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1,25-Dihydroxyvitamin D Induces Lipoprotein Lipase Expression in 3T3-L1 Cells in Association with Adipocyte Differentiation*

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ABSTRACT

1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is known to modulate the development of bone and other mesenchymal cell types. Since osteoblasts and adipocytes are thought to arise in bone marrow from a common progenitor, this work examined the effects of 1,25-$(OH)_2D_3$ on adipocyte development, and in particular on the expression of lipoprotein lipase (LPL), which is an early marker for the differentiated adipocyte. 3T3-L1 preadipocytes were cultured in the presence of 1,25-$(OH)_2D_3$ (10^{-9} to 10^{-7} M) for up to 7 days. LPL activity was measured in the medium and cell extracts, and LPL messenger RNA levels were measured by Northern blotting. When compared to control cells, 10^{-7} M 1,25-$(OH)_2D_3$ increased medium LPL activity by 2- to 3-fold and cellular LPL by 1.5-fold. Significant increases in medium and cellular LPL were observed at 10^{-9} M and were maximal at 10^{-7} M. Along with the increase in LPL activity, there was an increase in LPL messenger RNA by 2-fold at 5 days, and by 5-fold at 7 days. In addition to an increase in LPL, 1,25-$(OH)_2D_3$ increased expression of aP2, an adipocyte-specific marker associated with differentiation. After the addition of 1,25-$(OH)_2D_3$, there was a decrease in 3T3-L1 cell number, which is consistent with differentiation, and a decrease in vitamin D receptors. Finally, these cells developed a different morphology. 1,25-$(OH)_2D_3$-treated cells assumed a rounded appearance, although without detachment from the dish and without the degree of lipid accumulation usually associated with the addition of insulin, isobutylmethylxanthine, and dexamethasone. It is concluded that 1,25-$(OH)_2D_3$ induced LPL expression in 3T3-L1 cells through an induction of differentiation-dependent mechanism(s). These findings suggest an important role for 1,25-$(OH)_2D_3$ in normal adipocyte differentiation. (*Endocrinology* **137:** 1540–1544, 1996)

ADIPOCYTES are terminally differentiated cells that are derived from preadipocyte precursors. Previous studies have identified specific genes that are sequentially activated at different stages during adipocyte development, and a number of these genes have established roles in adipocyte lipid storage and release (1, 2). Lipoprotein lipase (LPL) is one such enzyme and represents a useful early marker for adipocyte differentiation. LPL is found primarily in adipocytes and hydrolyzes the triglyceride core of circulating chylomicrons and very low density lipoproteins into nonesterified fatty acids (NEFA), which are then taken up by the adipocyte. Hence, LPL is a central enzyme in lipoprotein metabolism and is important to the development of obesity.

Hormones and growth factors regulate adipocyte differentiation by modulation of specific adipocyte developmental genes (3). A combination of insulin, isobutylmethylxanthine (IBMX), and dexamethasone, when added to cultures of adipose stromal cells or 3T3-L1 cells (4, 5), induces phenotypic features compatible with the terminal differentiation of adipocytes. Recent evidence suggests that the sterol hormone 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] influences the developmental program of several cell types including hematopoietic cells (6) and bone osteoblasts (7). In addition, 1,25-$(OH)_2D_3$ also modulates adipocyte differentiation in bone organ cultures (8). Since preosteoblasts and preadipocytes are believed to arise from a common mesenchymal stromal cell within bone marrow (9), their developmental programs might share common molecular determinants. The present studies were therefore conducted to examine the effects of 1,25-$(OH)_2D_3$ on 3T3-L1 cells, a preadipocyte cell line that can normally be induced to differentiate into adipocytes by the addition of insulin, dexamethasone, and IBMX. We found that the 1,25-$(OH)_2D_3$ induced differentiation of 3T3-L1 cells in association with an increase in LPL, a key enzyme in adipocyte differentiation.

Materials and Methods

Cells and culture

3T3-L1 cells were purchased from the American Type Tissue Collection and grown in DMEM containing 10% FBS. After no more than three passages, cells were distributed into culture plates and grown to near confluence. Cells were then exposed to 1,25-$(OH)_2D_3$ at the indicated concentrations or induced to fully differentiate with 1 μg/ml insulin, 0.5 mM IBMX, and 0.25 μM dexamethasone for 72 h. Cells were then maintained in DMEM containing 10% serum and 1 μg/ml insulin. Each experiment contained control cells that were incubated in DMEM with 10% serum for the same number of days, to control for the occurrence of spontaneous differentiation. Cells were treated with 1,25-$(OH)_2D_3$ at different times such that all cultures were harvested on the

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same day. Control cells for 1,25-$(OH)_2D_3$ experiments were cultured with 0.01% ethanol for the same number of days.

Measurement of LPL activity

LPL was measured in the medium and in the cells after extraction with nonionic detergent. After removal of the medium, cells were washed with PBS, and the cells were transferred to a tissue grinder and disrupted in the presence of a solution containing deoxycholate/NP-40, as described previously (10). Aliquots of the medium and cell extracts were then assayed promptly for LPL activity.

LPL catalytic activity was measure using an emulsified [^{3}H]triolein substrate as described previously (11). After incubation of 100 μl sample with 100 μl substrate for 30 min, liberated ^{3}H-labeled NEFA were separated from glycerides using the method of Belfrage and Vaughn (12). The cell extracts were diluted 10-fold to remove any inhibitory effects of the detergent. For LPL activity, 1 unit represented 1 neq NEFA released/min/10^6 cells. Cell number was determined as described below.

RNA extraction and Northern blotting

RNA was extracted from 3T3-L1 adipocytes using the method of Chomczynski and Sacchi (13). RNA was quantitated spectrophotometrically, and the quality of RNA was verified by ethidium bromide staining of ribosomal RNA bands on a minigel. After analysis on a 1% agarose gel, total RNA (20 μg) was transferred to nylon and blotted with the random-primed (14) ^{32}P-labeled human LPL complementary DNA (15), followed by the ^{32}P-labeled probe for γ-actin (16), as described previously (17).

Measurement of cell number

3T3-L1 cells were plated in 8-cm^2 dishes and allowed to attach for 24 h. Cells were then treated with 1,25-$(OH)_2D_3$ or 0.01% ethanol. Fresh 1,25-$(OH)_2D_3$ was added with medium changes every 3 days. At selected times during treatment cells were trypsinized and aliquots counted using a Coulter counter.

Measurement of vitamin D receptor (VDR) numbers

The effect of 1,25-$(OH)_2D_3$ treatment on VDR numbers was estimated in 3T3-L1 cells before and after treatment with 1,25-$(OH)_2D_3$ using a whole cell internalization assay (18). Briefly, cells were preincubated in serum-free DMEM for 16 h, then harvested by trypsinization, pelleted, and resuspended in Ham's F-10 medium containing 1% FCS and 25 mM HEPES (pH 7.4). Aliquots (200 μl) of the cell suspension were incubated with labeled and unlabeled 1,25-$(OH)_2D_3$ in a shaking water bath at 37 C for 1 h. Cell suspensions were placed on ice for 15 min and then pelleted by centrifugation of 5 min at 500 × g, the medium was aspirated, and the cells were rinsed twice with 200 μl Ham's F-10 medium. The pellet was resuspended in 200 μl TKM buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 300 mM KCl, and 10 mM sodium molybdate] containing 0.5% Triton X-100 and vortexed. After incubation at 4 C for 15 min, the samples were diluted to 1.0 ml with TM buffer (TKM buffer without KCl) to yield a final potassium concentration of 60 mM in the sample. Receptor-bound [^{3}H]1,25-$(OH)_2$-D_3 was separated from unbound hormone on diethylaminoethyl cellulose filters, which were then counted using a scintillation counter. Because the cells had not been treated with 1,25-$(OH)_2D_3$ for 2 days before VDR assay and were changed to serum-free medium 16 h before assay, the VDR would be expected to be unoccupied.

Statistics

All data are expressed as the mean ± SEM and were analyzed nonparametrically using the Wilcoxon matched-paired signed-rank test for paired data.

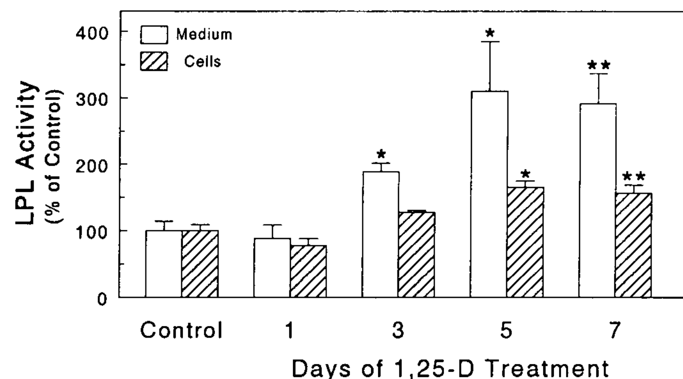

FIG. 1. Time course of effect of 1,25-$(OH)_2D_3$ on LPL activity. 1,25-$(OH)_2D_3$ (10^{-8} M) was added to the medium of confluent 3T3-L1 cells at the indicated number of days before cell harvest. LPL was measured in the medium, and in cell extracts, as described in *Materials and Methods*. Data are expressed in relation to the activity in control cells. Mean control cell LPL was 0.104 neq/min/ml in the medium, and 1.324 neq/min/10^6 cells for cellular LPL. *, $P < 0.05$. **, $P < 0.01$.

RESULTS

After reaching confluence, 1,25-$(OH)_2D_3$ was added to the medium at a concentration of 10^{-8} M, and LPL was measured on 1, 3, 5, and 7 days after the addition of 1,25-$(OH)_2D_3$. As shown in Fig. 1, when compared to the LPL activity in control cultures, there was a progressive increase in LPL activity. This increase in LPL activity was noted in the medium, which represents LPL spontaneously secreted from the cells, as well as in the cellular fraction.

The stimulation of LPL activity by 1,25-$(OH)_2D_3$ was time- and dose-dependent. Treatment of cells with as little as 10^{-9} M 1,25-$(OH)_2D_3$ for 5 days resulted in significant increases in medium LPL, with lesser effects on cellular LPL activity (Fig. 2). Treatment of cells with a maximum dose (10^{-7} M) resulted in significant increments in medium LPL activity by day 3; maximum LPL activity was seen 5 days after 1,25-$(OH)_2D_3$ treatment (Fig. 1).

The increase in LPL activity that was induced by 1,25-$(OH)_2D_3$ was associated with an increase in mRNA levels, as shown in Fig. 3. Northern blots of total RNA from cells exposed to 1,25-$(OH)_2D_3$ for up to 7 days showed a progres-

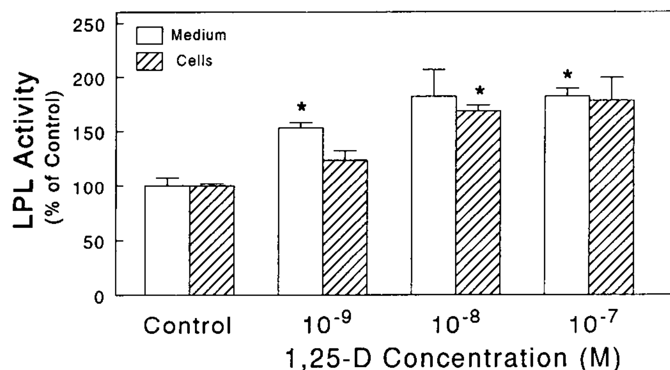

FIG. 2. Effect of increasing 1,25-$(OH)_2D_3$ concentration on LPL. 1,25-$(OH)_2D_3$ was added to confluent cultures of 3T3-L1 cells for 5 days at concentrations of 10^{-9} M to 10^{-7} M. LPL activity in the medium and cells was measured. *, $P < 0.05$. Mean control cell LPL was 0.074 neq/min/ml in the medium, and 1.223 neq/min/10^6 cells for cellular LPL.

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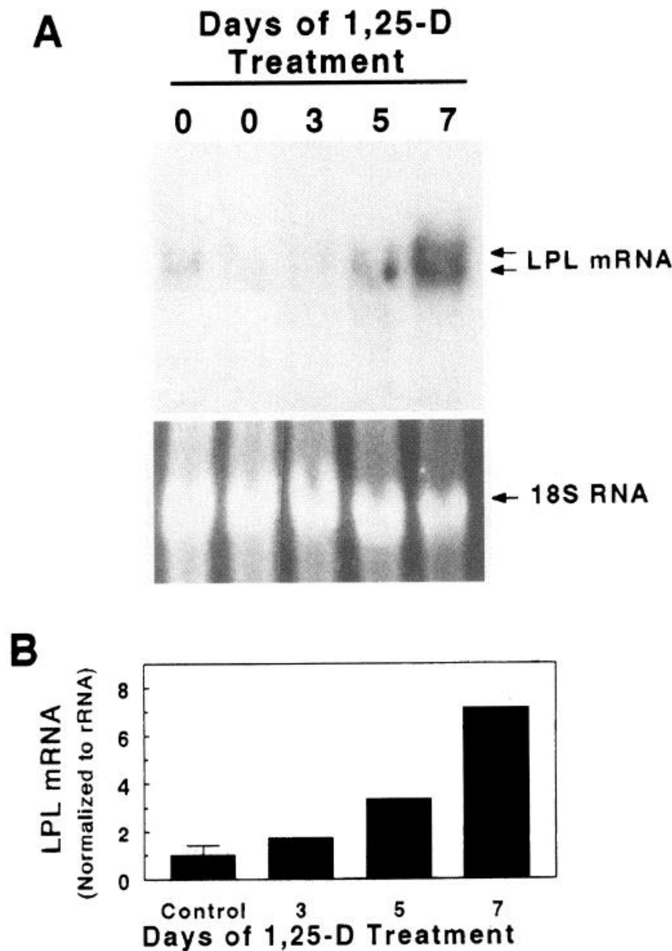

FIG. 3. Effect of 1,25-$(OH)_2D_3$ on LPL mRNA levels. A, Cells were cultured as described for Fig. 1, except RNA was extracted and Northern blotting was performed. Gels were loaded with equal quantities (20 μg) of total RNA, and this was confirmed by minigel with ethidium bromide staining. Shown is one of three representative Northern blots. B, Densitometric quantification of the image in panel A, expressed in relation to the scanned image of ribosomal RNA subunits.

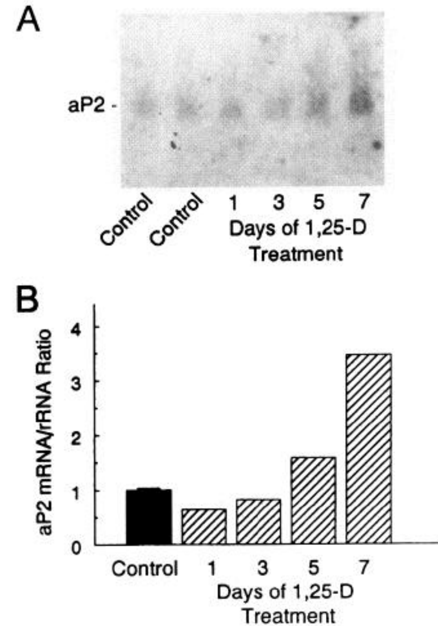

FIG. 4. Effect of 1,25-$(OH)_2D_3$ on aP2 expression. Cells were cultured as described for Fig. 3, and Northern blots were performed to examine the expression of aP2. A. One of two representative Northern blots. B, Densitometric analysis of the image in part A.

sive increase in the LPL mRNA. Gels were loaded with equal quantities of total RNA, and the quantity of RNA was confirmed by ethidium bromide staining of the RNA samples (Fig. 3).

The increase in LPL in response to 1,25-$(OH)_2D_3$ could have been a direct effect of the sterol on LPL gene expression or a secondary response to induction of 3T3-L1 cell differentiation. To determine whether there was an induction of adipocyte differentiation, we determined the effect of 1,25-$(OH)_2D_3$ on the expression of aP2, an adipocyte-specific marker known to increase with preadipocyte differentiation (19). Cultures of 3T3-L1 cells were treated with 10^{-7} M 1,25-$(OH)_2D_3$ for up to 7 days, and the expression of aP2 was assessed by Northern blotting. As shown in Fig. 4, an increase in aP2 expression was apparent by 5 days in culture, indicating that an increase in aP2 occurred over a similar time course as the increase in LPL.

1,25-$(OH)_2D_3$-induced differentiation of hematopoietic cells is usually accompanied by a retardation of cell proliferation and a down-regulation of VDR expression (6). 1,25-$(OH)_2D_3$ treatment of 3T3-L1 cells produced dose- and time-dependent decreases in cell proliferation, as shown in Fig. 5. Treatment with 10^{-7} M 1,25-$(OH)_2D_3$ resulted in a progressive decrease in 3T3-L1 cell number ($P < 0.05$ at day 7) in close temporal agreement with the induction of LPL activity (Fig. 5A). In response to increasing concentrations of 1,25-$(OH)_2D_3$, there was a trend for decreased cell number, although these data did not reach statistical significance (Fig. 5B). In addition, VDR number, as determined by [^{3}H]1,25-$(OH)_2D_3$ uptake, was markedly decreased after 7 days' treatment with 1,25-$(OH)_2D_3$, as compared with untreated control cells (Fig. 6).

3T3-L1 cells can be induced to differentiate by the addition of insulin, IBMX, and dexamethasone. We wished to compare this method of differentiation with 1,25-$(OH)_2D_3$-mediated adipocyte differentiation. Cells were plated into six-well clusters and induced to differentiate with either 1,25-$(OH)_2D_3$ at 10^{-7} M or the combination of insulin, IBMX, and dexamethasone, as described in *Materials and Methods*. As shown in Fig. 7, both combinations led to an increase in LPL activity over 7 days. However, 1,25-$(OH)_2D_3$ exerted a predominant increase in secreted (medium) LPL activity, whereas insulin, IBMX, and dexamethasone primarily increased cellular LPL. In addition to these differences in LPL activity, there were differences in morphology. Cells induced to differentiate with insulin, IBMX, and dexamethasone demonstrated typical massive lipid accumulation. However, cells that were exposed to 1,25-$(OH)_2D_3$ became rounder and accumulated some cytoplasmic vesicles but did not accumulate as much lipid as did the cells exposed to insulin, IBMX, and dexamethasone (data not shown).

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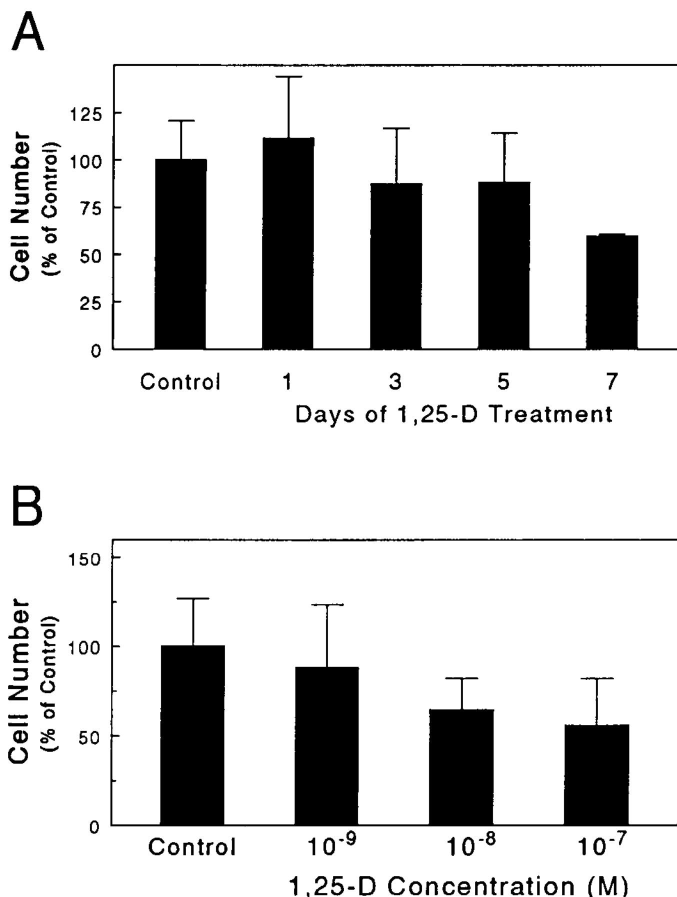

FIG. 5. Effect of 1,25-$(OH)_2D_3$ on 3T3-L1 cell number. Cells were cultured as described above, followed by cell counting, as described in *Materials and Methods*. A, Time course of 1,25-$(OH)_2D_3$ on cell number. *, $P < 0.05$. B, Dose-response relationship of 1,25-$(OH)_2D_3$ on cell number.

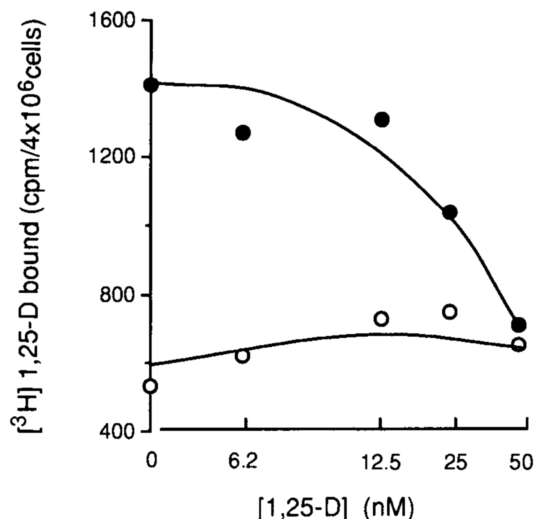

FIG. 6. Assessment of VDR levels in 3T3-L1 cells after 1,25-$(OH)_2D_3$-induced differentiation. Cells were treated with 100 nM 1,25-$(OH)_2D_3$ in 0.01% % ethanol (●) or ethanol alone (○) for 7 days. VDR numbers were then estimated by specific uptake of [^{3}H]1,25-$(OH)_2D_3$ using a competitive displacement assay as descried in *Materials and Methods*. 1,25-$(OH)_2D_3$ treatment caused a marked decrease in the apparent receptor number.

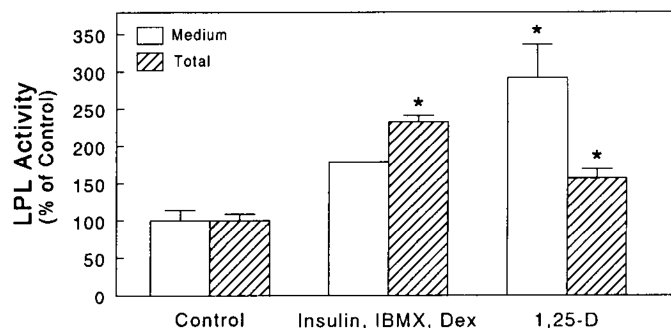

FIG. 7. LPL activity in response to different methods of differentiation. Cells were grown to confluence and induced to differentiate with either 1,25-$(OH)_2D_3$ or the combination of insulin, IBMX, and dexamethasone, as described in *Materials and Methods*. LPL activity was measured in the medium and the cells 7 days after the addition of the differentiation agents and was compared with the activity in control cells.

DISCUSSION

In response to a number of different hormones, preadipocytes can be induced to differentiate into mature, terminally differentiated adipocytes. This process is characterized by the induction of a number of specific genes, many of which are unique to adipocytes and play a key role in establishing the metabolic characteristics of the adipocyte. These genes may either enhance or suppress the activity of key enzymes, which initiate and sustain a cascade of molecular events that ultimately consummate adipocyte differentiation. LPL is one such gene that is central to adipocyte lipogenesis and is expressed at high levels in this cell. Although low levels of LPL are found in a variety of cells, only adipose tissue and muscle express high levels of LPL.

In the present study, we showed that the sterol hormone 1,25-$(OH)_2D_3$, which is known to regulate the differentiation of hematopoietic and bone cells, is also capable of modulating the differentiation of adipocyte-like cells. The marked induction of LPL by 1,25-$(OH)_2D_3$ was associated with an increase in LPL mRNA, suggesting an effect on LPL transcription or mRNA stabilization. Although it is possible that 1,25-$(OH)_2D_3$ directly activated LPL expression, our data suggested that the increase in LPL was part of a generalized induction of adipocyte differentiation. There are several reasons for this conclusion. First, much of the LPL gene promoter has been described (20), and this sequence contains no consensus vitamin D response element (21). Furthermore, induction of differentiation by 1,25-$(OH)_2D_3$ was accompanied by induction of expression of aP2, which is an adipocyte-specific gene that increases in expression during adipocyte differentiation (19). In addition, the induction of LPL by 1,25-$(OH)_2D_3$ was accompanied by a decrease in cell number, which is a feature of 1,25-$(OH)_2D_3$ effects on differentiation of monocytes into macrophages (6). Finally, 1,25-$(OH)_2D_3$ induced a change in adipocyte morphology, although these morphological changes were different than those induced by insulin, IBMX, and dexamethasone. Previous studies by Bellows *et al.* (22) have shown that 1,25-$(OH)_2D_3$ increased adipocyte formation in cultured fetal rat calvaria cells. Importantly, these investigators found that

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once the cells were committed to the adipocyte pathway, they maintained their differentiated state even in the absence of hormone. Taken together, these data suggest that 1,25-$(OH)_2D_3$ induced a cascade of cellular events that lead to an increase in LPL as part of the differentiation process.

The ability of 1,25-$(OH)_2D$ to induce adipocyte differentiation is especially intriguing considering that precursors of several different mesenchymal cells, including preosteoblasts and preadipocytes, are found in bone marrow and are thought to derive from a common stem cell precursor (9). The mature adipocytes residing in bone marrow express LPL, can be induced to differentiate with insulin, IBMX, and dexamethasone, and are similar to adipocytes found elsewhere (23). The exact function of adipocytes in bone marrow is not fully understood, but it is likely that they provide a source of fuel for other cells that reside in bone marrow.

In summary, the effects of 1,25-$(OH)_2D_3$ on LPL in 3T3-L1 cells were studied. The addition of 1,25-$(OH)_2D_3$ to cultures of preadipocytes resulted in an increase in LPL expression, along with an increase in expression of aP2 and a decrease in cell number, all of which suggest an induction of differentiation. The characterization of the molecular events that mediate differentiation of adipocyte precursors into highly specialized adipocytes during development is fundamental to a better understanding of lipid metabolism in health and disease. These studies suggest that 1,25-$(OH)_2D_3$ is one of many hormones and growth factors that play an important developmental role in adipose tissue.

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