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Molecular mechanism of 1,25-dihydroxyvitamin D₃ inhibition of adipogenesis in 3T3-L1 cells

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A large body of literature has demonstrated that adipocyte differentiation follows a well-defined program. Much of our current understanding of the molecular regulation of adipogenesis comes from in vitro studies of preadipocyte cell lines such as 3T3-L1, 3T3-F442A, and Ob1771 (12–14, 32). According to the current model, the adipogenic program involves several sequential stages over a period of 4–7 days (36, 38). Under the induction of proliferative agents, insulin, glucocorticoids, and phosphodiesterase inhibitor, growth-arrested cells undergo one or two rounds of cell division known as mitotic clonal expansion, which is accompanied by the induction of C/EBPβ and C/EBPδ. These earliest events are followed by increased expression of C/EBPα and PPARγ, the central transcriptional regulators of adipogenesis, which drive adipocyte-specific gene expression. Subsequently, in the final stage, the cells are terminally differentiated into mature adipocytes. These differentiated cells now express markers characteristic of adipocyte phenotype such as fatty acid synthase (FAS), lipoprotein lipase (LPL), acetyl-CoA carboxylase, GLUT4, and fatty acid-binding protein aP2, along with massive accumulation of triglyceride inside the cells.

1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the most active form of vitamin D metabolites, is an endocrine hormone that plays multiple physiological roles (46). This secosteroid hormone is known to be critical for the homeostasis of calcium and phosphate (27, 52). It also regulates sulfate transport (5), the renin-angiotensin system (26), the immune system (29), and normal organ development such as the mammary gland and skeletal muscle (9, 53). 1,25(OH)₂D₃ has previously been shown to regulates adipocyte differentiation; however, somewhat conflicting results have been reported regarding the role of vitamin D in adipogenesis. In early reports, vitamin D receptor (VDR)-like molecule was detected in 3T3-L1 cells (39), and 1,25(OH)₂D₃ was shown to pose an inhibitory effect on 3T3-L1 differentiation on the basis of its inhibition of glycerophosphate dehydrogenase activity and triglyceride content (21, 39) or its ability to counter the stimulatory effect of a peroxisome proliferator-activated receptor-γ (PPARγ) ligand on 3T3-L1 differentiation (18). 1,25(OH)₂D₃ was also able to inhibit adipocyte differentiation in mouse bone marrow stromal cells (22). On the other hand, 1,25(OH)₂D₃ was shown to promote adipocyte differentiation in primary rat calvaria cells (4) and rat bone marrow stromal cells (2) and to stimulate LPL expression in 3T3-L1 cells (35, 49). Other recent studies demonstrated that 1,25(OH)₂D₃ can stimulate FAS and suppress uncoupling protein 2 and leptin production in primary human adipocytes and adipose organ cultures (30, 41, 42).

Despite the conflicting conclusions reported in the literature, few studies have been carried out to systematically investigate the molecular mechanism underlying the effect of vitamin D on adipogenesis. Given the importance of the adipose tissue in the development of human diseases such as metabolic syndrome, delineation of the molecular events involved in vitamin D regulation of adipogenesis has become quite necessary. Here we report a systematic study aimed at understanding the molecular mechanism whereby 1,25(OH)₂D₃ inhibits adipogenesis in the 3T3-L1 cell model. Our data suggest that vitamin
D acts on multiple targets to block adipocyte differentiation in vitro.

EXPERIMENTAL PROCEDURES

Cell culture and treatment. 3T3-L1 cells (ATCC, Manassas, VA) were routinely cultured in growth medium (GM) consisting of DMEM supplemented with 10% FBS (HyClone, Logan, UT) and 2 mM glutamine. The cells were differentiated according to a well-established protocol described previously (44). Briefly, for differentiation, 3T3-L1 cells were cultured in GM to full confluence. Two days after confluence (referred to as day 0), the cells were switched to differentiation media (DM) consisting of DMEM supplemented with 10% FBS, 1 µg/ml insulin, 0.25 µM dexamethasone, and 0.5 mM isobutyl methylxanthine and cultured for 3 days. Next, the cells were maintained in DM but containing only 1 µg/ml insulin, and the medium was changed every 2–3 days. The cells normally differentiated into mature adipocytes on day 7 or 8. Depending on the purpose of the experiment, 1,25(OH)2D3 or ethanol (vehicle) was added to the DM at the indicated doses or at different times. In other experiments, troglitazone at indicated concentrations was also added to the cell culture system. The cells were harvested at indicated times during differentiation for RNA or protein extraction or stained with Oil Red O (Sigma, St. Louis, MO) according to the procedure described previously (40). In thymidine incorporation assays, 1 µCi/ml of [3H]thymidine was added to the culture after the DM switch, and the amount of [3H]thymidine incorporated in the cells was determined with a scintillation counter immediately after 48 or 72 h as described previously (50).

Recombinant adenovirus and infection. Recombinant adenovirus harboring human (h) VDR cDNA was generated using the AdEasy system according to the method described previously (17). The recombinant virus, Ad-hVDR, expresses the full-length hVDR protein. Ad-hVDR and Ad-GFP, the empty vector, were used to infect 3T3-L1 cells at confluence (day −2) at multiplicity of infection of ~10^5. Next, at day 0, the infected cells were switched to DM to initiate cell differentiation according to the standard protocol.

Isolation of mouse embryonic fibroblast. Mouse embryonic fibroblasts (MEF) were isolated from embryonic day 13.5 embryos generated from VDR^{+/−} × VDR^{−/−} mouse breeding (27). Briefly, the embryos were harvested and placed in PBS to remove the internal organs, head, and four limbs. The remaining embryo body was individually minced and digested with 0.5% trypsin and 10 mM EDTA for 0.5 h at 37°C. The digested materials were gently pipetted to a single cell suspension. The cells were cultured in DMEM supplemented with 10% FBS and used after three to four passages. Cells from each embryo were genotyped by PCR using genomic DNA isolated form the cells. VDR^{+/−} and VDR^{−/−} MEFs were differentiated according to the standard protocol described above.

Northern blot. Total cellular RNA was extracted using TRIzol reagents (Invitrogen, Grand Island, NY) according to instructions provided by the supplier. Northern blot analysis was carried out as described previously (25). Briefly, total RNAs (20 µg/lane) were separated on 1% agarose gels containing 0.6 M formaldehyde and transferred to Nyton membranes that were cross-linked in an ultraviolet cross-linker. Hybridization was carried out at 65°C in the hybridization buffer described by Church and Gilbert (7) with cDNA probe labeled with [32P]dATP using the Prime-a-Gene Labeling System (Promega, Madison, WI). After hybridization and being washed, the membranes were exposed to X-ray films at −80°C for autoradiography to visualize the mRNA transcript. The membranes were stripped and rehybridized with 32P-labeled 36B4 CDNA probe for internal loading control.

Western blot. Western blot analyses were carried out as described previously (25). Briefly, cells were lysed with the Laemmli buffer (23), and cell lysates were separated by SDS-PAGE. The separated proteins were then electroblotted on Immobilon-P membranes. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated secondary antibody, autoradiograms were prepared using the enhanced chemiluminescent system (Amersham, Piscataway, NJ) to visualize the protein antigen.

Cell transfection and luciferase reporter assays. 3T3-L1 cells were cultured in DMEM supplemented with 10% FBS. The cells, at ~60–80% confluency, were cotransfected in duplicate with C/EBP-PREX3-tk-Luc or PREX3-tk-Luc reporter plasmid (15) and other cDNA expression plasmids detailed in each experiment, using Lipofectamine reagent (Invitrogen) according to the instructions provided by the manufacturer. Cell luciferase activity was assayed after 24–48 h using the Firefly Luciferase Assay System (Promega) or Renilla Luciferase assay kit (Biotium, Hayward, CA). To normalize the transfection efficiency, a pCMV-β-gal plasmid was included in each transfection, and luciferase activity was normalized to β-galactosidase activity in each transfection. The β-galactosidase activity was determined by using the Gel-Screen Chemiluminescent Reporter System (Tropix, Bedford, MA).

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 pads were rinsed two times with cold PBS, transferred to cold DMEM containing 2% FBS and 1 µg/ml insulin, and cut with sterile scissors into small pieces. The fat pieces were cultured in six-well plates at 37°C and 5% CO2 in the presence or absence of 10^−8 M of 1,25(OH)2D3, and total RNAs were extracted at 24, 36, and 48 h. The RNAs were subject to Northern blot analyses.

RESULTS

Inhibition of adipocyte differentiation by 1,25(OH)2D3 is dose dependent but time sensitive. To confirm that 1,25(OH)2D3 inhibits 3T3-L1 cell differentiation into adipocytes, 1,25(OH)2D3 was added to the DM at day 0 when the differentiation was initiated. Adipocyte differentiation was evaluated with Oil Red O staining at day 7. As shown in Fig. 1, 1,25(OH)2D3 inhibited the formation of adipocytes in a dose-dependent manner, with few adipocytes seen at 10^−8 M (Fig. 1A). The expression of gene transcripts known to be associated with early and late stages of adipocyte differentiation, including C/EBPα, PPARγ, LPL, and aP2, was also blocked by 1,25(OH)2D3 in a dose-dependent fashion (Fig. 2A). Other genes, such as the sterol regulatory element-binding protein (SREBP)-1 and FAS, which were upregulated when the cells were turned to adipocytes, were also suppressed dose dependently by 1,25(OH)2D3 (Fig. 2C).

Interestingly, however, when 1,25(OH)2D3 (10^−8 M) was added to the DM 48 h after the adipogenic program was initiated, adipocyte differentiation could no longer be blocked (Fig. 1B). Consistently, the expression of the adipocyte differentiation markers, including C/EBPα, PPARγ, LPL, and aP2, were no longer inhibited by 1,25(OH)2D3 after 48 h (Fig. 2B). These data clearly indicate that the window in which 1,25(OH)2D3 can effectively inhibit 3T3-L1 adipogenesis is within the first 48 h after the differentiation program is initiated.

Blockade of adipogenesis by 1,25(OH)2D3 occurs after clonal expansion and C/EBPβ induction. It is well known that, within a few hours after the adipogenic program is initiated, the cells undergo one or two rounds of mitotic clonal expansion in the first 48 h, which is accompanied by an upregulation of C/EBPβ and C/EBPβ expression (48). To investigate whether 1,25(OH)2D3 affected these early events, 3T3-L1 cells were treated with 1,25(OH)2D3 (10^−8 M) at hour 0, and the cells were analyzed at different times after the differentiation process was initiated. As shown in Fig. 3, 4 h after 3T3-L1 cells
were switched to DM, C/EBPβ expression was markedly upregulated, whereas PPARγ upregulation was not detected until after 24 h. Treatment with 1,25(OH)₂D₃ completely blocked PPARγ expression but had no effect on C/EBPβ expression and luciferase activity was determined after 16 h when the reporter plasmid in GM. Next, the cells were switched to DM, cyte 3T3-L1 cells were transfected with a C/EBPβREx3-tk-Luc reporter plasmid in GM; (a) or in differentiation medium (DM, b-f) in the presence of ethanol (b) or increasing concentrations of 1,25(OH)₂D₃ (VD) as indicated (from 10⁻¹¹ to 10⁻⁶ M). 1,25(OH)₂D₃ was added to the DM at hour 0, and the cells were stained at day 7. B: blockade of 3T3-L1 differentiation by 1,25(OH)₂D₃ is time sensitive. 3T3-L1 cells were cultured in GM (a) or in DM (b-f), and 10⁻⁸ M of 1,25(OH)₂D₃ was added to the DM at 0 (c), 24 (d), 48 (e), or 72 (f) h after DM switch. The cells were stained at day 7. Note that 1,25(OH)₂D₃ fails to block 3T3-L1 cell differentiation 48 h after the cells were switched to the DM.

Inhibition by 1,25(OH)₂D₃ is reversible. To investigate whether the inhibition of adipocyte differentiation by 1,25(OH)₂D₃ is irreversible, 1,25(OH)₂D₃ (10⁻⁸ M) was added to the DM at day 0 and then removed by changing to fresh vitamin D-free DM at different times afterward. As shown in Fig. 5, even after 3 days of 1,25(OH)₂D₃ treatment, 3T3-L1 cells could still fully differentiate into adipocytes under the standard protocol (data not shown), and we used PPARγ as the marker for differentiation (expression of other downstream markers were relatively weak). When VDR⁺/⁺ and VDR⁻/⁻ MEFs were cultured in DM, PPARγ expression was markedly enhanced; 1,25(OH)₂D₃ treatment effectively blocked PPARγ upregulation in VDR⁺/⁺ cells, but not in VDR⁻/⁻ cells (Fig. 6B). Therefore, VDR is required for the 1,25(OH)₂D₃ inhibition.

Given the importance of VDR, the level of VDR protein in 3T3-L1 cells was determined at different stages of the adipogenesis process. As shown in Fig. 7, VDR protein was barely detectable in 3T3-L1 preadipocytes grown in GM; however, when the cells were switched to DM, VDR protein levels were...
drastically increased and peaked within 4–8 h, which gradually declined afterward along the progression of the differentiation process so that the VDR protein became barely detectable in mature adipocytes at day 8 (Fig. 7A). On the other hand, the protein level of retinoid X receptor (RXR)-α was gradually increased after differentiation (Fig. 7B). Interestingly, in the presence of 1,25(OH)2D3, VDR protein, particularly the upper slow-moving isoform, was markedly increased and stabilized at the late stages of differentiation (Fig. 7A), whereas RXR-α levels appeared to be repressed in the first 2 days (24–39 h; Fig. 7B). As a control, the level of extracellular signal-regulated kinase, a protein known to be important for 3T3-L1 differentiation (34), was unaltered in the presence or absence of 1,25(OH)2D3 (Fig. 7C).

To confirm the inhibitory activity of VDR in adipogenesis, hVDR was overexpressed in 3T3-L1 fibroblasts by infection with recombinant adenovirus harboring the full-length hVDR.

Fig. 2. Northern blot analyses of 3T3-L1 cell differentiation. A: 1,25(OH)2D3 dose dependently inhibits the expression of genes involved in adipogenesis. 3T3-L1 cells were cultured in DM in the presence of ethanol (E) or increasing concentrations (from 10−11 to 10−7 M) of 1,25(OH)2D3 added at hour 0. B: 1,25(OH)2D3 fails to inhibit the expression of differentiation markers 48 h after the adipogenic program is initiated. 3T3-L1 cells were cultured in GM or DM, and 1,25(OH)2D3 was added to the DM 0, 24, 48, or 72 h after the cells were switched to the DM. In both experiments, total cellular RNA was extracted at day 7 and subjected to Northern blot analyses (20 μg/lane). The membranes were sequentially hybridized with C/EBPα, peroxisome proliferator-activated receptor (PPAR)-γ, lipoprotein lipase (LPL), fatty acid-binding protein aP2 (aP2), and 36B4 probes as indicated. C: 1,25(OH)2D3 dose dependently inhibits fatty acid synthase (FAS) and sterol regulatory element-binding protein (SREBP)-1 expression. 3T3-L1 cells were cultured in GM or DM in the presence of different doses of 1,25(OH)2D3 as indicated. 1,25(OH)2D3 was added at hour 0, and total cellular RNAs were extracted at day 7 and subjected to Northern blot analyses with FAS and SREBP-1 probes.

Fig. 3. 1,25(OH)2D3 does not inhibit C/EBPβ mRNA induction or its trans-activating activity. A: 3T3-L1 cells were cultured in GM or DM in the absence or presence of 10−8 M 1,25(OH)2D3 as indicated. 1,25(OH)2D3 was added at hour 0, and total cellular RNAs were extracted at 4, 8, 16, 24, or 39 h. The RNAs were subject to Northern blot analyses with C/EBPβ, PPARγ, and 36B4 probes. B: 3T3-L1 cells cultured in GM were transfected with a C/EBPβ-ex3-tk-Luc reporter plasmid. After 24 h, the transfected cells were switched to DM in the presence of ethanol (DM + E) or 10−8 M 1,25(OH)2D3 (DM + VD). Luciferase activity was determined 16 h after the DM switch.

Fig. 4. 1,25(OH)2D3 does not inhibit the mitotic clonal expansion. A: determination of cell number. The same number of 3T3-L1 cells were seeded in 6-well plates and cultured in GM, DM, or DM containing 10−8 M 1,25(OH)2D3 for 48 or 72 h. The cell number was then determined with a Coulter counter in triplicate. B: [3H]thymidine incorporation assays. 3T3-L1 cells were cultured in GM, DM, or DM containing 10−8 M 1,25(OH)2D3 for 48 or 72 h in the presence of 1 μCi/ml of [3H]thymidine. The amount of [3H]thymidine incorporated in the cells was determined with a scintillation counter. *P < 0.01 vs. corresponding GM control.
When the hVDR-expressing 3T3-L1 cells were cultured in the DM, they ceased to differentiate into adipocytes, even in the absence of 1,25(OH)\(_2\)D\(_3\) (e.g., no PPAR\(\gamma\) and LPL induction); in contrast, cells infected with the empty adenovirus vector differentiated normally [as shown by the dramatic upregulation of PPAR\(\gamma\) and LPL, which was blocked by 1,25(OH)\(_2\)D\(_3\); Fig. 8B]. Therefore, overexpression of VDR in 3T3-L1 cells blocked adipogenesis, suggesting the inhibitory nature of VDR.

1,25(OH)\(_2\)D\(_3\) directly inhibits the expression of PPAR\(\gamma\) and C/EBP\(\alpha\). Data presented in Fig. 3 show that 1,25(OH)\(_2\)D\(_3\) inhibits PPAR\(\gamma\) and C/EBP\(\alpha\) expression not through suppressing the transacting activity of C/EBP\(\alpha\). However, inhibition of PPAR\(\gamma\) and C/EBP\(\alpha\) expression may well be secondary to inhibition of adipocyte differentiation by 1,25(OH)\(_2\)D\(_3\).
address this question, adipose tissues isolated directly from mice were treated with 1,25(OH)2D3 ex vivo for up to 2 days, and this treatment clearly decreased PPARγ cDNA levels in adipose tissue culture. Epididymal fat pads were dissected from normal mice, and the tissues were cultured in 6-well plates for 24, 36, and 48 h in the presence of ethanol or 10^{-8} M 1,25(OH)2D3. Total RNAs were extracted and analyzed by Northern blot with PPARγ (A) or C/EBPα (B) cDNA probe.

1,25(OH)2D3 antagonizes the transacting activity of PPARγ. Troglitazone is a PPARγ agonist and was able to induce 3T3-L1 cell differentiation in GM (Fig. 10A). Moreover, troglitazone was able to ameliorate the inhibition of adipocyte differentiation by 1,25(OH)2D3 or retinoic acid, as reflected by the higher LPL levels in the presence of troglitazone (Fig. 10B). Retinoic acid is a known inhibitor of 3T3-L1 cell differentiation (40). Given that both VDR and PPARγ share the same heterodimeric partner RXR and that RXR levels are considerably lower in the early phase of 3T3-L1 cell differentiation (Fig. 7B), we speculated that VDR may directly suppress the transacting activity of PPARγ by sequestering the limited amount of RXR in 3T3-L1 cells. To test this possibility, 3T3-L1 cells were transfected with a PPREx3-tk-Luc reporter plasmid. When the cells were cotransfected with PPARγ cDNA, the luciferase activity was increased by two- to threefold, as expected, and this induction was partially inhibited by 1,25(OH)2D3 treatment (Fig. 10C).

The cotransfection of hVDR cDNA inhibited the increase in luciferase activity induced by PPARγ, even in the absence of 1,25(OH)2D3; hVDR cDNA cotransfection also reduced the basal PPRE-luciferase activity (Fig. 10C). These observations were consistent with the data showing that adenoviral overexpression of hVDR inhibited 3T3-L1 cell differentiation in the absence of 1,25(OH)2D3 (Fig. 10D), suggesting that RXR may be a limiting factor in 3T3-L1 cells. To further test the possibility, a RXRα expression plasmid was included in the cotransfection experiment. RXR significantly increased the transacting activity of PPARγ (Fig. 10D). In the presence of RXR, hVDR was no longer able to inhibit the transacting activity of PPARγ regardless of the presence and absence of 1,25(OH)2D3 (Fig. 10D). We also found that 1,25(OH)2D3 treatment or cotransfection with hVDR had little effect on the transacting activity of C/EBPα (data not shown). These data suggest that VDR may specifically antagonize the transacting activity of PPARγ during 3T3-L1 cell differentiation.

DISCUSSION

Although 1,25(OH)2D3 has been reported to inhibit adipocyte differentiation in 3T3-L1 cells for more than a decade,
the molecular mechanism underlying this inhibition remains unclear. To address this important question, we have performed a systematic investigation aimed at delineating the molecular events surrounding the blockade of adipogenesis by 1,25(OH)2D3 in vitro. Our strategy is to take advantage of the well-defined adipogenic program and identify the molecular changes at each stage that are caused by 1,25(OH)2D3 treatment. The evidence obtained in the present study suggests that, in the 3T3-L1 cell model, 1,25(OH)2D3 inhibits adipogenesis probably by suppressing C/EBPα and PPARγ expression, antagonizing PPARγ transacting activity, and stabilizing the VDR protein. Together with a recent finding that 1,25(OH)2D3 in 3T3-L1 cells, 1,25(OH)2D3 appears to directly suppress the 

The data from the cotransfection studies suggest that 1,25(OH)2D3 also counters the transacting activity of PPARγ. This is likely achieved by competing for the limited amount of RXR through VDR, since RXR is the common heterodimeric partner of both VDR and PPARγ. Because the levels of RXR in 3T3-L1 cells are very low in the early stages of differentiation (relative to the late stages; Fig. 7B), VDR, once activated by 1,25(OH)2D3 binding, may sequester RXR from PPARγ in the early phase of adipogenesis, when the activity of PPARγ is crucial to advance the differentiation program. In the case of VDR overexpression, the large quantity of VDR in the cells may sequester RXR without ligand activation, and overexpression of RXR can apparently prevent the sequestering effect of VDR.

Clearly, the biological significance of the VDR profile during the course of adipocyte differentiation. Although the VDR protein level is barely detectable in the preadipocyte 3T3-L1 cells, it drastically increased within 4–8 h of the initiation of adipogenesis, which is followed by a gradual decline with the progression of differentiation. A similar VDR mRNA profile has also been reported in two other recent studies in which 3T3-L1 adipocyte differentiation was investigated by microarray analysis (6, 11). It is worth pointing out that the maximal induction of VDR takes place at approximately the same time as C/EBPβ induction and is much earlier than the upregulation of C/EBPα and PPARγ. Interestingly, ectopic expression of hVDR by adenovirus completely blocks adipogenesis even in the absence of the ligand, 1,25(OH)2D3, suggesting that VDR protein itself is inhibitory to adipogenesis. One possible mechanism is the sequestering of RXR by the excessive amount of VDR inside the cells. Therefore, naturally, the VDR level needs to be declined for the adipogenic process to proceed, but why it needs to be dramatically increased in the early phase remains an interesting question. Clearly, the biological significance of the VDR profile during adipogenesis requires further investigation.

In the presence of 1,25(OH)2D3, VDR protein in 3T3-L1 cells appears to be more stabilized, particularly in the late stages of the adipogenic program (Fig. 7A). In fact, stabilization of the VDR protein by 1,25(OH)2D3 has been reported in other cells (1). Therefore, because VDR is inhibitory, another possible mechanism that 1,25(OH)2D3 uses to inhibit adipocyte differentiation is to prevent the decline of the VDR protein concentration in the late stages. What is particularly intriguing is that 1,25(OH)2D3 appears to stabilize the slower-moving VDR species more (Fig. 7A). The VDR band of slower mobility may represent phosphorylated VDR (19, 20) or the VDR variant generated from the alternatively spliced first exon of the VDR gene that was identified recently (45). It will be interesting to ascertain the identity of this VDR isoform in future studies.

As an endocrine hormone, the in vivo effect of vitamin D on adipocytes/adipose tissue remains unclear, and whether the vitamin D status has any connection with human obesity is controversial (28, 33, 43). Given the link of obesity with metabolic syndrome, an increasingly epidemic problem char-
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