1,25-Dihydroxyvitamin D₃ upregulates leptin expression in mouse adipose tissue

Juan Kong¹,², Yunzi Chen¹,², Guojun Zhu¹, Qun Zhao³ and Yan Chun Li¹,²

¹Laboratory of Metabolic Disease Research and Drug Development, Shengjing Hospital, China Medical University, Shenyang, People's Republic of China
²Division of Biological Sciences, Department of Medicine, The University of Chicago, 900 East 57th Street, KCBD 9110, Chicago, Illinois 60637, USA
³Ministry of Health Key Laboratory of Congenital Malformation, Shengjing Hospital, China Medical University, 36 Sanhao Street, Heping District, Shenyang 110000, People's Republic of China

Abstract

Leptin is an adipose tissue-derived hormone that plays a critical role in energy homeostasis. Vitamin D has been shown to regulate energy metabolism, but the relationship between vitamin D and leptin is unclear. Leptin expression and secretion was reduced in vitamin D receptor (VDR)-null mice and increased in transgenic (Tg) mice overexpressing the VDR in adipocytes; however, as leptin is mainly determined by fat mass, it is unclear whether the vitamin D hormone directly regulates leptin expression. To address this question, we determined the effect of vitamin D on leptin expression in vivo and ex vivo. One-week treatment of WT mice with the vitamin D analog RO-27-5646 led to a significant increase in adipose leptin mRNA transcript and serum leptin levels. Moreover, in adipose tissue cultures, 1,25-dihydroxyvitamin D markedly stimulated mRNA expression and secretion of leptin, but not resistin, in adipose tissues obtained from WT mice, but not from VDR-null mice, and leptin upregulation induced by 1,25-dihydroxyvitamin D was more robust in adipose tissues obtained from VDR Tg mice compared with WT mice. These data demonstrate that 1,25-dihydroxyvitamin D stimulates adipose leptin production in a VDR-dependent manner, suggesting that vitamin D may affect energy homeostasis through direct regulation of leptin expression.

Introduction

White adipose tissue not only serves the purpose of an energy store but also functions as an endocrine organ for the regulation of energy homeostasis. Adipocytes release an array of endocrine signals that are integrated into the metabolic regulatory circuit and influence energy intake, storage and expenditure, and insulin sensitivity (Fruhbeck et al. 2001, Tilg & Moschen 2006). One important hormone secreted by the white adipose tissue is leptin, a 16 kDa peptide that plays a central role in energy homeostasis (Zhang et al. 1994). Leptin acts on its receptor in the hypothalamus to curb appetite and increase energy expenditure when body fat stores increase (Schwartz et al. 2000). As such, the level of plasma leptin is positively correlated with the body's adipose mass (Mantzoros 1999). High leptin in the circulation signals excessive energy storage to the CNS to suppress food intake and increase energy consumption. In addition, leptin is involved in the regulation of other functions...
such as reproduction and immunity (Donato et al. 2011, Procaccini et al. 2012).

The vitamin D hormone is a pleiotropic hormone that has a broad range of physiological functions (Bouillon et al. 2008). The active form of vitamin D is 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), which activates the vitamin D receptor (VDR), a member of the nuclear receptor superfamily, to exert biological activities (Haussler et al. 1998). The VDR is widely expressed in the body, including tissues not involved in calcium and phosphate homeostasis, which explains why 1,25(OH)$_2$D$_3$ plays multiple physiological roles beyond mineral metabolism (Nagpal et al. 2005). Previous studies from this and other laboratories have implicated a role for the VDR in the regulation of adipogenesis and energy metabolism. 1,25(OH)$_2$D$_3$ suppresses 3T3-L1 preadipocyte differentiation into mature adipocytes (Blumberg et al. 2006, Kong & Li 2006). Global VDR-null mice exhibit a lean phenotype that is resistant to high-fat diet-induced obesity, suggesting an involvement of VDR in the regulation of energy expenditure (Narvaez et al. 2009, Wong et al. 2009). Our recent studies demonstrated that transgenic (Tg) mice with targeted expression of human (h) VDR in the adipose tissue developed obesity due to reduced energy expenditure (Wong et al. 2011). Interestingly, an early study reported that 1,25(OH)$_2$D$_3$ suppressed leptin secretion in human adipose tissue (Menendez et al. 2001). Indeed, given the important role of leptin in energy metabolism, the relationship between vitamin D and leptin is of great interest. In the VDR-null and hVDR Tg mice, however, adipocyte leptin transcript levels were correlated with fat mass in these mice (Wong et al. 2009, 2011); therefore, it remains unclear whether vitamin D directly regulates leptin expression in the adipose tissue in vivo. The main goal of this study was to address this question using in vivo and ex vivo approaches. Our data indicate that 1,25(OH)$_2$D$_3$ upregulates leptin production in mouse adipose tissue.

Materials and methods

Animal studies

Global VDR(−/−) mice were described previously (Li et al. 1997). These mice were raised on a high-calcium diet containing 2% calcium, 1.5% phosphorus, and 20% lactose (Harlan, Teklad, WI, USA) right after weaning to normalize serum calcium levels as described (Li et al. 1998). aP2-hVDR Tg mice that specifically overexpress hVDR in adipocytes were reported previously (Wong et al. 2011). Three- to four-month-old mice were used for studies as mice at this age had adequate fat tissue for experiments. Mice were killed by exsanguinations under anesthesia, serum were collected and frozen at −20 °C until use, and epididymal fat pads were harvested for adipose tissue culture or for RNA isolation. To assess the in vivo effect of vitamin D on leptin, WT mice were treated daily with vehicle (propylene glycol:ethanol=90:10) or vitamin D analog RO-27-5646 (dissolved in vehicle; provided by Dr Uskokovic) by i.p. injection for 1 week at 1.5 μg/kg body weight (Qiao et al. 2005). Mice were killed 16 h after the last injection and epididymal fat pads and serum were harvested as earlier. All animal study protocols were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Adipose tissue culture

Epididymal fat pads were dissected immediately after mice were killed and placed in cold PBS (pH 7.2) kept on ice. The fat tissue was rinsed twice with cold PBS, transferred into cold DMEM containing 20% fetal bovine serum, and cut with sterile scissors into small pieces (0.1–0.3 mm). The fat pieces were cultured in six-well plates at 37 °C and 5% CO$_2$ for 24 and 48 h in the presence or absence of 1,25(OH)$_2$D$_3$ (0.1–100 nM), and total RNAs were extracted at the end of treatment. The media were harvested for leptin assays.

Cloning of leptin and resistin cDNA probes

Leptin and resistin cDNA probes were cloned by RT-PCR. Total RNAs (5 μg) were reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). PCR primers were designed according to mouse leptin and resistin cDNA sequence deposited in the GenBank database. Leptin primers are 5’-ATGTGCTGGA-GACCCCTGTG-3’ (forward) and 5’-TCAGCATTCAGGCT- TAACATCC-3’ (reverse), and resistin primers are 5’-CAACTCCCTGTTTCCAATGC-3’ (forward), and 5’-CTCAAGACTGCTGTGCCCTTCT-3’ (reverse). PCRs were carried out at the following condition: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s for 30 cycles. The PCR products were cloned into pSK(+) plasmid. The identity of mouse leptin and resistin cDNAs was confirmed by DNA sequencing.

Northern blot

Total RNAs were extracted from freshly dissected epididymal fat tissues or from cultured adipose tissues using TRIzol reagents (Invitrogen). Total RNAs (15 μg/lane) were
separated on 1% formaldehyde-agarose gels and transferred onto nylon membranes. The membranes were hybridized with \(^{32}P\)-labeled leptin and resistin cDNA probes as described previously (Kong & Li 2006). Transcripts were detected by autoradiography and quantified using a PhosphorImager (Molecular Dynamic, Sunnyvale, CA, USA). The membranes were striped and rehybridized with \(^{32}P\)-labeled 36B4 or GAPDH cDNA probe for the internal loading control.

**RT-PCR**

First-strand cDNAs were reverse-transcribed from 5 \(\mu\)g total RNAs using M-MLV reverse transcriptase (Invitrogen) and hexanucleotide random primers. The VDR transcript was amplified by PCR using primers 5'-GCCACGGGCTTCCACTTCAACG-3' (forward) and 5'-TAGCTCTGACAGAATTGGAGGC-3' (reverse), using a Bio-Rad DNA Engine (Bio-Rad). Leptin transcript was quantified by real-time RT-PCR using a LightCycler 480 Real-Time PCR System (Roche Applies Science) and SYBR green PCR reagent kits (Clontech) as described (Wong et al. 2009, 2011). Leptin PCR primers were 5'-TGTTGTCGCTTCCCTGTGCTTT-3' (forward) and 5'-CTGCGTGTGAATGTCATTG-3' (reverse). Beta-2 microglobulin was used as an internal control for normalization, and its PCR primers were 5'-ACCGCTGTATGCTATCCAGAAA-3' (forward) and 5'-ATTCAATGTGAGGCGGGTGGAAC-3' (reverse). The relative amount of leptin transcripts was calculated using the \(2^{-\Delta\Delta C_t}\) formula as described previously (Zhang et al. 2008).

**Serum leptin measurement**

Leptin concentrations in the serum and culture media were determined using a commercial mouse leptin ELISA kit (Diagnostic Systems Laboratories, Webster, TX, USA) according to the instruction provided by the manufacturer. The detection limit of this kit was 0.5 ng/ml.

**Statistical analysis**

Data were presented as the mean \(\pm\) S.E.M. and analyzed with the Student's \(t\)-test to assess significance. \(P\) values of 0.05 or lower were considered statistically significant.

**Results**

We first examined the effects of adipose VDR status on leptin production in mice. Northern blot analysis showed that adipose leptin mRNA transcript in the epididymal fat was downregulated (by 69 \(\pm\) 17\%) in VDR(-/-) mice compared with WT mice (Fig. 1A and B), which was confirmed by real-time RT-PCR quantitation (Fig. 1C). Similarly, serum leptin levels were markedly reduced (by 86 \(\pm\) 5\%) in the mutant mice (Fig. 1D). Resistin
mRNA levels were increased (by 75±8%) in VDR(−/−) mice (Fig. 1A and B). On the other hand, in aP2-hVDR Tg mice that carry targeted hVDR overexpression in adipocytes, adipose leptin transcript was upregulated (by 52±32%) (Fig. 2A and B), and this observation was confirmed by real-time RT-PCR quantitation (Fig. 2C). Serum leptin levels were also elevated (by 47±6%) (Fig. 2D), compared with WT mice. As VDR(−/−) mice were lean and aP2-hVDR Tg mice were obese (Wong et al. 2009, 2011), the changes in leptin production in these mice may just reflect the status of adiposity.

To address whether the vitamin D–VDR signaling affects leptin expression in the adipose tissue, we treated WT mice with a low-calcemic vitamin D analog RO-27-5646 for 1 week. Compared with vehicle-treated controls, RO-27-5646 treatment markedly upregulated adipose leptin transcript expression (by 290±84%) (Fig. 3A and B) in these mice, and this observation was confirmed by real-time RT-PCR quantitation (Fig. 3C). Serum leptin concentration in the treated mice was also markedly increased (by 210±128%) (Fig. 3D). This short-term treatment had no effects on body weight (data not shown). These results suggest that vitamin D signaling may directly upregulate adipose leptin expression.

We then used adipose tissue cultures to confirm the stimulatory effect of the vitamin D hormone on leptin expression. As shown in Fig. 4, VDR is expressed in white adipose tissue (Fig. 4A), providing a basis for 1,25(OH)2D3 signaling. Treatment of white adipose tissue with 1,25(OH)2D3 (10 nM) for 24 h significantly stimulated leptin secretion (by 41±19%) from the tissue (Fig. 4B). 1,25(OH)2D3 treatment dose dependently induced leptin mRNA transcript expression but had no effects on resistin expression (Fig. 4C and D). The latter indicates that the regulatory effect of 1,25(OH)2D3 on leptin is gene specific. Real-time RT-PCR quantitation confirmed the stimulation of leptin mRNA by 1,25(OH)2D3 (10 nM) treatment (Fig. 4E). These data provide direct evidence that 1,25(OH)2D3 upregulates leptin expression.

Finally, we assessed the requirement of VDR to mediate the stimulatory effect of 1,25(OH)2D3 on leptin. As shown in Fig. 5, whereas incubation of WT adipose tissue culture with 1,25(OH)2D3 upregulated leptin expression, 1,25(OH)2D3 had no effects on leptin in adipose tissue obtained from VDR(−/−) mice (Fig. 5A). As a control, 1,25(OH)2D3 had no effects on resistin in either WT or VDR(−/−) adipose tissue (Fig. 5A). On the other hand, 1,25(OH)2D3-induced upregulation of leptin was more robust in the adipose tissue obtained from aP2-hVDR Tg mice compared with the adipose tissue from WT mice (Fig. 5B), suggesting that this stimulatory action was determined by VDR status in adipocytes.

**Discussion**

In this study, we provided experimental evidence that the vitamin D hormone directly upregulates leptin expression...
and secretion in the white adipose tissue in mice. This regulation may contribute to the effect of the vitamin D endocrine system on energy metabolism in the body. We and other groups have previously reported a potential role of adipocyte VDR signaling in energy metabolism based on observations from VDR(-/-) mice (Narvaez et al. 2009, Wong et al. 2009). Using an adipocyte-specific hVDR Tg model, we recently demonstrated that adipocyte VDR suppresses energy expenditure and promotes obesity (Wong et al. 2011). Here, we showed that adipose leptin expression and serum leptin levels were positively correlated with adipose VDR status in VDR(-/-) and aP2-hVDR Tg mice (Figs 1 and 2); however, as the leptin status is mainly determined by adiposity, these animal data cannot prove whether leptin is regulated by the vitamin D hormone. By treating mice with a vitamin D analog, we provided evidence that vitamin D may directly induce adipose leptin expression (Fig. 3), as this short-term analog treatment did not significantly alter the status of mouse adiposity. More direct evidence for vitamin D upregulation of leptin came from experiments with adipose tissue cultures, in which we demonstrated that incubation of the adipose tissue with 1,25(OH)2D3 resulted in induction of leptin mRNA expression and protein secretion and that this regulation was VDR dependent (Figs 4 and 5). Our results are different from an early report by Menendez et al. (2001) showing that treatment of human adipose tissue cultures with vitamin D inhibited leptin secretion. In the study of Menendez et al., human adipose tissues were treated with 1,25(OH)2D3 for up to 96 h, and a marked suppression of leptin secretion was only observed at 72–92 h, suggesting that the inhibitory effects of 1,25(OH)2D3 may be indirect. Our data reported here were from mice, and we treated mouse adipose tissues for only 24–48 h ex vivo. The change of leptin within this short time frame suggests that the stimulatory effect of 1,25(OH)2D3 on leptin is direct. In fact, we showed that this effect is VDR dependent. The reason for the discrepancy between these two studies is unclear but could be due to species differences or different experimental conditions. Further investigations are needed to confirm or refute these human data regarding the effect of vitamin D on leptin production.

A growing number of epidemiological studies have shown an inverse correlation between adiposity or BMI and serum 1,25(OH)2D3 and 25-hydroxyvitamin D levels in humans (Wortsman et al. 2000, Arunabh et al. 2003, Parikh et al. 2004, Snijder et al. 2005), but whether low vitamin D levels play any roles in the development of obesity is unknown. Given the importance of leptin in energy homeostasis, there is a great interest to understand its potential relationship with vitamin D. Recently, a reverse correlation between serum 25-hydroxyvitamin D and leptin has been reported in obese human subjects.

Figure 3
Stimulatory effects of low-calcemic vitamin D analog on leptin expression in mice. WT mice were treated with vehicle or RO-27-5646 at a daily dose of 1.5 mg/kg for 1 week. (A and B) Northern blot analysis (A) and PhosphoImaging quantitation (B) of leptin mRNA in the epididymal fat of vehicle- or RO-27-5646-treated mice. (C) Real-time RT-PCR quantitation of leptin mRNA transcript in these mice. (D) Serum leptin levels determined by ELISA. **P<0.01; ***P<0.001 vs vehicle; n=5–7.
This relationship is consistent with the low vitamin D status observed in obesity by the other studies mentioned earlier and the high serum leptin level known in obesity. As vitamin D is highly fat soluble, the low-vitamin D status seen in the obese population is believed to be a result of excess vitamin D trapped in the fat tissue (Wortsman et al. 2000). In this case, the trapped vitamin D could increase the local concentration of vitamin D in the fat, resulting in enhancement of its actions on adipocytes including on leptin expression. Therefore, it is plausible that behind the observed inverse relationship between serum vitamin D and leptin status is the fact that high intra-adipose vitamin D concentration stimulates leptin secretion in obesity. This explanation is consistent with our results reported here. More studies are required to further examine this speculation.

The implication of vitamin D upregulation of leptin is unclear. While the body’s leptin status is mainly determined by adiposity, vitamin D may participate in fine-tuning leptin levels in the circulation. Interestingly, leptin has been shown to affect 1,25(OH)2D3 biosynthesis by downregulating 25-hydroxyvitamin D 1α-hydroxylase (Matsunuma & Horiuchi 2007), and this regulation seems to be mediated by fibroblast growth factor 23 (Tsuji et al. 2010). In this regard, upregulating leptin might be one of the multiple feedback loops by which the vitamin D hormone self-regulates its concentration in the body. Indeed, the complex interplay between vitamin D and leptin and its underlying biological implications warrant further investigations.

![Figure 4](image.png)

**Figure 4**
Vitamin D stimulation of leptin production in adipose tissue cultures. (A) RT-PCR detection of VDR mRNA expression in adipose tissue. Total RNAs isolated from epididymal white fat (WF) and kidney (K) of WT mice were subject to RT-PCR amplification. Arrow indicates the 392 bp VDR cDNA fragment. C, No RT control. (B) ELISA quantitation of leptin secretion into the cultured media at the dose of 10 nM define 1.25VD. *P<0.05 vs ethanol. (C and D) Dose-dependent stimulation of leptin in epididymal fat culture by 1,25-dihydroxyvitamin D3. Epididymal fat was cultured in the presence of ethanol (E) or different doses (0.1–100 nM) of 1.25VD for 24 h. Leptin and resistin mRNA levels were analyzed by northern blotting (C) and quantified by PhosphoImaging (D). *P<0.05 vs ethanol. (E) Real-time RT-PCR quantitation of leptin mRNA transcript in the epididymal fat culture treated with 10 nM 1.25VD. **P<0.01 vs E.

![Figure 5](image.png)

**Figure 5**
VDR-dependent stimulation of leptin expression by vitamin D. (A) Epididymal fat obtained from WT and VDR(−/−) mice was incubated with ethanol (E) or 10 nM 1,25-dihydroxyvitamin D3 (VD) for 24 and 48 h. Leptin and resistin mRNA levels were analyzed by northern blotting. (B) Epididymal fat obtained from WT and hVDR Tg mice was incubated with ethanol (E), 10 or 100 nM 1,25-dihydroxyvitamin D (1.25VD) for 24 h. Leptin mRNA transcript levels were quantified by real-time RT-PCR. **P<0.01 vs WT.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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