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

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Submitted 30 August 2005; accepted in final form 16 December 2005

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 current model, the adipogenic program involves several events of the adipogenic program. Treatment of 3T3-L1 cells with 1,25(OH)₂D₃ does not block the mitotic clonal expansion or C/EBPβ induction; rather, 1,25(OH)₂D₃ blocks the expression of C/EBPα, peroxisome proliferator-activated receptor-γ (PPARγ), sterol regulatory element-binding protein-1, and other downstream adipocyte markers. The inhibition by 1,25(OH)₂D₃ is reversible, since removal of 1,25(OH)₂D₃ from the medium restores the adipogenic process with only a temporal delay. Interestingly, although the vitamin D receptor (VDR) protein is barely detectable in 3T3-L1 preadipocytes, its levels are dramatically increased during the early phase of adipogenesis, peaking at 4–8 h and subsiding afterward throughout the rest of the differentiation program; 1,25(OH)₂D₃ treatment appears to stabilize the VDR protein levels. Consistently, adenovirus-mediated overexpression of human (h) VDR in 3T3-L1 cells completely blocks the adipogenic program, confirming that VDR is inhibitory. Inhibition of adipocyte differentiation by 1,25(OH)₂D₃ is ameliorated by troglitazone, a specific PPARγ antagonist; conversely, hVDR partially antagonizes the transactivating activity of PPARγ but not of C/EBPβ or C/EBPα. Moreover, 1,25(OH)₂D₃ markedly suppresses C/EBPα and PPARγ mRNA levels in mouse epididymal fat tissue culture. Taken together, these data indicate that the blockade of 3T3-L1 cell differentiation by 1,25(OH)₂D₃ occurs at the postclonal expansion stages and involves direct suppression of C/EBPα and PPARγ upregulation, antagonization of PPARγ activity, and stabilization of the inhibitory VDR protein.

vitamin D; vitamin D receptor; adipocyte differentiation

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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 confluency. 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 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 confluency (day –2) at multiplicity of infection of ~104. Next, at day 0, the infected cells were switched to the 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 then digested with 0.5% trypsin and 10 mM EDTA for 0.5 h at 37°C. The digested materials were gently pipetted into 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 subjected 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 at 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/EBPδ 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)_{2}D_{3} completely blocked PPARγ expression but had no effect on C/EBPβ upregulation (Fig. 3A). Because C/EBPβ is required for the induction of the downstream PPARγ and C/EBPα (47, 51), it is possible that 1,25(OH)_{2}D_{3} indirectly inhibits C/EBPα and PPARγ mRNA expression by inhibiting the transacting activity of C/EBPβ as in the case of retinoic acid inhibition of 3T3-L1 differentiation (40). To test this possibility, preadipocyte 3T3-L1 cells were transfected with a C/EBPβRE3-tk-Luc reporter plasmid in GM. Next, the cells were switched to DM, and luciferase activity was determined after 16 h when C/EBPα and PPARγ remained uninduced (Fig. 3A). As shown in Fig. 3B, the switch to DM led to an approximately twofold increase in luciferase activity resulting from C/EBPβ induction, and this activity increase was not affected in the presence of 1,25(OH)_{2}D_{3} (Fig. 3B). Therefore, 1,25(OH)_{2}D_{3} affects neither the induction of the C/EBPβ transcript nor its transacting activity.

Consistent with these observations, 1,25(OH)_{2}D_{3} treatment does not affect the mitotic clonal expansion stage. As shown in Fig. 4, the cell number almost doubled in 24 h after switching to the DM, and continued to increase at 72 h, regardless of 1,25(OH)_{2}D_{3} treatment (Fig. 4A); similarly, 1,25(OH)_{2}D_{3} treatment had no effect on the incorporation of [3H]thymidine into DNA stimulated by the DM (Fig. 4B). Taken together, these results indicate that 1,25(OH)_{2}D_{3} blocks the progress of the adipogenic program at a postclonal expansion stage, possibly by targeting C/EBPα and/or PPARγ.

Inhibition by 1,25(OH)_{2}D_{3} is reversible. To investigate whether the inhibition of adipocyte differentiation by 1,25(OH)_{2}D_{3} is irreversible, 1,25(OH)_{2}D_{3} (10^{-8} M) was added to the DM at hour 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)_{2}D_{3} treatment, 3T3-L1 cells could still fully differentiate into adipocytes in the absence of 1,25(OH)_{2}D_{3}, with only some temporal delay, indicating that the inhibition of adipogenesis by 1,25(OH)_{2}D_{3} is reversible.

VDR status appears to be critical for 1,25(OH)_{2}D_{3} inhibition. It is well known that VDR mediates most, if not all, actions of 1,25(OH)_{2}D_{3} (16). To address whether VDR is required for the inhibition of adipogenesis by 1,25(OH)_{2}D_{3}, we studied MEFs isolated from wild-type and VDR null mice (Fig. 6). As similarly reported in early studies (37), only 1–5% of MEFs were able to differentiate into mature 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)_{2}D_{3} treatment effectively blocked PPARγ upregulation in VDR^{+/−} cells, but not in VDR^{−/−} cells (Fig. 6B). Therefore, VDR is required for the 1,25(OH)_{2}D_{3} 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 RXRα 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/EBPα-expressing 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.
cDNA (Fig. 8A). 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\(\beta\). 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\(_3\). To inhibit PPAR\(\gamma\) and C/EBP\(\alpha\) expression not through suppressing the transacting activity of C/EBP\(\beta\).

Fig. 5. Inhibition by 1,25(OH)\(_2\)D\(_3\) is reversible. 3T3-L1 cells were cultured in DM or DM containing 10\(^{-8}\) M 1,25(OH)\(_2\)D\(_3\). At 12, 24, 36, 48, or 72 h, the DM + 1,25(OH)\(_2\)D\(_3\) media were replaced with fresh vitamin D-free DM, and the cells were continuously cultured to day 8. Total cellular RNAs were extracted on day 8 and subjected to Northern blot analyses with the cDNA probes indicated.

Fig. 6. Requirement of vitamin D receptor (VDR) to mediate 1,25(OH)\(_2\)D\(_3\) inhibition. A: Western blot showing VDR expression in VDR\(^{+/+}\) and VDR\(^{-/-}\) mouse embryonic fibroblasts. B: VDR\(^{+/+}\) and VDR\(^{-/-}\) mouse embryonic fibroblasts were differentiated in the DM in the presence or absence of 10\(^{-8}\) M 1,25(OH)\(_2\)D\(_3\) for 8 days, and total RNAs were subjected to Northern blot analyses with PPAR\(\gamma\) and 36B4 probes. Note that PPAR\(\gamma\) expression was not inhibited by 1,25(OH)\(_2\)D\(_3\) (+VD) in VDR\(^{-/-}\) cells.

Fig. 7. VDR protein profile during 3T3-L1 differentiation. 3T3-L1 cells were cultured in DM or DM containing 10\(^{-8}\) M 1,25(OH)\(_2\)D\(_3\), and total RNAs were extracted on day 7 and subjected to Northern blot analyses with indicated probes. Note the complete inhibition of DM-induced PPAR\(\gamma\) upregulation in Ad-hVDR-infected cells, even in the absence of 1,25(OH)\(_2\)D\(_3\). To inhibit PPAR\(\gamma\) and C/EBP\(\alpha\) expression not through suppressing the transacting activity of C/EBP\(\beta\).

Fig. 8. Inhibition of adipocyte differentiation by human (h)VDR overexpression. A: Western blot showing hVDR protein expression in 3T3-L1 cells. Cells were infected with adenoviral vector (Ad-V) or recombinant adenovirus harboring hVDR cDNA (Ad-hVDR) for 48 h, and cell lysates (20 \(\mu\)g/lane) were subjected to Western blot analysis with anti-VDR antibody. Kid, mouse kidney lysates for VDR standard. B: 3T3-L1 cells infected with Ad-Vector or Ad-hVDR were cultured in GM, DM, or DM containing 10\(^{-8}\) M 1,25(OH)\(_2\)D\(_3\) (DM + D), and total RNAs were extracted on day 7 and subjected to Northern blot analyses with indicated probes. Note the complete inhibition of DM-induced PPAR\(\gamma\) upregulation in Ad-hVDR-infected cells, even in the absence of 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 mRNA 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). 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. 10B), 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 upregulates C/EBPβ expression (24), we speculate that 1,25(OH)2D3 inhibits adipocyte differentiation by acting on multiple molecular targets, and the mechanism of inhibition is complex.

Upon initiation of the adipogenic program, preadipocytes undergo mitotic clonal expansion that is composed of one or two rounds of cell division. Interestingly, although 1,25(OH)2D3 is well known to have potent antiproliferative activity and inhibits cell division in many cell types, including normal and malignant cells, it does not affect the clonal expansion in 3T3-L1 cells. C/EBPβ upregulation is a very early event that is required for the mitotic clonal expansion (47) and mediates the downstream upregulation of PPARγ and C/EBPα expression (51). Interestingly, a recent study shows that 1,25(OH)2D3 stimulates C/EBPβ expression in kidney cells (8). However, we show here that 1,25(OH)2D3 has no effect on the upregulation of C/EBPβ mRNA expression in the early stage of adipocyte differentiation, nor does it affect the transacting activity of C/EBPβ protein. Therefore, 1,25(OH)2D3 suppression of the upregulation of C/EBPα and PPARγ is independent of C/EBPβ. This is different from retinoic acid inhibition of adipogenesis in 3T3-L1 cells, which inhibits C/EBPβ transactivating activity (40).

C/EBPα and PPARγ are the central transcriptional regulators of adipogenesis since they are required for the syntheses of many adipocyte functional proteins. C/EBPα and/or PPARγ have been reported to be the target of other adipogenic inhibitors such as calcineurin and Kruppel-like factor KLF2 (3, 31). In 3T3-L1 cells, 1,25(OH)2D3 appears to directly suppress the induction of C/EBPα and PPARγ and antagonize the transacting activity of PPARγ. Although it is possible that the blocking of C/EBPα and PPARγ by 1,25(OH)2D3 can be secondary to its inhibition of 3T3-L1 cell differentiation, the fact that 1,25(OH)2D3 also suppresses C/EBPα and PPARγ expression in mature adipose tissues argues against this possibility and supports a direct repression because the concern of adipocyte differentiation is not an issue for the epididymal fat. Therefore, inhibition of C/EBPα and PPARγ by 1,25(OH)2D3 is likely a cause, but not a consequence, of the in vitro adipogenic inhibition. This conclusion is consistent with the observation that 1,25(OH)2D3 can no longer block adipogenesis 48 h after the adipogenic program is initiated, because at this time the upregulation of C/EBPα and PPARγ has already taken place. Clearly, targeting C/EBPα and PPARγ is key to vitamin D’s effect on adipogenesis. Taken together with the evidence that 1,25(OH)2D3 does not affect C/EBPβ, the upstream regulator of C/EBPα and PPARγ, we conclude that 1,25(OH)2D3 blocks 3T3-L1 cell differentiation by directly suppressing the upregulation of C/EBPα and PPARγ. Given the important role of C/EBPα and PPARγ in adipogenesis, targeting C/EBPα and PPARγ may likely be the major mechanism of vitamin D’s inhibitory actions. More studies are needed to investigate the exact molecular mechanism underlying the suppression of these two genes by vitamin D.

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.

It is very intriguing that the levels of VDR protein change 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-
acetylated by insulin resistance and dyslipidemia (10), the role of the vitamin D endocrine system in adipocyte biology is definitely worth further investigations.

ACKNOWLEDGMENTS

We thank T. C. He for providing the AdEasy adenovirus system, Matthew Brady for providing 3T3-L1 cells, Reed Graves for providing troglitazone and cDNA plasmids, and Youfei Guan for providing the PPRE-tk-luciferase plasmid. We also thank Marc Bissonnette for critically reading the manuscript.

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-59327 and by an Innovation Award from the American Diabetes Association.

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