The Mechanism of 1,25-Dihydroxyvitamin D₃ Autoregulation in Keratinocytes*

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The synthesis of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) from its precursor, 25-dihydroxyvitamin D₃ (25(OH)D₃), is catalyzed by the mitochondrial cytochrome P450 enzyme 25-hydroxyvitamin D₃-1α-hydroxylase (1α-hydroxylase). It has been generally assumed that 1,25(OH)₂D₃ inhibits the activity of this enzyme by regulating its expression at the genomic level. We confirmed that 1,25(OH)₂D₃ reduced the apparent conversion of 25(OH)D₃ to 1,25(OH)₂D₃ while stimulating the conversion of 25(OH)D₃ to 1,24,25(OH)₃D₃ and 24,25(OH)₂D₃ respectively. However, 1,25(OH)₂D₃ failed to reduce the abundance of its mRNA or its encoded protein in human keratinocytes. Instead, when catabolism of 1,25(OH)₂D₃ was blocked with a specific inhibitor of the 25-hydroxyvitamin D₃-24-hydroxylase (24-hydroxylase) all apparent inhibition of 1α-hydroxylase activity by 1,25(OH)₂D₃ was reversed. Thus, the apparent reduction in 1α-hydroxylase activity induced by 1,25(OH)₂D₃ is due to increased catabolism of both substrate and product by the 24-hydroxylase. We believe this to be a unique mechanism for autoregulation of steroid hormone synthesis.

Vitamin D₃ is synthesized in the skin, undergoes 25-hydroxylation catalyzed by vitamin D₃-25-hydroxylase in the liver (1, 2), followed by 1α-hydroxylation in the kidney and other tissues to make the biologically active hormone 1,25(OH)₂D₃. The 1α-hydroxylase activity is catalyzed by mitochondrial P450c1α, encoded by a gene termed CYP27B1 (3–5). This is an activation pathway for vitamin D metabolites. 25(OH)D₃ and 1,25(OH)₂D₃ can then be converted to the less active forms 24,25(OH)₂D₃ and 1,24,25(OH)₃D₃, respectively, by the 24-hydroxylase activity of P450c24 encoded by a gene termed CYP24 (6, 7). These are inactivation pathways for vitamin D metabolites. The most important site of 1α-hydroxylation and 24-hydroxylase activities is in proximal renal tubular cells, but these enzymes are also found in various extrarenal tissues including epidermal keratinocytes (4, 8, 9). Keratinocytes synthesize and catabolize 1,25(OH)₂D₃ (4, 8, 10), contain 1,25(OH)₂D₃ receptors (11, 12), and respond to 1,25(OH)₂D₃ with accelerated differentiation (13, 14). It has been postulated that the conversion of 25(OH)D₃ to 1,25(OH)₂D₃ is inhibited by 1,25(OH)₂D₃ itself, through inhibition of 1α-hydroxylase gene expression (8, 15), just as the conversion of 25(OH)D₃ to 24,25(OH)₂D₃ or of 1,25(OH)₂D₃ to 1,24,25(OH)₃D₃ is increased by 1,25(OH)₂D₃ through induction of 24-hydroxylase (16). However, recent studies (17, 18) have suggested that 1,25(OH)₂D₃ may not regulate the 1α-hydroxylase by such a mechanism. To test this hypothesis, we examined the effect of exogenous 1,25(OH)₂D₃ on the abundance of 1α-hydroxylase mRNA and protein in cultured normal human keratinocytes compared with the apparent activity of the 1α-hydroxylase under conditions in which 24-hydroxylase was inhibited. We found that 1,25(OH)₂D₃ did not inhibit 1α-hydroxylase enzyme activity or the abundance of its mRNA or protein. Rather, the feedback regulation of 1,25(OH)₂D₃ involves its catabolism by 24-hydroxylase induction instead of its synthesis by 1α-hydroxylase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as previously described (19). Briefly, keratinocytes were isolated from newborn human foreskins by trypsinization (0.25% trypsin, 4 °C, overnight), and primary cultures were established in KGM containing 0.07 mM calcium. First and second passage keratinocytes were plated with KGM containing 0.03 mM calcium and used in the subsequent experiments described.

**Enzyme Activity Assays—**To measure 1α-hydroxylase and 24-hydroxylase activities, we used the method of Bikle et al. (8). Briefly, 0.05 μCi of [³H]25(OH)D₃ or [³H]1,25(OH)₂D₃ (Amersham Biosciences) was added to cultured normal human keratinocytes in 6-well plates. Following 1 h of incubation at 37 °C, the reaction was stopped with 1 ml of methanol. Both cells and medium were extracted by the method of Bligh and Dyer (20). Metabolites in the chloroform extract were separated and quantitated by a Waters high performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA) linked to a Flow Scintillation Analyzer (Packard, Meriden, CT). HPLC utilized a DuPont Zorbax Sil column (4.6 × 25 cm) and a non-linear concave gradient from 97.3 to 90:10 hexane:isopropanol for 1α-hydroxylated steroids or 90:10 hexane:isopropanol for 24-hydroxylated steroids. Output was monitored by radioactivity with a Flow Scintillation Analyzer (Packard, Downers Grove, IL). Chemically synthesized standards were used to determine the elution volumes of the metabolites.

**RNA Analysis—**Total RNA was isolated from the keratinocytes using the STAT-60® kit (Tel-Test “B”, Inc., Friendswood, TX), according to the procedures recommended by the manufacturer. The isolated RNA (20 mg per lane) was electrophoresed through a 0.8% agarose-formaldehyde gel, transferred to a nylon membrane (Hyb-n-N; Amersham...
Biosciences) using PosiBlot 30–30 Pressure Blotter (Stratagene, La Jolla, CA), and immobilized by baking the membrane at 80 °C for 2 h. Complementary DNA probes for human 1α-hydroxylase (4) and human involucrin (gift from Dr. Howard Green, Harvard Medical School) were labeled with [32P]dCTP (Amersham Biosciences) by Random Prime-IT, II labeling kit (Stratagene), and purified by NucTrap Probe Purification Columns (Stratagene). The membrane was prehybridized and hybridized in 5× SSC, 0.5% SDS, and 20 mg/ml salmon sperm DNA with the 32P-labeled human 1α-hydroxylase and involucrin cDNAs. After hybridization at 65 °C overnight, the membrane was washed in solutions with decreasing ionic strength and increasing temperature to a final stringency of 0.1× SSC and 0.1% SDS at 65 °C. The [32P]cDNA-mRNA hybrids were visualized by exposing to x-ray film. The 18 S ribosomal RNA on the same RNA blot hybridized with a 32P-labeled cDNA for 18 S RNA was used as a control.

Immunoblotting—Keratinocytes were washed twice with PBS and then incubated in lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, and 2 mg/ml aprotinin for 5 min. Cells were scraped into microcentrifuge tubes, incubated on ice for 15 min, and pelleted by centrifugation. The supernatant was collected. The protein concentration of the lysate was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were then electrophoresed through 7.5% polyacrylamide gels at 200 V for 30 min and electroblotted onto polyvinylidene difluoride membranes (0.2 micron, Bio-Rad Laboratories, Hercules, CA) in an electroblotting buffer (25 mM Tris, 192 mM glycine, 5% methanol) at 130 V for 2 h. After incubation in blocking buffer (100 mM Tris base, 150 mM NaCl, 5% non-fat milk, and 0.5% Tween 20), the blot was incubated with the appropriate primary antibodies overnight at 4 °C. 1α-hydroxylase protein was detected with a polyclonal rabbit anti-human antibody (21) at a dilution of 1:20,000 in blocking buffer. Involucrin protein was detected with a monoclonal mouse anti-human involucrin antibody (Sigma) at a dilution of 1:2000 in blocking buffer. After washes in the blocking buffer, the membranes were incubated for 1 h.

FIG. 1. The effect of 1,25(OH)2D3 on 25(OH)D3 metabolism in keratinocytes. Second passage human keratinocytes cultured in KGM containing 0.03 mM calcium were treated with 1,25(OH)2D3 at concentrations indicated for 16 h. The medium was removed and cells washed with fresh medium and then incubated with [3H]25(OH)D3 for 1 h. The concentration of 1,25(OH)2D3, 24,25(OH)2D3, and 1,24,25(OH)3D3 were measured. Data are presented as means ± range of duplicate determinations.

FIG. 2. The effect of 1,25(OH)2D3 on 1α-hydroxylase mRNA and protein abundance. Second passage human keratinocytes grown to 70% confluence in KGM containing 0.03 mM calcium were treated with vehicle (ethanol) or 1,25(OH)2D3 at the concentrations indicated. The cells were harvested for RNA 24 h later or for protein 48 h later. The 1α-hydroxylase mRNA, involucrin mRNA, and 18 S ribosomal RNA levels were analyzed by Northern analysis (A). 1α-hydroxylase and involucrin protein levels were analyzed by immunoblotting (B).

FIG. 3. Metabolism of [3H]1,25(OH)2-D3 in keratinocytes was affected by prior incubation with unlabeled 1,25(OH)2D3. Second passage human keratinocytes cultured in KGM containing 0.03 mM calcium were treated with unlabeled 1,25(OH)2D3 at concentrations indicated for 16 h. The medium was removed and cells washed with fresh medium and then incubated with [3H]1,25(OH)2D3 for 1 h. The data are shown as the percent recovery of radioactivity in the various peaks following HPLC of the chloroform extract or in the aqueous extract. Data are presented as means ± range of duplicate determinations.
16 h later. The apparent production of [3H]1,25(OH)2D3 from [3H]1,25(OH)2D3 to [3H]1,24,25(OH)3D3 and [3H]25(OH)D3 to [3H]1,25(OH)2D3 were measured using HPLC. The conversion rates of varying concentrations (from 10⁻⁸ to 10⁻⁶ M) of 1,25(OH)2D3 were not regulated by 1,25(OH)2D3 production by 1,25(OH)2D3 is due to [3H]1,25(OH)2D3 in keratinocytes was dramatically reduced by a VID400. VID400 inhibited 24-hydroxylase activity in a dose-dependent manner with essentially no [3H]1,25(OH)2D3 recoverable in the cells pretreated with 10⁻⁸ M 1,25(OH)2D3. The increase in [3H]24,25(OH)3D3 was biphasic with maximal concentration of 1,24,25(OH)3D3 observed in keratinocytes pretreated with 10⁻⁹ M 1,25(OH)2D3.

These results indicate that 1,25(OH)2D3 inhibited 1α-hydroxylase activity and stimulated 24-hydroxylase activity. We then determined whether the abundances of 1α-hydroxylase mRNA and protein were regulated by 1,25(OH)2D3. A 2.4-kb mRNA transcript and a 56-kDa protein were detected by Northern and Western analysis, respectively. The levels of 1α-hydroxylase mRNA and its protein in human keratinocytes were not regulated by 10⁻¹⁰ to 10⁻⁶ M 1,25(OH)2D3. In contrast, the level of mRNA and protein for involucrin (22), a marker for keratinocyte differentiation and analyzed on the same blots, were stimulated in a dose-dependent manner by 1,25(OH)2D3 (Fig. 2, A and B). These data indicate that [3H]1,25(OH)2D3 does not regulate 1α-hydroxylase mRNA or protein expression.

These results led us to hypothesize that the apparent inhibition of 1,25(OH)2D3 production by 1,25(OH)2D3 is due to increased catabolism of 1,25(OH)2D3 via induction of the 24-hydroxylase. To test this hypothesis, we incubated human keratinocytes with [3H]1,25(OH)2D3 for 1 h after exposing them to 10⁻⁹ M 1,25(OH)2D3 for 16 h. The results showed that 1,25(OH)2D3 markedly enhanced the ability of keratinocytes to metabolize [3H]1,25(OH)2D3 to both [3H]1α,23(S)-25(OH)D3 and [3H]1,24,25(OH)3D3. Only 20% of the added [3H]1,25(OH)2D3 was recovered in the cells preincubated with 1,25(OH)2D3, whereas 60% of the added [3H]1,25(OH)2D3 was recovered in the cells pretreated with vehicle (Fig. 3). These data demonstrate that exogenous 1,25(OH)2D3 stimulates the catabolism of endogenously produced 1,25(OH)2D3 in human keratinocytes. To test this hypothesis further, we examined the effect of an inhibitor of the 24-hydroxylase (VID400) (18, 23) on 1α-hydroxylase and 24-hydroxylase activities in keratinocytes. Cells were preincubated overnight with either 1,25(OH)2D3 or vehicle, and the conversion of [3H]1,25(OH)2D3 to [3H]24,25(OH)2D3 or [3H]25(OH)D3, to [3H]1,25(OH)2D3 was measured in the absence or presence of 25, 50, 100, and 200 nM VID400. VID400 inhibited 24-hydroxylase activity in a dose-dependent manner with complete inhibition at 200 nM (Fig. 4A). However, 200 nM VID400 inhibited 1α-hydroxylase activ-

![Fig. 4. The effect of VID400 on 24-hydroxylase and 1α-hydroxylase activities.](image)

![Fig. 5. The effect of 1,25(OH)2D3 on 1α-hydroxylase activity.](image)
Regulation of 25-Hydroxyvitamin D-1α-Hydroxylase

...by more than 30% (Fig. 4B). In contrast, 100 nM VID400 inhibited 24-hydroxylase activity by more than 90% (Fig. 4A) but only slightly inhibited 1α-hydroxylase activity (Fig. 4B). Thus, we used 100 nM VID400 to determine the degree to which induction of 24-hydroxylase contributed to the apparent inhibition of 1α-hydroxylase activity by 1,25(OH)₂D₃. Keratinocytes were preincubated for 24 h with 1,25(OH)₂D₃, and the conversion of [³H]25(OH)D₃ to [³H]1,25(OH)₂D₃ was measured with and without the presence of VID400. Preincubation with 10⁻⁹ M 1,25(OH)₂D₃ reduced the apparent 1α-hydroxylase activity by 90%. However, addition of 100 nM VID400 restored most of the apparent reduction in 1α-hydroxylase activity (Fig. 5). When the concentration of VID400 was increased to 200 nM, no reduction in apparent 1α-hydroxylase activity was observed in the cells treated with 1,25(OH)₂D₃, although basal activity was reduced (data not shown). In agreement with recent data (18), these results indicate that the 1α-hydroxylase activity is not regulated by 1,25(OH)₂D₃. The apparent reduction in 1,25(OH)₂D₃ production is due to increased catabolism of [³H]1,25(OH)₂D₃ and [³H]25(OH)D₃ via the 24-hydroxylase, induced by 1,25(OH)₂D₃.

The intracellular concentration of 1,25(OH)₂D₃ in keratinocytes is controlled by 1α-hydroxylase and 24-hydroxylase, which are responsible for 1,25(OH)₂D₃ synthesis and degradation, respectively. Transcription of the gene encoding 24-hydroxylase is induced by 1,25(OH)₂D₃, and the promoter of this gene contains two vitamin D-responsive elements (24) that mediate the ability of 1,25(OH)₂D₃ to induce this gene in keratinocytes (25) and other cells. Studies purporting to demonstrate negative regulation of 1α-hydroxylase by 1,25(OH)₂D₃ need to be reconsidered in light of our observations. For example, mice lacking the vitamin D receptor have increased amounts of 1α-hydroxylase mRNA (3), suggesting a genomic mechanism of regulation by 1,25(OH)₂D₃. However, parathyroid hormone can induce transcription of the gene for 1α-hydroxylase (26), and parathyroid hormone is quite elevated in mice lacking the vitamin D receptor. Thus, increased serum concentrations of parathyroid hormone, rather than decreased 1,25(OH)₂D₃, could well be responsible for the increased abundance of 1α-hydroxylase mRNA in these animals. The present data support the conclusion that the regulation of 1,25(OH)₂D₃ levels by 1,25(OH)₂D₃ itself involves induction of its catabolism rather than inhibition of its production. This mechanism appears to be a unique mode for autoregulation of steroid hormone synthesis.

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