Published in final edited form as:

Eur J Nutr. 2018 March ; 57(2): 723–730. doi:10.1007/s00394-016-1359-x.

Maternal vitamin D deficiency during pregnancy affects expression of adipogenic–regulating genes peroxisome proliferator–activated receptor gamma (PPAR γ) and vitamin D receptor (VDR) in lean male mice offspring

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Abstract

Purpose—Maternal vitamin D deficiency during pregnancy is a widespread issue that may have long-lasting consequences on offspring adiposity. We sought to determine how maternal vitamin D deficiency during the *perinatal* period would affect offspring adipose tissue development and gene expression.

Methods—Female C57BL/6 J mice were fed either a vitamin D deficient (VDD) or control diet from 4 weeks before pregnancy (periconception) until 7 days postparturition. Male offspring were weighed and euthanized at 75 days of age (early adult period), at which point serum was collected for biochemical analyses, and perigonadal and subcutaneous white adipose tissue (PGAT and SQAT, respectively) were excised, weighed, then flash-frozen for later histology and analyses of adipogenic gene expression.

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Conflicts of interest All authors declare that they have no conflicts of interest.

Compliance with ethical standards

Human and animals rights The animal experiments were approved by and in accordance with the University of Missouri Institutional Animal Care and Use Committee (IACUC, protocol 4031). Additionally, all procedures followed the NIH Guidelines for the Care and Use of Laboratory Animals.

Results—All adult male offspring were nonobese; there were no significant differences in body weight, adipose pad weight, or adipocyte size. However, VDD-exposed offspring had greater expression of the adipogenic-regulating genes peroxisome proliferator-activated receptor gamma (*Pparg*) and vitamin D receptor (*Vdr*).

Conclusions—This study suggests that exposure to vitamin D deficiency during the perinatal period can directly affect genes involved in the development of adipose tissue in non-obese offspring. These novel findings invite further investigation into the mechanisms by which maternal vitamin D status during pregnancy affects adipose development and metabolic health of offspring.

Keywords

Vitamin D; Pregnancy; Adipogenesis; Gene expression; Fetal programming; DOHaD

Introduction

Maternal vitamin D deficiency during pregnancy is a major global public health concern. Recent data suggest that up to 82% of pregnant women in the USA have suboptimal (<32 ng/ml) circulating concentrations of 25-hydroxyvitamin D (25(OH)D), the vitamin D metabolite used to assess status [1]. Vitamin D deficiency during pregnancy has been linked with adverse health outcomes, such as gestational diabetes and insulin resistance in the mother [2, 3], and several other negative effects in the offspring, including low birth-weight and autoimmune disease [4–8].

It is well established that maternal nutrition impacts the intrauterine environment, and consequently, the fetal epigenome [9, 10]. Alterations in the expression of specific genes during this period of fetal growth and development can potentially lead to lifelong health consequences for the offspring [11]. To date, the majority of studies examining the role of nutrition in fetal programming have focused specifically on macronutrients and total energy intake. However, it has become increasingly clear that alterations in individual micronutrients, such as vitamin D, have the ability to cause the epigenetic modifications that characterize fetal programming [12]. Among the proposed health consequences influenced by maternal nutrition are obesity and the metabolic complications that arise largely due to the chronic low-grade inflammation in white adipose tissue (WAT) that accompanies obesity [13–15].

Obesity is a disease of multiple etiologies that is characterized by excess adiposity. Increases in fat mass may occur via either the growth of existing adipocytes (hypertrophy) or the formation of new adipocytes (adipogenesis), the latter occurring largely during fetal development and early life [14]. While the production and secretion of inflammatory chemokines and cytokines that accompany adipocyte expansion are most often associated with the adverse consequences of obesity on health, increases in adipocyte number early in life lay the foundation for greater increases in fat mass. Therefore, exploring factors that may modify the adipogenic process may be of great importance in combating obesity.

Over 100 transcriptional factors have been identified in adipocytes, of which several have been implicated in regulating aspects of adipogenesis [16]. The factors considered most

influential include peroxisome proliferator-activated receptors- γ (PPAR γ) and CCAAT/enhancer-binding proteins α and β (C/EBP α & β) [17, 18]. The nuclear factor PPAR γ is well established as a prime regulator of adipogenesis, as it is the convergence point of most adipogenic pathways [18, 19]. Additionally, it is the only known factor that can independently induce adipogenesis in the absence of other transcription factors. Mice lacking one copy of the PPAR γ gene (i.e., are heterozygous) demonstrate hypoplasia and smaller adipocytes [20]. C/EBPs are a family of binding proteins that serve as transcriptional factors. Three members of the family (C/EBP- α , β , and δ) act in concert with PPAR γ to exert key roles in promoting the differentiation of preadipocytes to adipocytes, each acting at different time points during the adipogenic process [19, 21, 22]. Previous studies reveal that reduced expression of any of the three adipogenic-C/EBPs greatly inhibits adipogenesis [23–26].

The vitamin D receptor (VDR) and its ligand, 1,25-dihydroxyvitamin D, have also been shown to affect adipocyte proliferation [27, 28]. However, the specific effects and mechanisms by which VDR signaling exerts its adipogenic control are unclear, as in vivo and in vitro studies reveal both stimulatory and inhibitory effects [28–30]. Furthermore, the majority of in vivo studies aiming to examine the connections between VDR and adipogenic genes have utilized a VDR-knockout model, which may not fully replicate the consequences of naturally occurring vitamin D deficiency [31–33].

Although the importance of vitamin D receptor signaling in normal fetal and adipose tissue development is undeniable, little is known about how maternal status impacts offspring. In the current pilot study, we sought to use a mouse model to: (1) explore the impact of maternal vitamin D deficiency during the perinatal period on the development of adipose tissue in male offspring; (2) elucidate the potential mechanisms by which vitamin D exerts its adipogenic control by examining several genes associated with adipogenesis and adipose function; (3) investigate how potential vitamin D-mediated changes in adipose tissue development and function impact the systemic inflammation linking obesity to metabolic abnormalities.

Materials and methods

Animals and diet

The animal experiments were approved by and in accordance with the University of Missouri Institutional Animal Care and Use Committee (IACUC, protocol 4031). All procedures followed the NIH Guidelines for the Care and Use of Laboratory Animals. All experiments were conducted as part of a larger study examining the effects of maternal vitamin D deficiency during pregnancy on neurological development and behavior of offspring. Twelve-week-old female C57BL/6 J mice acquired from Jackson Labs (Jackson Labs; Bar Harbor, ME) and raised on control diet (TD.10695; Harlan Teklad; Madison, WI) were randomly assigned to either a vitamin D deficient diet (TD.10694; Harlan Teklad; Madison, WI) or control diet (Table 1) three weeks prior to mating and maintained on this diet throughout pregnancy up until postnatal day 7, at which point dams from the vitamin D deficient group were switched to the control diet. In order to prevent hypocalcaemia secondary to vitamin D deficiency, both diets contained increased amounts of calcium and

phosphorous relative to standard AIN-93G (Table 1). Peripheral maternal vitamin D status was assessed 5 days after parturition and at time of euthanasia (approximately 10 weeks after returning to the control diet) via serum sampling to confirm the intended effect of the diet provided (data not shown). At weaning (postnatal day 21), male offspring ($n = 8$ per each maternal diet group) were maintained on a control diet until (postnatal day 75); whereupon, they were weighed and humanely euthanized for tissue collection.

Tissue collection

Following euthanasia, subcutaneous white adipose tissue (SQAT), perigonadal white adipose tissue (PGAT), and liver were excised from adult male offspring and weighed. A portion of each depot was either A) flash-frozen in liquid nitrogen for later RNA extraction and gene expression analysis or B) placed in neutral-buffered 10% formalin for histology. Serum was collected and stored at -80°C until analysis.

RNA extraction and cDNA synthesis

Perigonadal adipose tissue samples ($n = 5$ per group) were homogenized in QIAzol reagent (Qiagen Inc.; Valencia, CA). Total RNA was then isolated by RNeasy lipid tissue kit (Qiagen; Valencia, CA). RNA purity and concentration were assessed by Nanodrop spectrophotometer (ThermoScientific; Wilmington, DE). cDNA was synthesized from RNA using high-capacity cDNA reverse transcription kit (Applied Biosystems; Carlsbad, CA).

Real-time PCR

Quantitative real-time PCR (qPCR) was performed using the ABI StepOne Plus (Applied Biosystems; Carlsbad, CA). Primer sequences (Table 2) were designed using the NCBI Primer Design tool and checked using BLAST. Primers were obtained from the University of Missouri DNA Core (Columbia, MO). 10 μl of Maxima SYBR Green/ ROX qPCR Master Mix (ThermoScientific; Wilmington, DE), an appropriate amount of gene-specific primers, and 2.5 μl of cDNA template were loaded onto a 96-well plate in duplicate. PCR was performed with the thermal conditions according to manufacturer recommendations. Dissociation melt curve analyses were performed to verify the specificity of the PCR products. The expression levels of *Vdr*, *Cebpa*, *Pparg*, and *Srebf1* were compared using the $2^{-\text{CT}}$ method relative to *Actb*.

Histological analysis

Formalin-fixed samples from perigonadal adipose tissue ($n = 8$ per group) were sent to IDEXX BioResearch (Columbia, MO) for processing. Samples were embedded in paraffin, sectioned into 5 μm segments, and stained with hematoxylin and eosin for morphometric determinations. Sections were visualized by an Olympus BX60 photomicroscope (Olympus; Melville, NY) and photographed at 10 $\times$ magnification using a Spot Insight digital camera (Diagnostic Instruments; Sterling Heights, MI). At least five fields of view were selected for each sample, from which the diameters of 100 adipocytes were measured using ImageJ software (National Institute of Health; Bethesda, MD). The average value of these 100 adipocytes was used to represent each mouse.

Blood biochemistry

Whole blood was collected in serum-separator tubes by cardiac puncture immediately following euthanasia, centrifuged according to manufacturers' instructions, and stored at -80°C . Circulating serum 25(OH)D concentrations were determined by ELISA (Immunodiagnostik; Germany). Fasting insulin and circulating concentrations of cytokines, chemokines, and adipokines were determined by a commercially available multiplex kit (Millipore Milliplex; Billerica, MA) on a MAGPIX instrument (Luminex Technologies; Austin, TX).

Statistical analysis and presentation

For all outcome variables, a Student's *t* test was used to test for differences between experimental groups. In order to control for false discovery rate (FDR) due to multiplicity, the Benjamini–Hochberg procedure was performed post hoc using *PROC MULTTEST*. Data are represented as mean $\pm$ SE. *p* values represent raw values obtained from *t* test. All analyses were carried out using SAS 9.2 statistical software (Cary, NC).

Results

Body and adipose weight

All animals were nonobese; there was no significant difference in total body weight on postnatal day 75 ($p = 0.837$; Table 3). Additionally, there were no differences in the weight of either SQAT or PGAT between the two groups ($p = 0.510$ and $p = 0.388$, respectively; Table 3). Likewise, the ratio of PGAT to SQAT did not differ between male offspring born to vitamin D deficient dams relative to controls (1.61 vs. 1.71; $p = 0.869$). PGAT mean adipocyte size also did not differ between the two groups ($p = 0.695$).

Adipogenic gene expression

Analysis of PGAT mRNA expression showed that 75-day-old male mice born to vitamin D deficient dams had greater expression of the *Vdr* (1.73-fold increase; $p = 0.032$) and *Pparg* (1.49-fold increase; $p = 0.043$; Fig. 1). However, there were no differences in the expression of *Srebf1* or *Cebpa* between the two groups ($p > 0.05$). Similarly, there were no differences in the expression of resistin (*Retn*), leptin receptor (*Lepr*), or adiponectin (*Adipoq*) mRNA between the two groups ($p > 0.05$).

Blood biochemistry

Male mice born to vitamin D deficient dams did not have any significant differences in circulating concentrations of serum fasting insulin or leptin relative to controls ($p = 0.274$ and $p = 0.889$, respectively; Table 4). While there were no differences in the majority of cytokines and chemokines measured, levels of both resistin and IL-2 were significantly elevated in males born to vitamin D deficient dams compared to controls ($p = 0.036$ and $p = 0.042$, respectively; Table 4).

Discussion

This investigation sought to explore the impact of maternal vitamin D deficiency during pregnancy on adipogenesis and associated inflammation, as well as adipogenic gene expression, in male offspring. We found that although there were no differences in total body weight, individual fat pad weight, or adipocyte size, there were differences in PGAT *Pparg* and *Vdr* mRNA expression in adult male offspring born to vitamin D deficient dams relative to controls. This novel finding suggests that maternal vitamin D status during pregnancy may have lasting effects on factors regulating adipose tissue development in subsequent adult offspring.

Visceral adipose tissue is often considered to be more metabolically significant than subcutaneous adipose tissue [34–36]; therefore, the visceral fat pad, PGAT, was used to examine the expression of several genes known to be important in adipogenesis and adipocyte function. Of the seven measured, only *Pparg* and *Vdr* were differentially expressed. The increased expression of PPAR γ in the offspring of vitamin D deficient dams is a particularly remarkable observation as it is widely considered to be *the* primary driver of adipogenesis, a highly complex process involving myriad transcriptional factors [37]. Hara et al. [38] described PPAR γ as a “thrifty gene” based on their findings that heterozygous PPAR γ -deficient mice were protected from high-fat diet-induced obesity and adipocyte hypertrophy. Furthermore, the role of PPAR γ as an insulin sensitizer is well documented and led to the development of a class of drugs (thiazolidinediones) designed to improve insulin sensitivity through direct activation of the receptor [39]. There is evidence suggesting that one of the ways in which these drugs improve insulin sensitivity is through the stimulation of adipogenesis, which produces smaller, more insulin-sensitive adipocytes [40]. The result of this, however, is that these adipocytes are susceptible to hypertrophy and possess the same harmful potential.

While not considered one of the key regulators, the importance of VDR signaling in adipogenesis is undeniable, as evidenced by the observation that VDR knockout mice fail to develop grossly detectable amounts of adipose tissue [31]. In vivo studies have shown that 1,25-dihydroxyvitamin D, the bioactive form of vitamin D and VDR ligand, upregulates the expression of VDR in multiple tissues by acting as a protein stabilizer [41, 42]. Increases in *VDR* in response to treatment with 1,25-dihydroxyvitamin D have also been observed in human adipose tissue explants; however, the degree of upregulation appears to be dependent on the degree of obesity [43]. The observed increase in PGAT *Vdr* expression of mice born to vitamin D deficient dams was surprising, as the offspring had circulating concentrations of 25(OH)D that were sufficient, and therefore, likely had sufficient concentrations of 1,25-dihydroxyvitamin D as the two are highly correlated [44, 45]. Several in vivo studies using preadipocyte cell lines have shown that VDR-mediated inhibition of adipogenesis is associated with decreases in both PPAR γ expression and PPAR γ transactivating activity [29, 46]. Additionally, there is strong proof of cross talk and reciprocal inhibition between VDR and PPAR γ , as both require heterodimerization with retinoic x receptor (RXR) in order to exert their activity through response element binding [47, 48].

Our findings that visceral adipose tissue of male offspring born to vitamin D deficient mothers have increased expression of *PPAR γ* and *Vdr* should be interpreted in the context of the animal model used. The lack of changes to total body weight or adipose tissue pads that we observed were not surprising, as the mice were young and lean. While the model is susceptible to diet-induced obesity, the C57BL/6 J mouse does not typically develop obesity on a normal caloric diet [49]. The two diets used in the current study were isocaloric, differing only in micronutrient composition. It is possible that had we used a model prone to obesity or a hypercaloric diet, the differences in *Pparg* and/ or *Vdr* expression would stimulate greater adipose tissue mass in male offspring born to Vitamin D deficient females.

Vitamin D metabolites are known to influence adipokine production and the inflammatory response in adipose tissue [50–52]. As adipocytes expand, they produce and secrete several inflammatory cytokines and chemokines such as TNF- α , interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and leptin [53, 54], and there is evidence for chronic inflammation as the causal link between obesity and its related metabolic conditions [55]. Therefore, these molecules were measured in our study to indirectly assess the indirect effects of the metabolic health of offspring adipose tissue. The observed absence of widespread inflammation, like the absence of effects on body or adipose pad weights, is also likely due to the animal model we used. Notwithstanding, we noted greater serum concentrations of resistin and IL-2 in the offspring of vitamin D deficient mothers. Similar to leptin, circulating concentration of the adipocyte-derived protein resistin increases proportionally with adiposity [56]. In both rodent models and humans, resistin has also been associated with the progression of insulin resistance. However, the degree to which it influences the development of insulin resistance in humans is uncertain, with current evidence suggesting a likely indirect effect [57–59]. The relationship between vitamin D and resistin is unclear, as studies have shown both positive and negative associations [60, 61]. As mentioned above, the animals in the current study were all lean and vitamin D sufficient, and thus, the observed increases in circulating resistin concentrations were surprising. We do not currently have a good explanation for this finding. The pro-inflammatory cytokine IL-2 is primarily associated with T-cell function and protection against autoimmune disease [62, 63]. While a positive association between obesity and circulating concentrations of IL-2 has been noted, IL-2's role in obesity or its subsequent metabolic complications remain elusive. Previous studies have demonstrated that VDR signaling inhibits the expression of *IL2* in T-cells [64]. Since the mechanisms governing these processes are dependent on the formation of a VDR-RXR heterodimer, the local availability of 1,25-dihydroxyvitamin D is essential. As the offspring were vitamin D sufficient, it is unlikely that this was a complicating factor.

The primary limitation of this pilot study was the small group sizes. Another key limitation of our study was the lack of protein expression quantification, which we were unable to perform due to limited adipose tissue collected from these nonobese animals. Future experiments should use animal models of obesity to determine if the modest but significant changes we observed in adipogenic gene expression translates to significant metabolic health consequences in offspring exposed to vitamin D deficiency during development. Investigations should also focus on whether alteration of these genes may be considered biomarkers for individuals at risk for obesity due to maternal nutritional changes. Continued research efforts will aim to establish the mechanisms by which fetal exposure to vitamin D

deficient environments impacts adipose tissue development. Additionally, due to the fact that this was part of a larger experiment with the primary overarching goal to examine in utero programming risk for later obesity in males, only this sex was available to us for the current suite of studies. In future work, we will examine whether male vs female offspring demonstrate different susceptibility to maternal vitamin D deficiency.

In conclusion, we show for the first time that perinatal vitamin D deficiency, an easily correctable state impacting a considerable fraction of human pregnancies, can have lasting effects on the expression of important regulators of adipogenesis, namely PPAR- γ and VDR. The observed changes did not correlate with any obvious risks to the metabolic health of the nonobese offspring at the time they were examined. However, they do demonstrate the potential long-lasting consequences of inadequate vitamin D perinatal exposure, which may be a critical period in terms of metabolic programming.

Acknowledgments

Funding This work was funded by the Department of Nutrition & Exercise Physiology and the University of Missouri SIRC research grant.

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Fig. 1.

Adipogenic mRNA expression in PGAT of male offspring born to control or vitamin D deficient dams. The expression levels were quantified by RT-PCR. Gene expression was normalized using β -actin as an internal control. Data are expressed as the fold difference relative to male offspring of dams fed control diet during pregnancy. Vdr, vitamin D receptor; PPAR γ , peroxisome proliferator-activated receptor gamma; C/EBP α , CCAAT/enhancer-binding protein alpha; SREBP1, sterol regulatory element-binding transcription factor 1. Values are means, and *error bars* are SEM ($n = 5$ per group). *between group $p < 0.05$

Table 1

Composition of diets

Nutrient	Control	D deficient
Phosphorous	0.6%	0.6%
Calcium	1.00%	1.00%
Vitamin D	1.0 IU/g	No added
Protein	19.6% kcal	19.6% kcal
CHO	59.4% kcal	59.4% kcal
Fat	16.9% kcal	16.9% kcal
Energy density	3.7 kcal/g	3.7 kcal/g

Table 2

RT-PCR Primers

Gene	Forward 5' → 3'	Reverse 5' → 3'
<i>Vdr</i>	GACCGCCTATCCAAACACACT	ATCTCATTGCCGAACACCTC
<i>Pparg</i>	GCATGGTGCCTTCGCTGA	TGGCATCTCTGTGTCAACCATG
<i>Srebf1</i>	CACTCCCTCTGTATGCTACGG	CTTGTTTGGCGATGTCTCCAG
<i>Cebpa</i>	AGAAAGACGGTGGACAAGCT	GGGCAGCTGCTTTGAACAAGTT
<i>Retn</i>	GTACCCACGGGATGAAGAACC	GCAGACCCACAGGAGCAG
<i>Lepr</i>	GTGTGAGGAGGTACGTGGTG	TGGACTGTTGGGGAAGTTGGTAG
<i>Adipoq</i>	GACACCAAAAGGGCTCAGGAT	TGGGCAGGATTAAAGAGGAACA
<i>Actb</i>	ACGGCCAGGTCATCACTATTG	TGGAAAAGAGCCCTCAGGGC

Vdr Vitamin D receptor, *Srebf1* sterol regulatory element-binding transcription factor 1, *Cebpa* CCAAT/enhancer-binding protein alpha, *Retn* resistin, *Lepr* leptin receptor, *adipoq* adiponectin, *Actb* beta-actin

Table 3

Effect of maternal vitamin D deficiency on offspring body weight, tissue weight, adipocyte size, and serum 25(OH)D of male offspring

	Control	D deficient	<i>p</i> value
Whole-body weight (g)	26.3 ± 2.5	25.7 ± 1.4	0.465
SQAT weight (mg)	325 ± 67	306 ± 69	0.510
PGAT weight (mg)	556 ± 196	494 ± 132	0.388
Liver weight (mg)	753 ± 257	721 ± 223	0.754
Mean adipocyte area (µm ²)	3162 ± 507	3381 ± 439	0.711
Serum 25 (OH) D (nmol/L)	44.6 ± 2.7	43.9 ± 3.2	0.546

Data are represented as mean ± SE

Table 4

Between group differences in offspring circulating insulin, inflammatory cytokines, and chemokines

	Control	D deficient	P value
Serum insulin (ng/ml)	0.83 ± 0.12	1.1 ± 0.19	0.274
Serum leptin (ng/ml)	3.6 ± 0.7	3.7 ± 0.6	0.889
Serum resistin (ng/ml)	4.9 ± 0.7	7.3 ± 0.8	0.036 [*]
Serum M-CSF (pg/ml)	0.19 ± 0.13	0.23 ± 0.11	0.280
Serum MCP-1 (pg/ml)	25.2 ± 5.3	32.4 ± 4.6	0.315
Serum PAI-1 (ng/ml)	1.3 ± 0.14	1.54 ± 0.12	0.126
Serum TNF-α (pg/ml)	11.4 ± 1.3	12.0 ± 1.4	0.273
Serum IFN-γ (pg/ml)	8.3 ± 2.5	20.7 ± 11.6	0.320
Serum IL-1a (pg/ml)	169.4 ± 17.7	151.5 ± 20.3	0.514
Serum IL-1b (pg/ml)	0.61 ± 0.18	0.74 ± 0.16	0.601
Serum IL-2 (pg/ml)	1.19 ± 0.14	2.19 ± 0.46	0.042 [*]
Serum IL-4 (pg/ml)	0.52 ± 0.12	0.64 ± 0.21	0.634
Serum IL-6 (pg/ml)	8.5 ± 1.8	8.1 ± 1.2	0.851
Serum IL-10 (pg/ml)	1.67 ± 0.73	1.73 ± 0.94	0.957

Data are represented as mean ± SE

^{*} Indicates a significant between group difference ($p < 0.05$)