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Vitamin D deficiency decreases adiposity in rats and causes altered expression of uncoupling proteins and steroid receptor coactivator3

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ABSTRACT

The vitamin D endocrine system is functional in the adipose tissue, as demonstrated *in vitro,* in cultured adipocytes, and *in vivo* in mutant mice that developed altered lipid metabolism and fat storage in the absence of either 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] or the vitamin D receptor. The aim of the present study was to examine the role of vitamin D and calcium on body adiposity in a diet-induced vitamin D deficient rat model. Vitamin D-deficient rats gained less weight and had lower amounts of visceral fat. Consistent with reduced adipose tissue mass, the vitamin D-deficient rats had low circulating levels of leptin, which reflects body fat stores. Expression of vitamin D and calcium sensing receptors, and that of genes involved in adipogenesis such as peroxisome proliferator-activated receptor, fatty acid synthase and leptin were significantly reduced in white adipose tissue of deficient rats compared to vitamin D-sufficient rats. Furthermore, the expression of uncoupling proteins (Ucp1 and Ucp2) was elevated in the white adipose tissue of the deficient rat indicative of higher energy expenditure, thereby leading to a lean phenotype. Expression of the p160 steroid receptor coactivator3 (SRC3), a key regulator of adipogenesis in white adipose tissue was decreased in vitamin D-deficient state. Interestingly, most of the changes observed in vitamin D deficient rats were corrected by calcium supplementation alone. Our data demonstrates that dietary vitamin D and calcium regulate adipose tissue function and metabolism.

1. Introduction

The biologically active hormonal form of vitamin D, $1,25(OH)_2D_3$ acts through the vitamin D receptor (VDR) [1], a member of the super family of nuclear receptors and transcription factors [1]. The principal function of $1,25(OH)_2D_3$ is maintenance of calcium homeostasis in the body, but the presence of VDR in several cell types that do not regulate calcium metabolism including keratinocytes, monocytes, brain glial cells and pancreatic β cells, implies that the hormone is involved in a broader spectrum of biological processes [2,3]. Adipose tissue is yet another target tissue which expresses VDR and responds to its ligand $1,25(OH)_2D_3$ [4,5]. For instance, *in vitro* studies using primary human adipocytes or mouse 3T3-L1 cell lines reported both stimulation and inhibition of adipogenesis by $1,25(OH)_2D_3$ [6,7]. Stimulation of adipocyte differentiation by $1,25(OH)_2D_3$ was underscored by the finding that VDR knockdown leads to inhibition of adipogenesis [4]. Gene array profiling of human pre-adipocytes demonstrated that $1,25(OH)_2D_3$ regulates genes involved in apoptosis, proliferation and inflammation [8]. However, only few studies examined the role of VDR and its ligand in adipocyte biology *in vivo*. One study with VDR knock-out (VDR-KO) mice demonstrates that lack of VDR results in lean phenotype and resistance to high-fat diet-induced obesity [9]. This study also reported similar changes in adiposity of mice lacking the Cyp27b1 gene (that encodes the enzyme needed for conversion of 25-hydroxyvitamin D3 to 1,25 $(OH)_2D_3$), suggesting that both VDR and its ligand play an important role in adipose tissue function. Another study with VDR-KO mice demonstrated the role of vitamin D in energy expenditure by regulation of uncoupling proteins [10].

Dietary calcium has also been reported to play a role in body adiposity [11,12]. Studies in vitamin D-sufficient rats have shown that low calcium diet increases adiposity [13]. Furthermore,

Abbreviations: AI, adiposity index; $[Ca^{+2}]i$, intracellular Ca^{+2} concentration; CaSR, calcium-sensing receptor; FFM, fat free mass; LBM, lean body mass; RIA, radioimmunoassay; SRC, steroid receptor coactivator; SREBP, sterol regulatory element binding protein; TAF, tissue associated fat; TOBEC, total body electrical conductivity; UCP, uncoupling protein; VDR, vitamin D receptor; WAT, white adipose tissue; $25(OH)D_3$, 25-hydroxycholecalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D.

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clinical studies in vitamin D-sufficient subjects demonstrate that high calcium diets decrease adiposity and this has been attributed to inhibition of systemic levels of $1,25(OH)_2D_3$, which causes a decrease in intracellular $[Ca^{+2}]i$ in adipocytes and an increase in lipolysis [11]. However, there are no published studies on the effect of a high calcium diet in vitamin D deficiency.

Nuclear receptors, including the VDR, regulate transcription through interaction with coactivators [14,15]. Since members of the nuclear receptor family (PPARγ, liver X receptor (LXR) regulate fat metabolism, the role of coactivators in this physiological process was also studied. For instance, gene ablation studies have shown that the steroid receptor coactivator-3 (SRC3), a member of the p160 family of nuclear receptor coactivators plays a critical role in white adipose tissue differentiation and function [16] However, expression and contribution of nuclear receptors coactivators in adipose tissue function has not been studied in the context of dietary changes in vitamin D and calcium. The goal of this study was to determine if the adiposity changes occur without genetic manipulation in a diet-induced vitamin D deficient rat model and to assess the effect of calcium *per se* in the absence of vitamin D on adiposity related parameters, including expression of nuclear receptors and their coactivators. Our data indicates that vitamin D deficiency accompanied by hypocalcaemia leads to reduced body adiposity which was associated with (a) alterations in adipokines and adipogenic genes, including Pparγ and Fasn, (b) up regulation of the uncoupling proteins (Ucp1 and Ucp2), and (c) down regulation of SRC3 protein. All the changes measured in the deficient state are reversed by supplementation with vitamin D, whereas high calcium diet partially corrected them.

2. Materials and methods

2.1. Animal experimentation

Sprague Dawley male weanling rats ($n = 24$) were obtained from the National Center for Laboratory Animal Sciences (NCLAS) at the National Institute of Nutrition, Hyderabad, India. The experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publ No: 85-23) and with the approval of the 'Institute 's Ethical Committee on Animal experiments' at the National Institute of Nutrition, Hyderabad, India. Rats were housed individually in wire mesh – bottomed polypropylene cages, and maintained under incandescent lighting conditions (12 h light/dark cycles). Temperature and relative humidity were kept constant at $22 \pm 2\,^{\circ}C$ and $55 \pm 10\%$, respectively. Rats were randomly divided into two groups of 6 and 18 and feeding was initiated at weanling (21 days old). One group (Con; $n = 6$) was fed ad libitum on a synthetic AIN-93 based formulation control diet (65 kcal% carbohydrate, 20 kcal% protein, 15 kcal% fat with 0.57% Ca, 0.43% P and 1000 IU/kg of vitamin D_3), while the other group (Def; $n = 18$) was given the same diet but devoid of any vitamin D_3. The diets were procured from Research Diets Inc., New Jersey, USA. Animals were provided free access to diet and deionized distilled water. The animals were fed initially for 12 weeks on their respective diets. Food intake and body weights were monitored daily and weekly respectively. The vitamin D deficient status of the rats was confirmed by measuring the serum 25-hydroxycholecalciferol $[25(OH)D_3]$ and calcium levels, after which the rats in the deficient group were further subdivided into three groups: one group (Def; $n = 6$) was continued on the deficient diet, the second group (SD; $n = 6$) was shifted onto the control diet containing vitamin D and the third group (HCa; $n = 6$) was shifted to a rescue diet with high calcium (4.97% Ca and 3.74% P) devoid of vitamin D_3. The Ca/P molar ratio was maintained at 1.03 in all the diets used for the study. The initial group of control rats (Con; $n = 6$) continued to be on the control diet till the end of the experiment. All the four groups of rats were fed on the respective diets for a further 6 weeks. At the end of 18 weeks post-weaning (21 weeks of age) the serum $25(OH)D_3$ and calcium levels were checked again and rats were sacrificed by CO_2 inhalation; blood, liver and different fat pads (retroperitoneal, epididymal and mesenteric) were collected, weighed and then frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ till processed.

2.2. Serum parameters

Fasting blood samples were collected on the day of sacrifice, serum separated and stored at $-80\,^{\circ}C$ until analysis. Serum calcium was measured using atomic absorption flame emission spectrophotometer (AAS-7000 Shimadzu, Japan) using oxy-acetylene flame [17]. Serum $25(OH)D_3$ levels were measured by HPLC [18], while serum $1,25(OH)_2D_3$ levels were measured by an ELISA kit (Immunodiagnostics (IDS), Ltd., UK), serum leptin measured by rat leptin kit (Merck Millipore, USA) and serum insulin was measured by RIA using a kit (BRIT-DAE, Mumbai, India). Serum adiponectin was done using Lincoplex research kit (Linco Research, St. Louis, MO) on a BIOPLEX platform (Bio-Rad). Total cholesterol, HDL cholesterol and triglyceride levels were determined in plasma using enzymatic assay kits from Biosystems (Barcelona, Spain).

2.3. Total body electrical conductivity (TOBEC)

The lean body mass (LBM) was determined at 21 weeks of age using TOBEC- small animal body composition analysis system (EM-SCAN, Model SA-3000 Multi detector), an instrument which determines total body electrical conductivity of small animals in a non-destructive manner. The difference between the impedance measured when the animal is inside the electromagnetic field and when the chamber is empty is an index of the total electrical conductivity of the body, which in turn is proportional to the animal's LBM. The LBM and Fat free mass (FFM) were computed mathematically [19].

2.4. Adiposity index (AI) and tissue associated fat (TAF)

AI, an index of visceral adiposity, was computed mathematically [20]. The wet weights of the retroperitoneal/perirenal, mesenteric, and epididymal/gonadal fat pads were determined, and the adiposity index was computed using the following formula: adiposity index = (sum of the weights of the three fat depots/body weight) × 100. The LBM is the weight of the animal excluding visible fat and FFM is the weight without any form of fat (i.e., visible and tissue associated). Therefore, the difference between LBM and FFM gives an estimate of the TAF. Both LBM and FFM were calculated after TOBEC analysis. Total fat was calculated as the difference between the body weight and LBM.

2.5. Histology of WAT, liver and adipose tissue triglyceride mass

Retroperitoneal fat tissue and liver samples were collected at necropsy and preserved in 10% buffered (pH 7.4) neutral formalin. Paraffin wax sections (4–5 μm) were prepared from pieces of retroperitoneal fat pad using a microtome (Leica RM 2155). Sections were deparaffinised, rehydrated, and stained with hematoxylin/eosin. Adipocyte number and area were measured using an Olympus 1X-51 inverted microscope and captured by Jenoptik CCD digital camera (prog RESR C3) at 40X magnification. Total number of cells in ten different fields was counted and average was taken. Similarly, area of individual cells from ten different fields was measured using ProgRes® capture pro software

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