The Involvement of Mitogen-Activated Protein Kinases in the 1α,25-Dihydroxy-Cholecalciferol-Induced Inhibition of Adipocyte Differentiation In Vitro

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Summary The present study was undertaken to investigate the mechanism by which 1α,25-dihydroxy-cholecalciferol [1α,25-(OH)2-VD3] modulates the differentiation of mouse 3T3-L1 preadipocytes into mature adipocytes. Treatment with 1α,25-(OH)2-VD3 in the presence of insulin, dexamethasone and 3-isobutyl-1-methyl-xanthine significantly inhibited the triacylglycerol accumulation, and mRNA expressions of adipocytokines (adiponectin and tumor necrosis factor-α) and plasminogen activator inhibitor-1 in the picomolar concentration range, indicating that 1α,25-(OH)2-VD3 under physiological conditions inhibits the differentiation of 3T3-L1 cells. 1α,25-(OH)2-VD3 potently reduced the mRNA and/or protein expressions of CCAAT-enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ), and the nuclear translocation of PPARγ. Furthermore, it inhibited the mRNA expression and phosphorylation of extracellular signal-regulated kinase (ERK), one of mitogen-activated protein kinases. These results indicate that 1α,25-(OH)2-VD3 can be an inhibitor of adipocyte differentiation, and suggest, in addition to C/EBPα and PPARγ, an important role of ERK in mediating 1α,25-(OH)2-VD3-induced alteration in adipocyte differentiation.

Key Words 1α,25-dihydroxy-cholecalciferol, adipocyte differentiation, 3T3-L1 cells, mitogen-activated protein kinases

Obesity is an important disease in the realm of preventive medicine because it is regarded as a risk factor for a broad spectrum of cardiometabolic disturbances, including dyslipidemia, hypertension, glucose intolerance, cardiovascular disease and type 2 diabetes mellitus (1, 2).

Adipocyte precursor cells (i.e., preadipocytes) are present throughout life. Accordingly, obesity may be partially mediated by stimulating the differentiation of preadipocytes into adipocytes or by increasing fat accumulation in the differentiated adipocytes (3). Furthermore, adipose tissue has recently been identified as an endocrine organ that secretes various kinds of bioactive molecules called adipocytokines (4, 5). The differentiation of preadipocytes into adipocytes is accompanied by many changes in gene expression, e.g., a dramatic increase in the expression of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) (6, 7). Recently, it has been suggested that the mitogen-activated protein kinase (MAPK) signaling pathway regulates the expression of C/EBPα and PPARγ mRNA during adipogenesis (8–10). Murine 3T3-L1 preadipocytes have been frequently used to study the differentiation of preadipo-

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Materials and Methods

Materials. Mouse 3T3-L1 preadipocytes were obtained from the European Collection of Cell Cultures, Wiltshire, UK. PPARγ Transcription Factor Assay kit was purchased from Cayman Chemical Co., Ann Arbor,
MI, USA. Rabbit polyclonal anti-human PPARγ antibody and goat HRP-linked anti-rabbit IgG antibody were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA, and Cell Signaling Technology, Beverly, MA, USA, respectively. A phospho-MAPK family antibody sampler kit was obtained from Cell Signaling Technology, Japan, Tokyo, Japan. A transcriptor First Strand cDNA Synthesis kit and LightCycler FirstStart DNA Masterplus SYBR green reagent were obtained from Roche Diagnostics, Indianapolis, IN, USA, TRizol reagent and the primers for β-actin, adiponectin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1 (PAI-1), PPARγ, C/EBPα, p38MAPKα, p38MAPKβ, extracellular signal-regulated kinase 1/2 (ERK1/2) and c-jun N-terminal kinase (JNK) were purchased from Invitrogen, Carlsbad, CA, USA. 1α,25-(OH)2-VD3, 1α-Oh-VD and 25-OH-VD3 were obtained from Sigma Chemical Co., St. Louis, MO, USA. Triglyceride E-test Wako was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were of analytical grade.

Adipocyte differentiation. 3T3-L1 preadipocytes were cultured at 37°C in a humidified atmosphere of 5% CO2/95% air. The cells were maintained in growth medium (DMEM with 10% FBS and 1% penicillin-streptomycin). Differentiation was induced according to the protocol enclosed with the 3T3-L1 preadipocytes from the European Collection of Cell Cultures: differentiation of the cells was initiated 2 d after confluence for 3 d in a growth medium containing 1 μg/mL insulin, 0.25 μM dexamethasone and 0.5 mM IBMX. This was followed by 2 d in a growth medium containing 1 μg/mL insulin. Thereafter, the cells were cultured in the growth medium for 2 d.

Treatment with 1α,25-(OH)2-VD3. 1α,25-(OH)2-VD3 was prepared in Me2SO and added to the medium from day 3 (time of addition of insulin, dexamethasone and IBMX). The Me2SO concentration was maintained at 0.25% of the total volume, and preliminary experiments demonstrated no significant effects of 0.25% Me2SO on cell differentiation.

Measurement of TG. The TG contents in the cells were measured as described previously by us (21). The amount of TG, an index of lipid accumulation, was quantitatively measured using a Triglyceride E-test Wako kit following normalization by protein amounts and expressed as TG contents (μg/mg protein).

Measurement of PPARγ and phosphorylated (p-) MAPK protein expressions. Untreated or 1α,25-(OH)2-VD3-treated 3T3-L1 cells up to day 5 were washed with ice-cold PBS, and then lysed. The prepared lysates were mixed with an equal volume of solubilization buffer [20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 125 mM Tris/HCl (pH 6.8)] and boiled for 10 min. Cell lysates were analyzed using a 7.5% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes by electroblooting and the membranes were incubated overnight in TBS-T (0.14 M NaCl, 20 mM Tris and 0.1% Tween 20, pH 7.4) containing the respective primary antibody (PPARγ, p-p38MAPK, p-ERK or p-JNK antibody) and 3% nonfat dry milk. After incubation, the membranes were incubated with secondary goat HRP-linked anti-rabbit IgG antibody for 1 h, followed by ECL (Amersham-Phamacia Corp., Buckingham, UK).

Measurement of nuclear bioactive PPARγ. Bioactive PPARγ in nuclear extract of 3T3-L1 cells was determined as described in our previous paper (21). Briefly, the nuclear fractions from 3T3-L1 cells were separated using the Nuclear Extraction kit (Marligen Bisciences Inc., Rockville, MD, USA). Bioactive PPARγ in the nucleus was measured using the PPARγ Transcription Factor Assay kit.

Measurement of mRNA expressions. Untreated or 1α,25-(OH)2-VD3-treated 3T3-L1 cells up to day 5 were washed with ice-cold PBS. Total cellular RNA was prepared using TRizol reagent. One microgram of total RNA was reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit. The concentration and quality of the purified total RNA were determined spectrophotometrically at 260 nm and by the OD260:280 ratio. mRNA expression was measured by a real time RT-PCR using a LightCycler FirstStart DNA Masterplus SYBR green reagent and LightCycler instrument (Roche). Results were expressed as relative amount to the target mRNA to β-action mRNA, and the values in the presence or absence of drugs were expressed as relative amount to the None value (no addition of insulin, dexamethasone or IBMX).

The primers for β-actin, adiponectin, TNF-α, PAI-1, PPARγ, C/EBPα, p38MAPKα, p38MAPKβ, ERK1/2 and
Fig. 2. Alterations in TG contents of 3T3-L1 cells treated with 1α,25-(OH)₂-VD₃ analogs. The differentiation of 3T3-L1 preadipocytes was initiated 2 d after confluence for 3 d in growth medium containing 1 μg/mL insulin, 0.25 μM dexamethasone and 0.5 mM IBMX. This was followed by 2 d in growth medium containing 1 μg/mL insulin. Thereafter, the cells were cultured in the growth medium for 2 d. VD₃, 25-OH-VD₃, 1α,25-(OH)₂-VD₃, and 1α,25-(OH)₂-VD₃ (0.5 and 1.0 nM) was added to the medium from day 3 (time of addition of insulin, dexamethasone and IBMX) to day 9 (end point of the experiment). The treated cells were lysed with lysis buffer, and the TG contents were measured using a Triglyceride E-test Wako kit. The data represent the means±SE of five experiments. *p<0.05, **p<0.01; significantly different from Control (Cont.).

Fig. 3. Alterations in TG contents of 3T3-L1 cells treated with 1α,25-(OH)₂-VD₃. The differentiation of 3T3-L1 preadipocytes was initiated 2 d after confluence for 3 d in growth medium containing 1 μg/mL insulin, 0.25 μM dexamethasone and 0.5 mM IBMX. This was followed by 2 d in growth medium containing 1 μg/mL insulin. Thereafter, the cells were cultured in the growth medium for 2 d. VD₃, 25-OH-VD₃, 1α,25-(OH)₂-VD₃, 1α,25-dihydroxy-cholecalciferol; TG, triacylglycerol; IBMX, 3-isobutyl-1-methyl-xanthine.

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**RESULTS**

Figure 1 shows the effect of 1α,25-(OH)₂-VD₃ on the TG contents, a marker of lipid accumulation, in 3T3-L1 cells. An incubation of 3T3-L1 preadipocytes with insulin, dexamethasone and IBMX (Cont.) increased the TG contents by 4–5 fold compared to the None value (no addition of insulin, dexamethasone or IBMX) (data not shown). At concentrations ranging from 0.1 to 100 nM, 1α,25-(OH)₂-VD₃ dose-dependently inhibited the TG contents. The reduction of TG accumulation was apparent.

Statistical analysis. Results are the means±SE. The significance of differences between two groups was assessed by one-way analysis of variance (ANOVA) followed by Scheffe’s multiple range test. p-values less than 0.05 were considered significant.
ent even at a concentration of 0.2 nM (17% inhibition). Yoshimoto and Norman (22), and Tuohimaa et al. (23) have reported that the physiological concentration of $1\alpha,25-(OH)_2VD_3$ in the plasma of chicks and humans is about 125 or 100 pM, respectively. Thus, it seems likely that $1\alpha,25-(OH)_2VD_3$ in the physiological concentration range acts as an inhibitor of TG accumulation in adipocytes.

Figure 4 illustrates the effects of VD$_3$, 25-OH-VD$_3$ and $1\alpha$,OH-VD$_3$ in comparison with $1\alpha,25-(OH)_2VD_3$ on

Fig. 4. Alterations in PPAR$_\gamma$ and C/EBP-$\alpha$ mRNA levels of 3T3-L1 cells treated with VD$_3$ or $1\alpha,25-(OH)_2VD_3$. The differentiation of 3T3-L1 preadipocytes was initiated 2 d after confluence for 3 d in growth medium containing 1 µg/mL insulin, 0.25 µM dexamethasone and 0.5 mM IBMX. This was followed by 2 d in growth medium for 2 d. VD$_3$ or $1\alpha,25-(OH)_2VD_3$ (0.5 and 1.0 nM) was added to the medium from day 3 (time of addition of insulin, dexamethasone and IBMX) to day 5. Total RNA was extracted with TRIzol reagent, and mRNA levels were measured by a real time RT-PCR using a Transcriptor First Strand cDNA Synthesis kit and LightCycler Firststart DNA Master SYBR Green I kit. Results are expressed as relative amount of the target mRNA to $\beta$-actin mRNA, and the values in the presence or absence of drugs are expressed as relative amount to the None value (no addition of insulin, dexamethasone and IBMX). The data represent the mean±SE from four experiments. $^*p<0.01$; significantly different from None. $^p<0.01$; significantly different from Control (Cont.). VD$_3$, cholecalciferol; $1\alpha,25-(OH)_2VD_3$, $1\alpha,25$-dihydroxy-cholecalciferol; PPAR$_\gamma$, peroxisome proliferator-activated receptor $\gamma$; C/EBP-$\alpha$, CCAAT-enhancer binding protein $\alpha$; IBMX, 3-isobutyl-1-methyl-xanthine.

Fig. 5. Alterations in protein expression of PPAR$_\gamma$ in cell lysates (A) and in bioactive PPAR$_\gamma$ in nuclear extract (B) of 3T3-L1 cells treated with VD$_3$ or $1\alpha,25-(OH)_2VD_3$. The differentiation of 3T3-L1 preadipocytes was initiated 2 d after confluence for 3 d in growth medium containing 1 µg/mL insulin, 0.25 µM dexamethasone and 0.5 mM IBMX. This was followed by 2 d in growth medium containing 1 µg/mL insulin. Thereafter, the cells were cultured in the growth medium for 2 d. VD$_3$ or $1\alpha,25-(OH)_2VD_3$ (1.0 nM) was added to the medium from day 3 (time of addition of insulin, dexamethasone and IBMX) to day 5. (A) Detection of protein expression was performed with Western blotting. The density of PPAR$_\gamma$ band was divided by that of the respective $\beta$-actin band. Data represent ratios to the None value (no addition of insulin, dexamethasone or IBMX). (B) The nuclear fractions from 3T3-L1 cells were separated using the Nuclear Extraction kit. Bioactive PPAR$_\gamma$ in the nucleus was measured using the PPAR$_\gamma$ Transcription Factor Assay kit. Data represent ratios to the None value. The data represent the mean±SE from three or four experiments. $^*p<0.01$; significantly different from None; $^p<0.01$; significantly different from Control (Cont.). VD$_3$, cholecalciferol; $1\alpha,25-(OH)_2VD_3$, $1\alpha,25$-dihydroxy-cholecalciferol; PPAR$_\gamma$, peroxisome proliferator-activated receptor $\gamma$; IBMX, 3-isobutyl-1-methyl-xanthine.
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TG contents in 3T3-L1 cells. At a concentration of 10 nM, VD3, 25-OH-VD3 and 1α-OH-VD3 showed no significant effect on the TG accumulation, whereas 1α,25-(OH)2-VD3 inhibited it by 88%. Although 1α-OH-VD3 at a concentration of 100 nM reduced the TG accumulation (56% inhibition), the inhibitory effect was weaker than that of 1α,25-(OH)2-VD3 (92% inhibition). VD3 is produced in the skin in response to ultraviolet B radiation and then converted in the liver to 25-OH-VD3, which acts as a precursor for the renal production of 1α,25-(OH)2-VD3. Therefore, the results of Fig. 2 indicate that, of the two hydroxyl groups, the 1α-hydroxyl position is important for the 1α,25-(OH)2-VD3 effect, and that only 1α,25-(OH)2-VD3 formed naturally in the cell body, but not an intermediate, can down-regulate the TG accumulation.

Figure 3 illustrates the effects of VD3 and 1α,25-(OH)2-VD3 on the mRNA expressions of adiponectin, TNF-α and PAI-1. After incubation with insulin, dexamethasone and IBMX, 3T3-L1 adipocytes produced more mRNA expressions of adiponectin, TNF-α and PAI-1. VD3 at concentrations of 0.5 and 1.0 nM had no significant effect on these three mRNA expression levels, but 1α,25-(OH)2-VD3 at 0.5 and/or 1.0 nM inhibited all of them (adiponectin mRNA, 94 and 96% inhibition; TNF-α mRNA, 8 and 41% inhibition; PAI-1 mRNA, 69 and 86% inhibition). Adipose tissue has been identified as an endocrine organ that secretes various kinds of bioactive molecules called adipokines (4, 5). After differentiation, mature 3T3-L1 adipocytes secrete adiponectin and TNF-α (6, 7). On the other hand, PAI-1 is an important component of the plasminogen/plasmin system as it is the main inhibitor of tissue-type and urokinase-type plasminogen activator. Its plasma level is strongly correlated with parameters that define insulin resistance syndrome, in particular the Body Mass Index and visceral accumulation of body fat, suggesting that PAI-1 may be an adipose tissue-derived circulating peptide (24). PAI-1 has been reported to be produced in 3T3-L1 cells after differentiation (25). Thus, the results of Fig. 3 demonstrate that 1α,25-(OH)2-VD3 under physiological conditions inhibits the differentiation of 3T3-L1 preadipocytes into mature adipocytes.

In an attempt to clarify the mechanism by which 1α,25-(OH)2-VD3 inhibits the adipocyte differentiation,
we measured the mRNA and/or protein expressions of transcriptional regulators involved in adipocyte differentiation (Figs. 4–7). Figures 4 and 5 illustrate the effects of VD3 and 1α,25-(OH)2-VD3 on the mRNA and/or protein expressions of PPARγ and C/EBP-α. As shown in Fig. 4, the addition of insulin, dexamethasone and IBMX increased PPARγ and C/EBP-α mRNA levels. VD3 at 0.5 and 1.0 nM had no significant effect on PPARγ or C/EBP-α mRNA levels, whereas 1α,25-(OH)2-VD3 at the same concentrations inhibited these two mRNA levels by 89 and 95% for PPARγ, and 79 and 85% for C/EBPα. Also, as depicted in Fig. 5, the addition of insulin, dexamethasone and IBMX significantly enhanced the PPARγ in cell lysates and the bioactive PPARγ in the nucleus of 3T3-L1 cells. The co-addition of 1α,25-(OH)2-VD3 (1 nM), but not VD3 (1 nM), with insulin, dexamethasone and IBMX decreased them by 86 and 89%, respectively. These results show that 1α,25-(OH)2-VD3 inhibits adipocyte differentiation via the C/EBP-α and PPARγ pathway. This is in accordance with previous findings reported by Blumberg et al. (26).

Furthermore, Figs. 6 and 7 show the relationship between the MAPK pathway and 1α,25-(OH)2-VD3-induced inhibition of adipocyte differentiation. As shown in Fig. 6, after incubation with insulin, dexamethasone and IBMX, 3T3-L1 adipocytes produced more mRNA expressions of p38MAPKα, p38MAPKβ, ERK1/2 and JNK. The increases in mRNA levels of p38MAPKα, p38MAPKβ and ERK1/2 were inhibited by the addition of 1α,25-(OH)2-VD3 (0.5 and 1.0 nM) p38MAPKα, 52 and 57% inhibition; p38MAPKβ, 36 and 48% inhibition; ERK1/2, 43 and 51% inhibition) but not VD3. In contrast, neither VD3 nor 1α,25-(OH)2-VD3 had a significant effect on the increase in JNK mRNA level induced by insulin, dexamethasone or IBMX. 1α,25-(OH)2-VD3 at 0.5 and 1.0 nM also decreased p-ERK protein expression level, whereas there was no reduction in the protein expression levels of p-p38MAPK and p-JNK (Fig. 7). The results of Figs. 6 and 7 indicate that the decreases in mRNA level and phosphorylation of ERK are closely related to the 1α,25-(OH)2-VD3-induced inhibition of adipocyte differentiation.

**DISCUSSION**

There are some reports concerning the modulatory role of 1α,25-(OH)2-VD3 on adipocyte differentiation (14–20). However, the molecular mechanism of 1α,25-(OH)2-VD3-induced modulation remains unclear. The differentiation of preadipocytes into mature insulin-responsive adipocytes involves exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors commencing with C/EBP-β and C/EBP-δ, which ultimately induce the expression of C/EBP-α and PPARγ (6, 7). Although, several laboratories have investigated the role of ERK1/2 MAPK in regulating adipogenesis, their conclusions are somewhat controversial. Some studies claim that activation of ERK1/2 MAPK by various effectors blocks adipogenesis (27, 28), whereas others suggest that it promotes preadipocyte differentiation (8, 9). The distinguishing factor might involve the precise timing of MAPK activation during the initial stages of differentiation. For instance, effectors that activate the ERK1/2 pathway at late stages of adipogenesis are likely to block adipogenic gene expression due to a MAPK-dependent phosphorylation of PPARγ (29, 30). Activation of the pathway early in adipogenesis prior to PPARγ expression might, on the other hand, promote differentiation by activating transcription factors operating to initiate C/EBP-α and PPARγ expression (10).

In the present study, 1α,25-(OH)2-VD3 potently reduced the mRNA and/or protein expressions of C/EBPα and PPARγ, and the nuclear translocation of PPARγ. Furthermore, it inhibited the mRNA expression and phosphorylation of ERK, one of the MAPKs. These results suggest a possible role of ERK in mediating 1α,25-(OH)2-VD3-induced alteration in adipocyte differentiation.

Sato and Hiragun (17) have reported that 1α,25-
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(OH)_2-VD_3 inhibited morphologic and enzymatic expression during differentiation of 3T3-L1 preadipocytes to mature adipocytes, and that the action of 1α,25-(OH)_2-VD_3 on the adipocyte differentiation may result from a receptor-mediated event. Blumberg et al. (26) have shown that in the presence of 1α,25-(OH)_2-VD_3, VDR blocks adipogenesis of 3T3-L1 cells by down-regulating the C/EBP-α-PPARγ pathway. Thus, it appears that the potent reduction of adipocyte differentiation induced by 1α,25-(OH)_2-VD_3 shown in the present study is mediated by the VDR.

Previously, ERK has been reported to regulate the action of C/EBP-α and/or PPARγ through a direct (29, 30) or indirect (10) mechanism. However, in the present study, it is unclear whether the 1α,25-(OH)_2-VD_3-induced inhibition of adipocyte differentiation can be ascribed to a depression on the ERK effect. Further studies are needed to clarify this problem.

The present findings may provide new information to extend the ongoing debate as to the mechanisms through which 1α,25-(OH)_2-VD_3 plays functional roles both physiologically and pharmacologically in animal and human bodies.

REFERENCES


