generally found in obese Zucker rats (7) usually develops in the ZDF rats between 7

and 9 weeks of age with insulin levels increasing until approximately 20 weeks. At that point, insulin levels begin to fall, which is consistent with the progression of human NIDDM pathology (46, 110), indicating that altered leptin signaling in β cells does not significantly impair disease progression in the ZDF, as in other rodent models of obesity with leptin receptor defects (131). Female ZDF rats do not normally develop hyperglycemia or NIDDM symptoms, but they maintain measures of obesity and insulin resistance comparable to males. However, there is evidence that a high-fat diet can induce hyperglycemia, indicating that with appropriate dietary modulation, the female ZDF rat may also be an adequate rodent model of NIDDM (185). In addition, at later stages these animals develop symptoms consistent with neuropathy (28, 147), retinopathy (51), nephropathy (45, 77), and hypertension (111, 122), which are common complications of NIDDM in humans, confirming the usefulness of this rodent model for studying pathologies associated with NIDDM.

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CHAPTER 2: VITAMIN D HOMEOSTASIS IS COMPROMISED DUE TO INCREASED URINARY EXCRETION OF THE 25-HYDROXYCHOLECALCIFEROL-VITAMIN D-BINDING PROTEIN COMPLEX IN THE ZUCKER DIABETIC FATTY RAT

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Abstract

Altered serum concentrations of the major circulating form of vitamin D (25-hydroxycholecalciferol, 25D3) and its active hormone derivative (1,25-dihydroxycholecalciferol, 1,25D3) have been linked to non-insulin dependent diabetes mellitus (NIDDM). However, a mechanistic basis for this occurrence has not been fully elucidated. Normally, renal reabsorption of vitamin D-binding protein bound 25D3 absolutely requires receptor-mediated endocytosis via a receptor complex containing megalin, cubilin, and disabled-2 (Dab2), whereas an absence of megalin or its endocytic partners can lead to a marked urinary loss of 25D and severe vitamin D deficiency.

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Therefore, we hypothesized that reduced serum vitamin D status in NIDDM may be due to reduced expression of megalin and/or its endocytic partners and increased urinary excretion of protein-complexed 25D3. In the present study, we utilized Zucker diabetic fatty Rats (ZDF) to demonstrate that renal reuptake of the 25D3-DBP complex was compromised in ZDF animals, which was reflected by a reduction in expression of megalin and Dab2. Moreover, serum levels of both 25D3 and 1,25D3 were reduced and urinary 25D3, 1,25D3, and DBP excretion were elevated in the ZDF animals compared to their lean controls, regardless of vitamin D levels in the diet. Taken together, these are the first reports to our knowledge that associate compromised renal reabsorption of the 25D3-DBP complex with expression of megalin and its endocytic partners in NIDDM, which in turn can lead to compromised vitamin D status.

Keywords diabetes · vitamin D · vitamin D binding protein · kidney · rat

INTRODUCTION

Optimal vitamin D status has been associated with improved long-term health outcomes in cardiovascular disease and cancer, complications that occur at higher incidences in individuals with noninsulin-dependent diabetes mellitus (NIDDM). Poor renal function, also a consequence of poorly controlled type 2 diabetes, results in hypertension, increased epithelial cell damage, and increased risk for CVD (3, 11). In addition to data from case control studies that indicates that maintenance of optimal serum 25-hydroxycholecalciferol (25D3) concentrations (>90 nmol/L) is preventative against many types of cancer, research has suggested that optimal vitamin D status may be protective against hypertension and nephron damage through the suppression of renin production in the kidney (13, 36). Furthermore, clinical and epidemiological studies have suggested that type 2 diabetics and

individuals with chronic kidney disease are more likely to be in what is considered the suboptimal range (< 80 nmol/L) with respect to serum vitamin D status (7, 15), though the mechanistic basis for this occurrence has not been elucidated. Therefore, fully understanding all factors influencing vitamin D homeostasis in this population may reveal opportunities to improve outcomes and comorbidities associated with type 2 diabetes, especially those with renal complications.

Cells of the renal proximal tubule are responsible for reabsorption of the major circulating form of vitamin D (25D3) from the glomerular filtrate and are the primary site of activation of 25D3 to the active hormone, 1,25-dihydroxycholecalciferol (1,25D3), which is released into the blood for systemic use. For 25D3 to be reabsorbed and/or activated by CYP27B1 in the kidney, the proximal tubule must internalize the 25D3-vitamin D-binding protein (DBP) complex due to strong binding of DBP to 25D3 in circulation (21, 22). This process is absolutely dependent on the actions of the membrane receptor megalin and its endocytic partners cubilin and disabled-2 (Dab2). In support of this concept, researchers showed that when megalin, cubilin, or Dab2 expression is absent, loss of 25D3-DBP in the urine was dramatically elevated and severe vitamin D deficiency resulted (21, 22). Moreover, renal megalin and cubilin levels were markedly reduced in humans and rats with diabetes (30, 31), which resulted in increased urinary excretion of albumin, another known ligand of megalin.

In the present study, we tested our hypothesis that uncontrolled NIDDM would lead to decreased renal expression of megalin, cubilin, and/or Dab 2 in Zucker Diabetic Fatty Rats (ZDF), a well-established model of NIDDM (34). Furthermore, we hypothesized that reduced expression of megalin or any of its endocytic partners would lead to increased urinary

excretion of the 25D3-DBP complex and compromised vitamin D status. Our first objective was to characterize vitamin D homeostasis and expression of megalin, cubilin, and Dab 2 in ZDF rats. Secondly, we determined whether vitamin D deficiency in ZDF rats was due to hormonal changes or reduced endocytosis of the 25D3-DBP complex, and whether increased concentrations of cholecalciferol in the diet could influence renal vitamin D reabsorption.

MATERIALS AND METHODS

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee and were performed according to Iowa State University Laboratory Animal Resources Guidelines. Male Zucker diabetic fatty (ZDF) and lean control rats were obtained from Charles River Laboratories at 6 wks of age and individually housed in plastic cages in a 12-hour light-dark cycle with free access to food and water until 14 wks of age.

Study 1: characterization of vitamin D excretion in ZDF rats. ZDF rats (n=6) and lean rats (n=6) were fed a commercial high-energy rodent diet (Purina Formulab Diet 5008) over the course of the study to induce a diabetic state in the ZDF animals and sacrificed at 14 wks.

Study 2: assessment of vitamin D homeostasis in ZDF rats. Male Zucker Diabetic Fatty [ZDF, n=24) and lean (n=16)] rats were fed a high-energy diet (Purina Formulab Diet 5008) until 11 wks, when the ZDF rats were randomly assigned to one of three diets (n=8), vitamin D deficient (0 IU cholecalciferol/kg, VD), vitamin D sufficient (1000 IU/kg, VS), or vitamin D supplemented (10000 IU/kg, VDS), and the lean rats to one of two diet treatments (n=8), VD or VDS. Diets were formulated based on the AIN-93G purified diet. Animals were sacrificed at 14 wks of age.

For both studies, all animals were fasted twenty-four hours prior to sacrifice and placed in metabolic cages for urine collection. Animals were anaesthetized with an intraperitoneal injection of ketamine-xylazine (90 and 10 mg/kg body wt). Whole kidneys were excised and snap frozen in liquid nitrogen for subsequent RNA isolation. Whole blood was collected by cardiac puncture and serum was separated by centrifugation and stored at -80°C until analysis.

Assessment of Renal Function. Serum and urinary creatinine levels were measured via a commercial kit (QuantiChrom™ Creatinine Assay Kit, Bioassay Systems, Hayward, CA).

Assessment of Blood Glucose and Serum Insulin. To confirm the presence of diabetes in ZDF rats, blood glucose was measured by glucometer (Bayer Healthcare LLC) at the time of sacrifice. Serum insulin was analysed using an ELISA specific for rat insulin (Millipore, Billerica, MA).

Assessment of Urinary Albumin and DBP. Urinary albumin and DBP concentrations were measured via commercial ELISA kits (Exocell, Philadelphia, PA and Life Diagnostics Inc, West Chester, PA, respectively). Sample dilutions used for ELISAs were as directed or empirically determined, as appropriate.

Assessment of 25D3 and 1,25D3 Status. Total 25D3 and 1,25D3 were assessed in both serum and urine samples using a commercial enzyme immunoassay kit (Immunodiagnostic Systems, Scottsdale, AZ). Urinary excretion of vitamin D metabolites were assessed relative to urine creatinine.

Real-time PCR. Total kidney RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI), quantified by UV detection, and used for first strand cDNA

synthesis (5 ug/50ul reaction) using a High Capacity cDNA synthesis kit with RNase inhibitor (Applied Biosystems, Foster City, CA). Three stocks of cDNA were generated per kidney sample, and each cDNA stock was quantified by UV detection and independently analyzed for megalin, cubilin, Dab2, and CYP27B1 by real-time PCR. Real time PCR reactions were performed in duplicate using iScript SYBR Green Detection reagents (Bio-Rad, Hercules, CA), 200 ng cDNA/well, and primer sets specific for rat megalin (Forward Primer-AACGGTCAGTGTATTCCGAGCGAA), Reverse Primer-TTGGCAGTCGTCATCTCCATCACA), Cubilin (Forward primer-AGGGACACAAGGAACCTTTGCCTA, reverse primer-GTCTTTGCTGCAGTCATTGTGGCT), Dab2 (Forward primer-AGGTTGAAGAAGCCAACAAAGCGG), Reverse primer-AGTCCTGCTTTACGCCATTCGGTA), and CYP27B1 (Forward primer-AAGTTCCTCCCGACACAGAAACCT), Reverse primer-GCTTCTGGGCAAAGGCAAACATCT) and were normalized against 18S mRNA (Forward Primer-CCAGAGCGAAAGCATTGCAAGA), Reverse Primer-AATCAACGCAAGCTTATGACCCGC). Gene expression was determined as fold-induction relative to lean control animals.

Histology and Immunohistochemistry. Formalin-fixed kidneys were embedded in paraffin, sectioned at 5 μ M, and stained with Hematoxylin and Eosin Y for routine histological assessment. To detect megalin and Dab2, paraffin wax embedded sections were placed in 1 mol/L urea and microwaved for 10 min then cooled for 1 h. Slides were washed 3 \times for 5 min in ddH₂O, then washed in phosphate-buffered saline (PBS) for 5 min. Slides were then soaked in 90% methanol containing 30% hydrogen peroxide for 15 min at RT

followed by 3 washes with PBS (5 min/wash). Following an overnight incubation with blocking buffer (3% bovine serum albumin/0.1% Tween in PBS) slides were incubated overnight with either a 1:50 dilution (in blocking buffer) of a polyclonal antibody directed against megalin (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:50 dilution (in blocking buffer) of monoclonal antibody directed against Dab2 (BD Pharmingen, San Jose, CA). Slides were then washed 3× in PBS and incubated with a 1:500 dilution in blocking buffer of the appropriate biotinylated secondary antibody for 1 h at RT. Following 3 washes in PBS for 10 min, 1 drop of ABC (Vector Laboratories, Burlingame, CA) was applied to slides for 30 min at RT. Slides were washed in PBS 3× for 5 min and DAB (Vector Laboratories, Burlingame, CA) was applied for 5 min followed by a 5 min wash in ddH₂O, counterstaining with Hematoxylin, and mounting with Permount.

Assessment of Serum Calcium and Parathyroid Hormone. To determine whether vitamin D status was influenced by calcium and parathyroid hormone levels, serum calcium and parathyroid hormone concentrations were measured using commercially available ELISA kits (Bioassay Systems, Hayward, CA and Immutopics, San Clemente, CA, respectively)

Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA), unpaired t-test, or Mann-Whitney test using InStat software (version 3.0b for Macintosh, GraphPad Software) or linear regression using Prism software (version 5.0a for Mac OSX, GraphPad Software), as appropriate. Differences between means and linear relationships were considered significant when p values < 0.05 were obtained.

RESULTS

Study 1: Characterization of Renal Vitamin D Reabsorption in ZDF Rats

Confirmation of NIDDM in ZDF rats. After 8 weeks on the Purina 5008 diet, fasting blood glucose and serum insulin levels in the ZDF rats were both elevated ~4-fold compared to the non-diabetic control animals (Figure 1). This confirmed hyperglycemia and hyperinsulinemia in ZDF rats.

Megalyn, cubilin, and Dab2 gene expression. To determine whether loss of the 25D3-DBP complex in the urine was due to decreased renal absorption, whole kidney lysates were utilized to measure megalin, cubilin, and Dab2 mRNA expression using real-time PCR. We found that both megalin and Dab2 expression was reduced in ZDF animals compared to the lean control animals (~50% and ~80%, respectively, Figure 2A and 2B), whereas we did not detect differences in cubilin mRNA expression (data not shown). Similarly, we found that immunohistochemical staining of tissue sections revealed that megalin and Dab2 protein expression was reduced in the renal proximal tubules in these animals (Figure 3). Moreover, from our histological observations of kidney tissue from ZDF rats, necrosis appeared to be present in the renal proximal tubules, which may explain, at least in part, the reduced staining of megalin and Dab2 in kidney sections.

Serum creatinine. Renal function was assessed by measuring serum creatinine. Serum creatinine levels were elevated ~80% in ZDF animals compared to lean controls, indicating that renal function was compromised in ZDF animals (Figure 4).

Albuminuria and increased DBP excretion in ZDF rats. We measured urinary albumin for two purposes. 1) albumin, like DBP, is a known ligand of megalin (30), and 2) severe albuminuria is a biological marker of nephropathy (8, 30, 31). We found that marked

albuminuria was present in the ZDF animals, which excreted ~20-fold greater amounts of albumin compared to lean control animals. Similarly, ZDF rats excreted large amounts of DBP in urine (8.9 $\mu\text{g}/\text{mL}$) (Figure 2C and 2D). In contrast, DBP was virtually undetectable in the urine of lean control animals, indicating that the ability of ZDF animals to reabsorb the 25D3-DBP complex was markedly compromised.

Urinary and serum 25D3 and 1,25D3 concentrations. Urine was examined for the presence of the 25D3 and 1,25D3 to determine whether loss of DBP in the urine was associated with increased urinary loss of vitamin D. Urine from ZDF animals contained elevated concentrations of total 25D3 (which is dependent on DBP for transport to the kidney) the major circulating metabolite of vitamin D, compared to lean control animals (31% higher in ZDF animals when normalized to urinary creatinine levels, Figure 5B and 5D). Likewise, urinary concentrations of 1,25D3 (normalized to urinary creatinine concentrations) were greater in ZDF animals compared to control animals (Figure 4D). Additionally, urinary DBP concentration strongly correlated with the urinary concentration of 25D3 (Figure 6) in the ZDF animals ($r^2=0.85$, $p<0.05$). Serum 25D3 and 1,25D3 levels did not differ between groups (Figure 5A and 5C); however, we determined that consumption of vitamin D/kg bodyweight was significantly higher in ZDF animals (Figure 7), an indication that loss of urinary vitamin D metabolites was compensated for through the diet, a question that we addressed below in the second set of our experiments.

Study 2: Assessment of Vitamin D Homeostasis in ZDF Rats

Assessment of 25D3 and 1,25D3 status. As in our first set of experiments, NIDDM was confirmed in all ZDF rats. Glucose and insulin levels were 200-400% greater in ZDF rats compared to lean animals (data not shown). After 3 weeks of being fed the experimental

diet both 14 wk old ZDF rats fed a vitamin D deficient (VD, 0 IU cholecalciferol/kg diet) or a 1000 IU cholecalciferol/kg (VS) diet exhibited reduced serum 25D3 levels compared to their lean counterparts (43- and 22% reduction, respectively) fed the same diets (Figure 8A). Moreover, urinary excretion of 25D3 in ZDF animals fed the VD levels was elevated compared to their lean counterparts by 30% after normalization to urinary creatinine (Figure 8B), indicating that vitamin D status was compromised to a greater extent in the ZDF animals than in lean animals after 3 wks on the VD diet. In support of this concept, urinary excretion of 25D3 by ZDF rats fed the VS was elevated 24% compared to lean rats and in contrast to study 1, we did not detect differences in cholecalciferol intake/kg bodyweight between the ZDF and lean control animals fed the VS diet (data not shown). Serum 1,25D3 levels were reduced 70% whereas urinary 1,25D3 levels were ~60-fold greater when normalized to urinary creatinine from ZDF animals fed the VD diet compared to lean controls (Figure 8C), ZDF animals fed the VS diet exhibited 64% lower serum 1,25D3 concentrations and excreted ~9-fold higher concentrations of 1,25D3 in urine (Figure 8D). When additional vitamin D (10000 IUs/kg) was added to the diets of the ZDF rats (VDS), large increases were seen in both serum and urinary 25D3 concentrations (70%- and 12-fold, respectively). However, serum concentrations of 1,25D3 remained markedly lower in ZDF animals fed the VDS diet, indicating that high-dose cholecalciferol supplementation of ZDF rats effectively elevated serum 25D3 concentrations but had no impact in improving serum 1,25D3 levels.

Serum creatinine. As in study 1, renal function was assessed by measuring serum creatinine. Serum creatinine levels were elevated ~76% in ZDF animals compared to lean controls (Figure 8D), indicating that renal function was compromised in ZDF animals.

Urinary albumin, DBP, and renal megalin expression in ZDF rats. Similar to what we observed in our first set of experiments, we detected severe albuminuria and urinary loss of DBP in all ZDF animals compared to their lean controls (FIG 9A and 9B). Urinary albumin concentrations ranged from 470-1250 mg/dL and urinary DBP concentrations ranged from 2200-3500 among all ZDF rats, whereas urinary albumin and DBP from lean animals were virtually undetectable. Moreover, as reported above, a reduction in renal mRNA megalin expression was observed in all ZDF groups compared to their lean counterparts (figure 9C).

CYP27B1, serum calcium, and PTH. Although the diabetic rats all exhibited decreased levels of serum 1,25D₃, serum calcium and PTH concentrations did not differ between ZDF and lean animals in any of the treatment groups (Figure 10A and 10B). Renal CYP27B1 mRNA expression was elevated only in ZDF animals fed the VD diet, a likely result of reduced serum 1,25D₃ concentrations and/or a lack of dietary cholecalciferol as we have previously reported (28). Similar to our previously published work (28), a reduction in renal CYP27B1 mRNA expression was observed when ZDF animals were fed the VDS diet.

DISCUSSION

In the present study, we found that NIDDM was associated with increased urinary excretion of 1,25D₃, 25D₃, and vitamin D-binding protein (DBP), which binds with high affinity >99% of circulating 25D₃ and with low affinity to 1,25D₃ for delivery to the renal proximal tubule for reabsorption (2, 14, 23, 29, 33, 35). Moreover, we found that serum levels of 25D₃ and 1,25D₃ were reduced in animals that not only excreted disproportionately larger amounts of 25D₃, 1,25D₃, and DBP in urine, but also exhibited reduced renal expression of megalin and Dab2. Furthermore, we found that compromised vitamin D status

was independent of both renal CYP27B1 expression and calcium homeostasis in diabetic animals. Rather, our data strongly suggest that the major contributing mechanism behind compromised vitamin D status in our animal model was inadequate megalin- and/or Dab2-mediated renal reabsorption of 25D₃ and 1,25D₃. These findings are consistent with a recent clinical study, where researchers found that urinary levels of megalin and cubilin were markedly elevated in albuminuric patients with type 1 diabetes (30), further indicating that as diabetes progresses the ability to maintain normal vitamin D homeostasis via megalin-mediated mechanisms is compromised.

A growing number of reports have convincingly linked low vitamin D status to the incidence of a number of chronic diseases, including diabetes, cancers of the colon, breast, prostate, and autoimmune disease, and osteoporosis (6, 19, 32). Additionally, researchers have estimated that the majority of the United States population exhibits serum vitamin D levels that are substantially lower than those required for reducing chronic disease risk (6). Hence, interest in utilizing vitamin D in dietary intervention strategies has markedly increased over the last decade. Moreover, the theory that low vitamin D status is a contributing factor to the incidence of chronic disease is at the heart of an ongoing debate about dietary recommendations for vitamin D. Based on data from observational studies, Garland, et al. (6) recently reported that in order to provide a 50% reduction in the risk of cancers of the colon and breast, serum levels of 25D₃ need to be ~80 and 110 nM, respectively. It is estimated that in order to reach these serum 25D₃ levels, the average individual must consume between 2000-3500 IU vitamin D₃/day, or 5-7 times the Adequate Intake defined in the Dietary Reference Intakes for vitamin D₃ (6). In diabetes, the vitamin D requirement for protection against secondary chronic diseases appears to be even greater.

This idea is supported by research that demonstrated that type 1 and type 2 diabetics exhibited reduced concentrations of the major circulating form of vitamin D [25D₃], and/or its active derivative 1,25-dihydroxyvitamin D₃ (1,25D₃) (23, 29, 33). There is little known about why vitamin D status is suboptimal in these individuals, but our data suggest that it may be due to compromised kidney function. In support of this concept, we found that rats with NIDDM exhibited a drastic reduction in the renal expression of the endocytic membrane proteins, megalin and Dab2, which are essential for reabsorption of protein-bound 25D₃ (30), and increased urinary loss of albumin and DBP, which are also absolutely dependent on megalin for reabsorption. In support of this concept knockout mouse studies revealed that animals lacking megalin or either of its endocytic partners, cubilin or Dab2, exhibited a marked urinary loss of 25D₃ and severe vitamin D deficiency (17, 18, 21, 22).

Though we and others have found that vitamin D metabolism can be dramatically altered under diabetic conditions (1, 27, 29), exploration of the role of megalin and its endocytic partners in maintaining optimal vitamin status is a new concept. Hence, these studies may offer new insight into whether vitamin D supplementation and monitoring urinary and serum vitamin D levels can help prevent or alleviate secondary complications stemming from compromised kidney function in diabetes. Our findings indicate that reabsorption of vitamin D by the kidney is a major contributing factor to suboptimal vitamin D status in diabetes, which has clear implications with respect to the development of secondary chronic diseases such as cardiovascular disease and many types of cancer. It is well-documented that non-insulin dependent diabetics are at a disproportionately high risk for the development of breast, prostate, and colorectal cancer, cancers that are arguably the most sensitive to the actions of vitamin D. The naturally occurring active form of vitamin D

(1,25D₃) has well-documented anti-proliferative actions, including cell cycle arrest, differentiation, and induction of apoptosis (4, 5, 12, 24, 37). Because low serum levels of 1,25D₃ and its precursors are often present in diabetes (1, 14, 27, 29), there may be a substantially larger dietary vitamin D requirement for these individuals. Numerous animal studies have outlined the potential role of increased vitamin D status with respect to the inhibition of tumor formation and promotion. Vitamin D supplementation prior to treatment with chemical carcinogens inhibited fat-induced colorectal tumor promotion in rats fed a high-fat diet (16, 25). Injection of 1,25D₃ or its analogues potently decreased the appearance of aberrant crypt foci and tumors, as well as the proliferation and metastasis of established tumors (9). Consistent with this concept, high tumor and polyp frequency, as well as increased tumor proliferation, have been reported in studies where rodents were fed low vitamin D diets (10, 20, 26). Furthermore, diabetes has been linked to an increased risk of developing cancer in countless human and animal studies, although the mechanism behind this phenomenon remains unclear. Interestingly, obesity and consumption of a high-fat diet, known risk factors for development of heart disease, cancer and type 2 diabetes, also appear to strongly affect an individual's vitamin D requirement (1, 14, 27, 29, 33).

In summary, these studies have provided evidence that megalin-mediated endocytosis plays a critical role in vitamin D homeostasis in NIDDM. Taken together, our data provide the first evidence, at least to our knowledge, that reduced renal reabsorption of circulating vitamin D via compromised receptor-mediated endocytosis is a key contributor to suboptimal vitamin D status in NIDDM.

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Disclosure

The authors disclose that there is no duality of interest associated with this manuscript.

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FIGURES

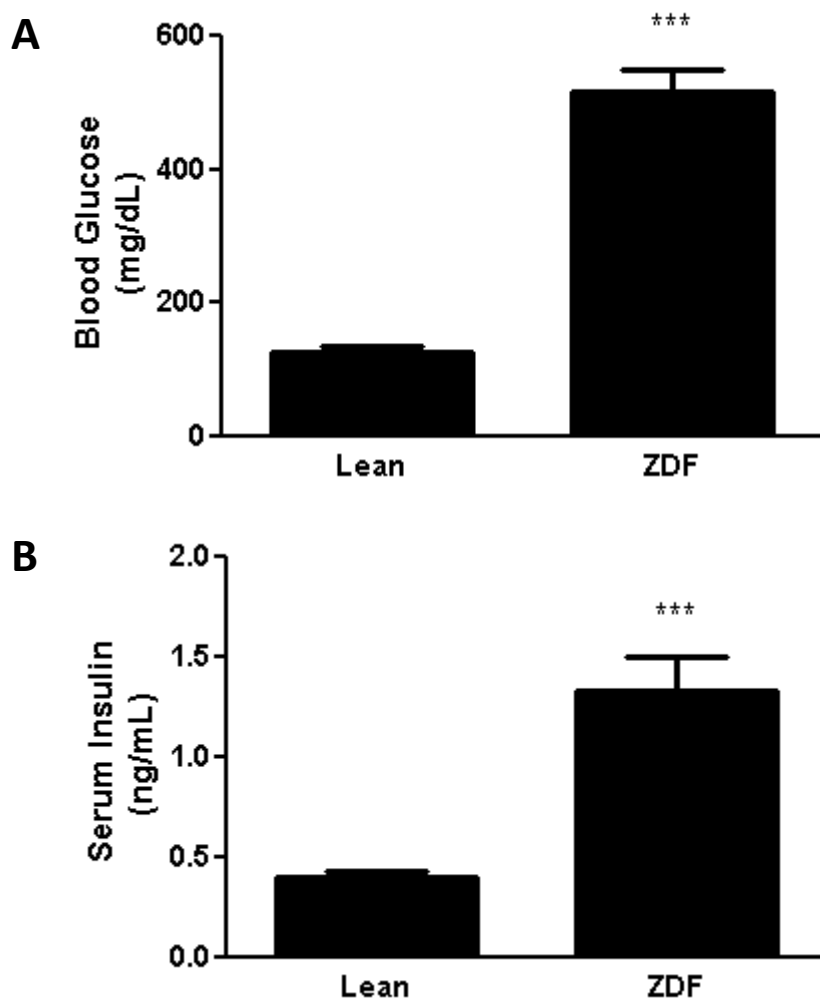


Fig. 1 Confirmation of NIDDM in ZDF rats. ZDF and lean rats were obtained at 6 wk of age and fed a commercial high-energy rodent diet (Purina Formulab Diet 5008) to induce a diabetic state sacrificed at 14 wks. To confirm the presence of diabetes in ZDF rats, blood glucose was measured by glucometer at the time of sacrifice. Serum insulin was analysed using an ELISA specific for rat insulin. Data are expressed as means \pm SEM (n = 6); ***P < 0.001.

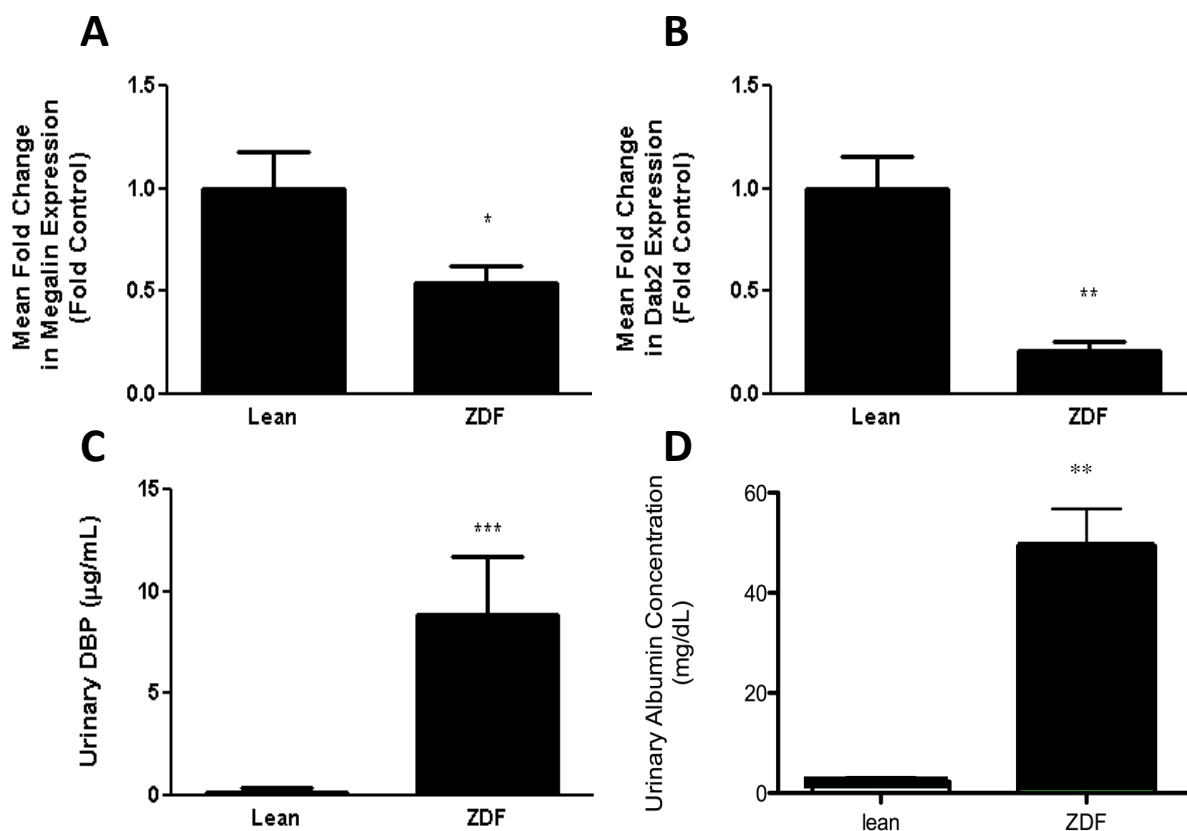


Fig. 2 Reduced megalin and Dab2 expression and renal reabsorption of DBP and albumin in ZDF rats. Renal tissue and urine was collected from the same animals as described for Fig. 1. **Fig. 2A and 2B.** Megalin and Dab2 mRNA were analyzed as described in Materials and Methods. A) Megalin mRNA abundance (determined by real-time PCR) in ZDF and lean rats. B) Dab2 mRNA abundance (determined by real-time PCR) in ZDF and lean rats. **Fig. 2C and 2D.** Urinary DBP and albumin were analyzed as described in Materials and methods. A) Urinary DBP concentrations from ZDF and lean rats. B) Urinary albumin concentrations from ZDF and lean rats. Data are expressed as means \pm SEM (n = 6); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

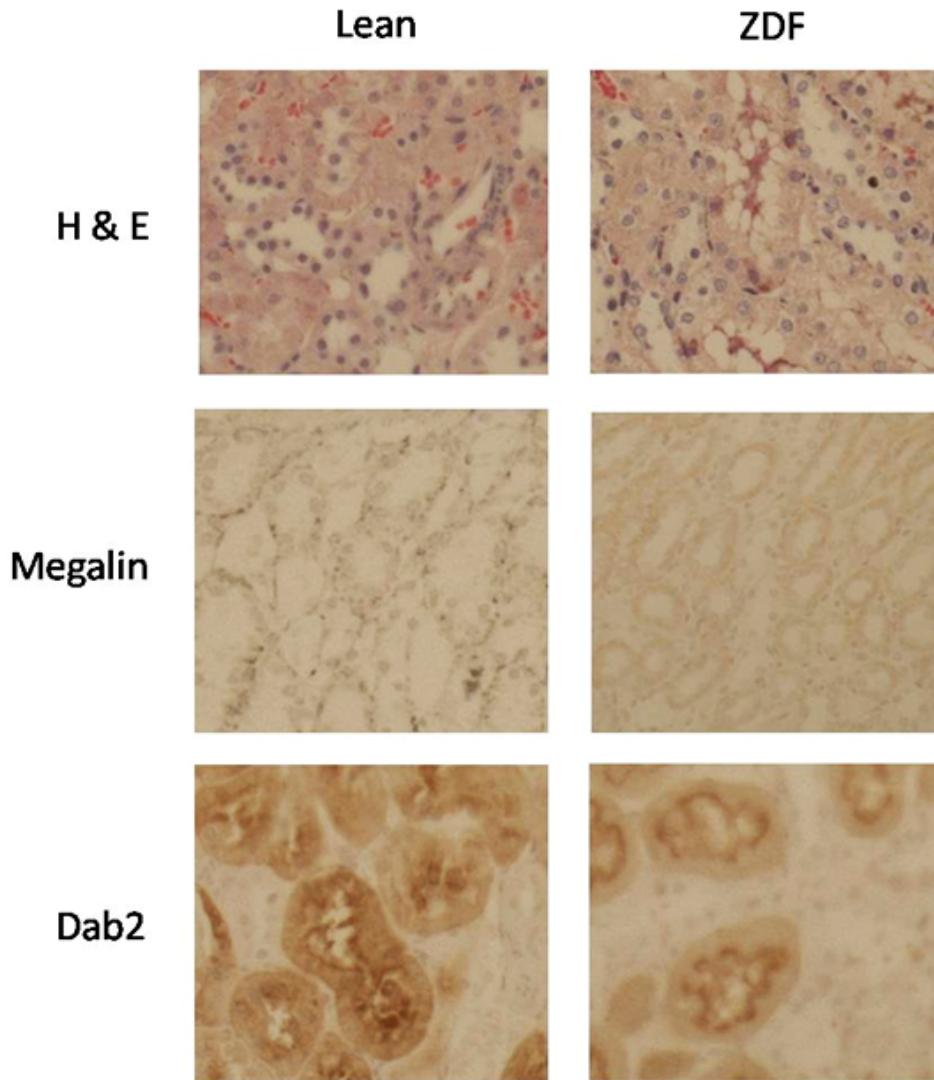


Fig. 3 Renal morphology and immunohistochemical analysis of megalin and Dab2 expression in kidney of lean and ZDF rats. Kidneys were excised from the same animals as described for Fig. 1, processed, and sectioned for staining with Hematoxylin and Eosin for routine histological assessment (top panels) or subjected to immunohistochemical staining for megalin and Dab2 as described in Materials and Methods. Megalin- (middle panels) and Dab2-positive cells (bottom panels) appear dark brown against the blue Hematoxylin counterstain.

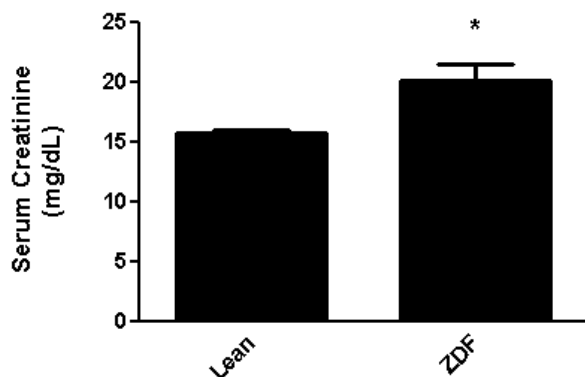


Fig. 4. Assessment of renal function in ZDF rats. Serum collected from the same animals as described for Fig. 1 was utilized to measure serum creatinine. Data are expressed as means \pm SEM (N=6); * $P < 0.05$.

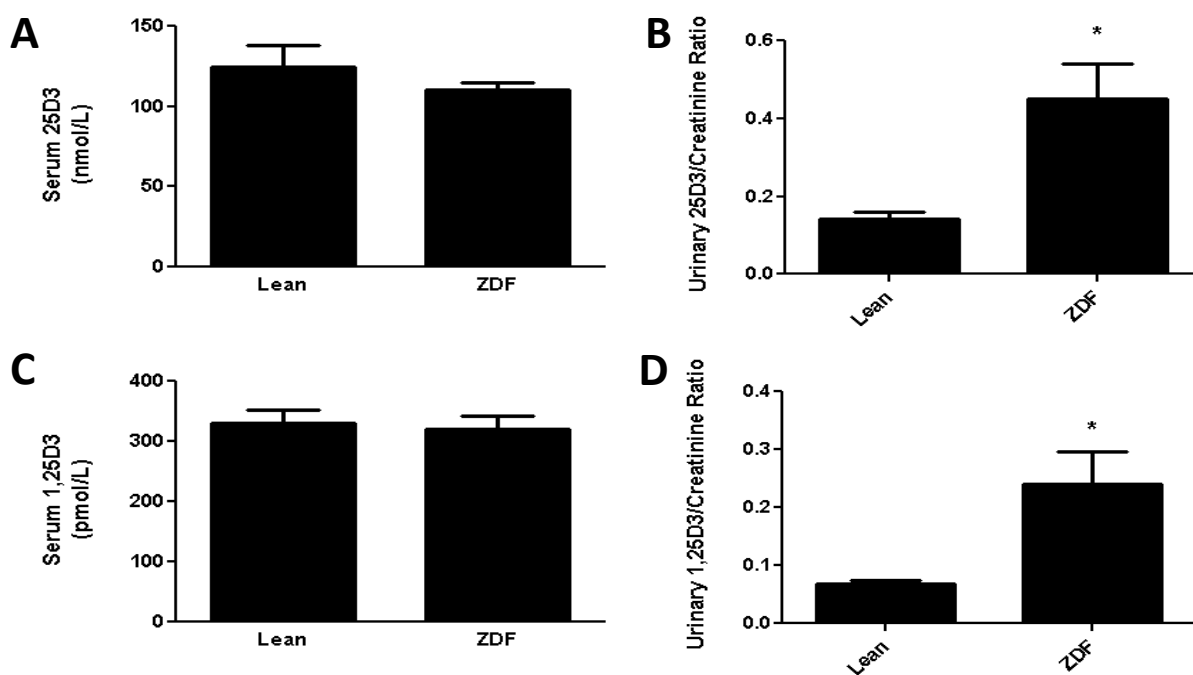


Fig. 5 Serum and urinary 25D3 and 1,25D3 concentrations in lean and ZDF rats. Serum and urine collected from the same animals as described for Fig. 1 were utilized to measure 25D3 and 1,25D3 concentrations in lean and ZDF rats by ELISA as described under Materials and Methods. A) Serum 25D3 concentrations. B) Urinary 25D3 concentrations. C) Serum 1,25D3 concentrations. D) Urinary 1,25D3 concentrations. Data are expressed as means \pm SEM (n = 6); * $P < 0.05$.

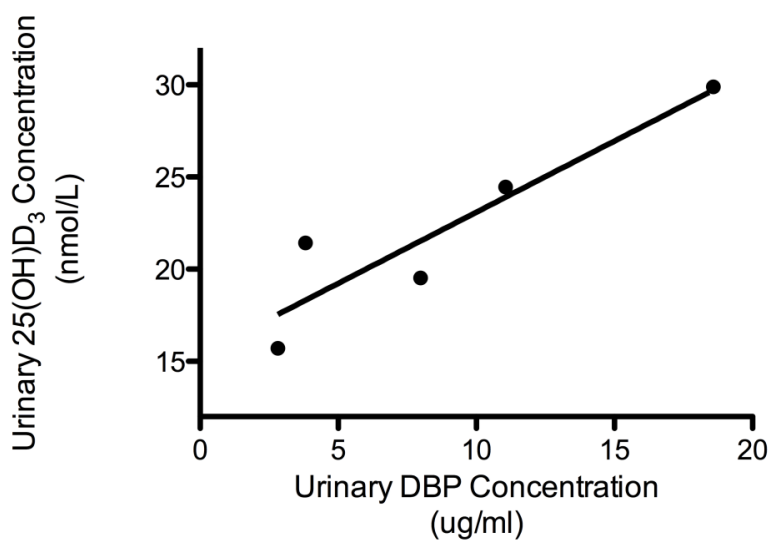


Fig. 6. Urinary excretion of DBP correlates with urinary 25D3 in ZDF rats. ZDF ($n = 5$) were obtained at 6 wks of age and fed a diabetogenic diet (Purina 5008) for 8 wks until diabetes was evident. 24 h urine output was collected and urinary DBP and 25D3 levels were assessed by ELISA and EIA, respectively, as described in Materials and Methods. Results of a Pearson correlation test are indicated by the solid line ($r = 0.921$, $P < 0.01$).

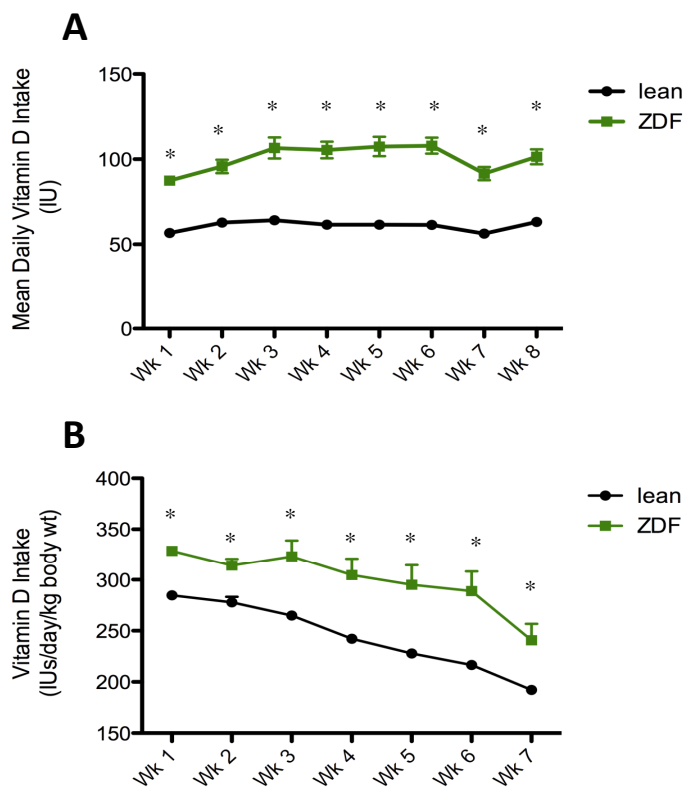


Fig. 7. Cholecalciferol intake in lean and ZDF rats. ZDF rats and their lean controls were acquired at 6 wk of age and fed a commercial high-energy rodent diet (Purina Formulab Diet 5008) until they were sacrificed at 14 wk of age. A) Total cholecalciferol intake in ZDF rats and their lean controls during the 8 wk study period. B) Cholecalciferol intake calculated as IU's/d/kg bodyweight for the 8wk study period. Data are expressed as means \pm SEM (n = 6); * $P < 0.05$.

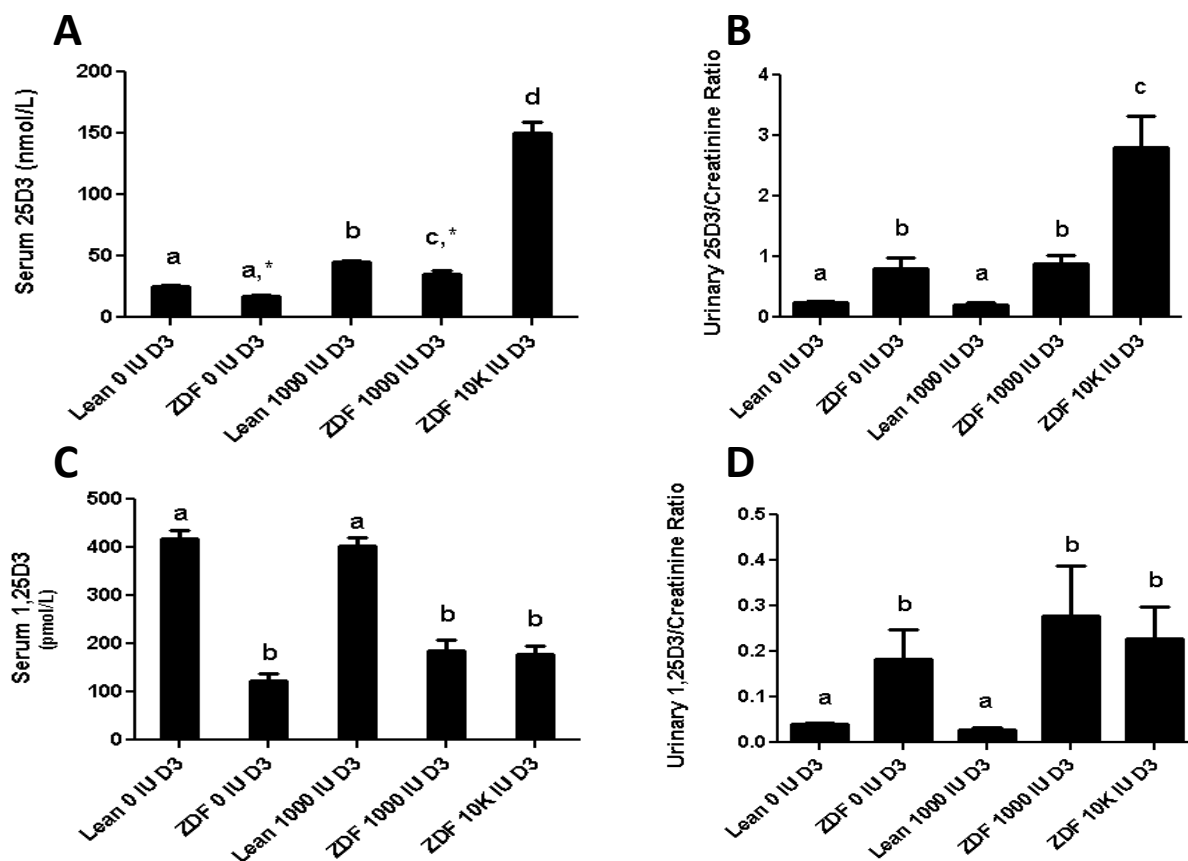


Fig. 8. Increased urinary output and compromised serum concentrations of 25D3 and 1,25D3 in ZDF rats. 11 wk old ZDF and lean control rats were fed either 0, 1000, or 10000 IU cholecalciferol/kg diet for 3 wk. 25D3 and 1,25D3 concentrations were measured in serum and urine collected from ZDF and lean control rats by ELISA as described under Materials and Methods. A) Serum 25D3 concentrations. B) Urinary 25D3 concentrations. C) Serum 1,25D3 concentrations. D) Urinary 1,25D3 concentrations. Data are expressed as means ($n = 8$) \pm SEM; bars with different letters are significantly different ($P < 0.05$). *Statistically significant, ZDF versus respective lean control ($P < 0.05$).

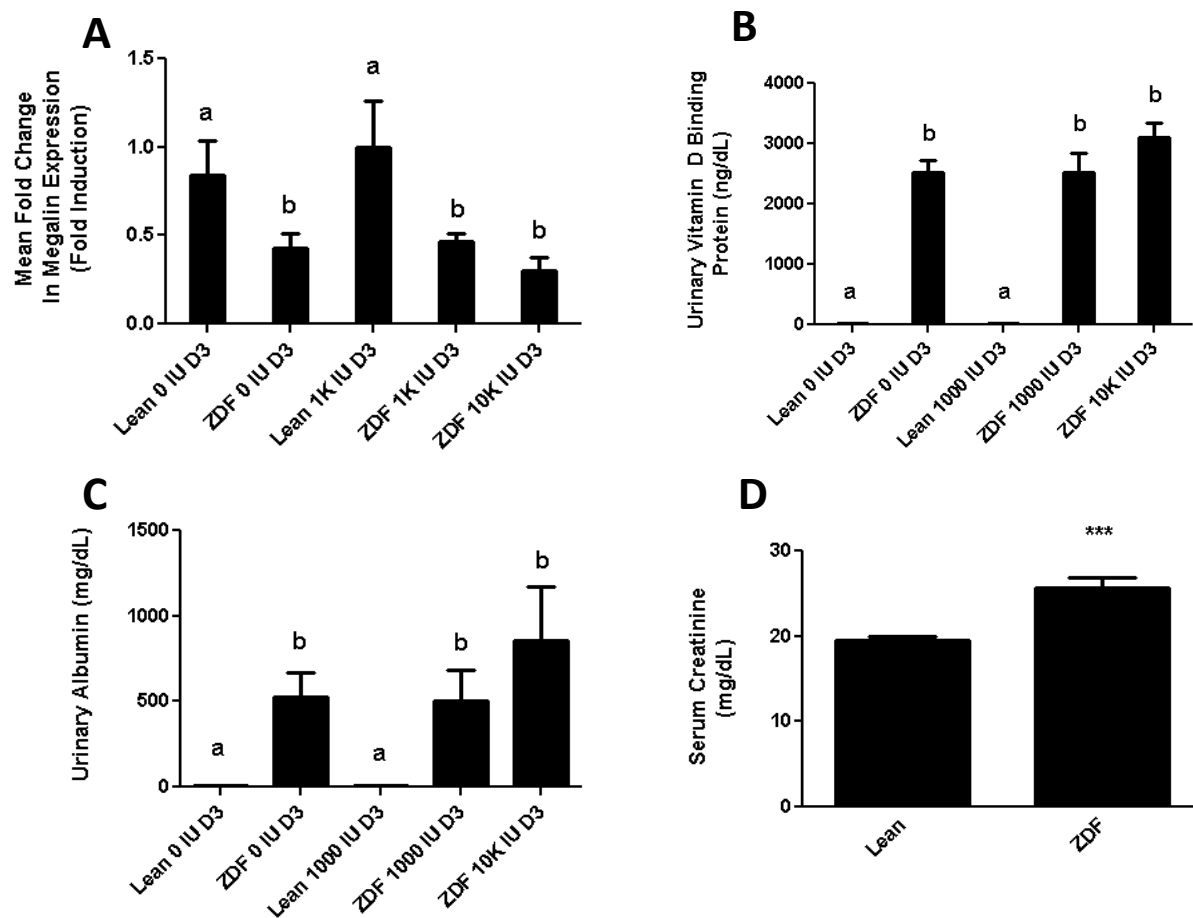


Fig. 9. Assessment of megalin expression, renal reabsorption of DBP and albumin, and kidney function in ZDF rats. Renal tissue and urine was collected from the same animals as described for Fig. 7. Renal megalin mRNA expression and urinary DBP and albumin concentrations were determined as described in Materials and Methods. A) Megalin mRNA abundance in kidney. B) Urinary DBP concentrations. C) Urinary albumin concentrations. Data are expressed as means \pm SEM ($n = 6$); bars with different letters are significantly different ($P < 0.05$). D) Serum creatinine levels of all lean and ZDF animals. Data are expressed as means \pm SEM ($n=12-18$); *statistically significant ($P < 0.05$).

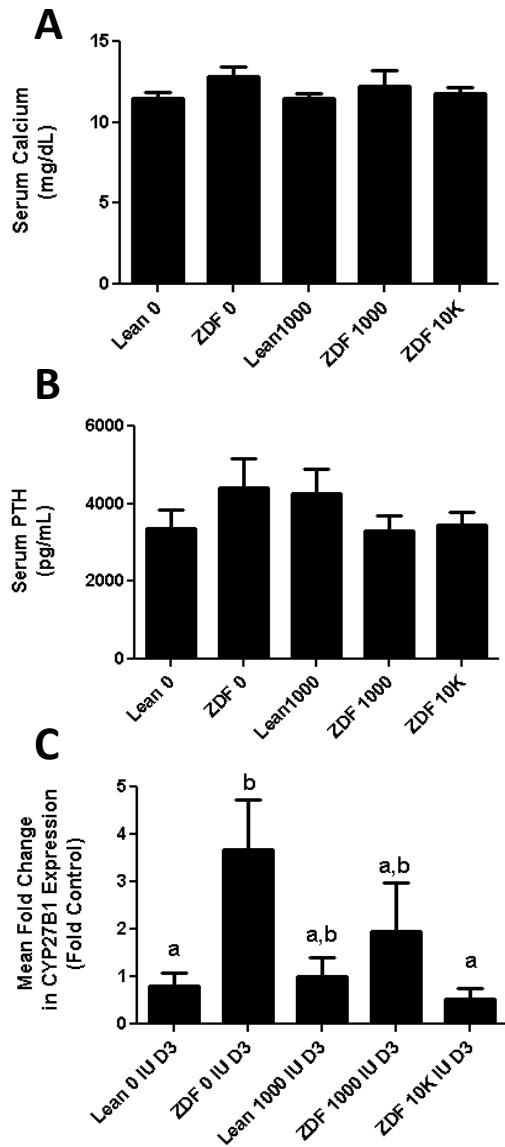


Fig. 10. The effect of NIDDM on serum calcium, serum PTH, and renal CYP27B1 expression. Serum calcium and parathyroid hormone concentrations were measured using and renal CYP27B1 expression was assessed by real-time PCR in the same animals as described for Fig. 7. A) Serum Calcium, B) serum PTH, and C) renal CYP27B1 expression in ZDF and lean control rats. Data are expressed as means ($n = 8$) \pm SEM; bars with different letters are significantly different ($P < 0.05$).

CHAPTER 3: GENERAL CONCLUSIONS

General Discussion

The clustering of complications arising out of NIDDM and/or the related metabolic syndrome show considerable overlap with research into “nonclassic” actions of vitamin D, specifically in its protective effects regarding cardiovascular disease and cancer. It is therefore not surprising to find that NIDDM patients tend to have suboptimal vitamin D status, and it is tempting to speculate that there may be a causal relationship existing between NIDDM, poor vitamin D status, and chronic diseases. However since there is no clear understanding of an underlying mechanism behind the NIDDM/vitamin D association (6, 21), it is unclear whether strategies for improving vitamin D status in NIDDM would provide the same protective effect on chronic disease risk that seems to be apparent in general populations. Therefore, elucidating this mechanism emerges as an important step in attempting to improve patient outcomes in NIDDM.

Since obesity and NDDIM are commonly associated, one proposed explanation behind suboptimal vitamin D status is that sequestration of 25D3 in adipose tissue is increased in NIDDM and decreases bioavailability of 25D3. Wortsman et al. (24) found that circulating serum 25D3 levels are reduced in obese versus non-obese subjects after either oral vitamin D supplementation or UVB skin exposure; however, neither urinary excretion of vitamin D nor kidney function were assessed. Furthermore, our results indicate that impaired renal function, specifically reduced proximal tubular reuptake of the vitamin D-DBP complex by megalin and its endocytic partner Dab2, contributes to reduced circulating

vitamin D in NIDDM. The reduced serum metabolites of vitamin D found in our diabetic rat model are consistent with many previous studies linking suboptimal vitamin D concentrations and NIDDM, obesity and metabolic syndrome in human populations (1, 4, 12, 17). Additionally, our rat model showed considerable proteinuria, including urinary DBP excretion. Albuminuria and microalbuminuria have been observed to occur more frequently in humans with metabolic syndrome or obesity (3, 11, 15). In early stage diabetic rats, megalin expression was reduced and marked albuminuria was observed, indicating that proximal tubule cell (PTC) reuptake impairment may occur early in disease progression (22). This impairment of protein reabsorption is known to predispose patients to glomerular hypertrophy and development of glomerulosclerosis (5, 8, 28). Moreover, the PTC itself is known to be a target for insulin (9, 16). In cultured opossum PTCs, megalin expression was decreased when PI3K inhibitors impaired insulin signaling. Additionally, angiotensin II receptor type 1 activation also decreased megalin expression (9), indicating RAS activation might also reduce PTC protein reabsorption. From its known inhibition of the RAS, vitamin D itself may be prevent glomerular degeneration (7, 13, 14, 19, 25, 27). In support of this concept, epidemiological studies indicate improved renal outcomes in CKD with 1,25D3 or analog treatment (2, 20, 26). Furthermore, diabetic mice with increased 1,25D3 production show protection from developing nephropathy normally seen in the disease (23). Taken together, it appears that maintaining proximal tubular reuptake and conversion of vitamin D may be an important target in preventing a progression of renal injury leading to reduced vitamin D status and disrupted renal vitamin D metabolism. Consistent with these studies, our findings highlight the role of the renal proximal tubule in maintaining overall vitamin D status, via megalin-mediated endocytosis of circulating DBP-bound vitamin D. This novel

concept in vitamin D homeostasis may be important in considering vitamin D requirements where known renal pathology exists or in populations at risk for developing renal complications, such as NIDDM.

Recommendations For Future Research

Although there is evidence that improved vitamin D status via oral supplementation improves insulin signaling in human NIDDM populations (10, 18), there have been no supplementation studies in these populations to assess long-term outcomes, such as cardiovascular disease or cancer. Additionally, although we demonstrated that megalin-mediated delivery of 25D3 to the PTC is reduced in NIDDM, we did not examine megalin expression in other tissues. Since other tissues sensitive to 25D3 action also require megalin and Dab2 for cellular delivery, research is needed to examine whether these tissues retain their ability to internalize and utilize circulating 25D3 in NIDDM. This knowledge would be important in assessing the physiological relevance of supplementation to improve serum 25D3 status, and the levels required to induce a protective effect.

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