Effect of vitamin D_3 derivatives on cholesterol synthesis and HMG-CoA reductase activity in cultured cells

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Abstract Treatment of logarithmically growing rat intestinal epithelial cells (IEC-6) in culture with vitamin D_3 (cholecalciferol), 25-hydroxy vitamin D_3 (25-hydroxy cholecalciferol), 1,25-dihydroxy vitamin D_3 (1,25-dihydroxycholecalciferol), and 24,25 dihydroxy vitamin D_3 (24(R),25-dihydroxycholecalciferol), caused an inhibition of the cholesterol biosynthetic pathway at two separate sites. At concentrations > 2 μg/ml, the hydroxylated forms of vitamin D_3 caused an accumulation of methyl sterols indicating an inhibition of lanosterol demethylation. Vitamin D_3, however, had little effect on lanosterol demethylation. A second site of inhibition occurs at 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate limiting enzyme in cholesterol biosynthesis at concentrations < 2 μg/ml. All vitamin D_3 compounds, except 1,25-dihydroxy vitamin D_3, inhibited HMG-CoA reductase activity in a concentration-dependent manner. The lack of inhibition of HMG-CoA reductase activity by 1,25-dihydroxy vitamin D_3 in IEC-6 cells was not due to impaired uptake, since 1,25-dihydroxy vitamin D_3 caused an accumulation of methyl sterols under similar conditions. The inhibition of HMG-CoA reductase activity and cholesterol synthesis by vitamin D_3 and 25-hydroxy vitamin D_3 was also observed in other cell culture lines such as human skin fibroblasts (GM-43), transformed human liver cells (Hep G2), and mouse peritoneal macrophages (J-774). On the other hand, 1,25-hydroxy vitamin D_3 showed effects on HMG-CoA reductase activity that varied with the cell line. In J-774 and human skin fibroblasts, 1,25-dihydroxy vitamin D_3 showed a biphasic effect on reductase activity such that at low concentrations reductase activity was inhibited but was restored to control values at high concentrations. In Hep G2 cells, 1,25-dihydroxy vitamin D_3 greatly stimulated HMG-CoA reductase activity which was attenuated by 25-hydroxy vitamin D_3. Treatment of cells with ketoconazole (30 μM), a known inhibitor of the cytochrome P-450-dependent hydroxylation of vitamin D_3, attenuated the effect of D_3 on reductase activity. However, the drug had no effect on the inhibition of reductase activity by 25-hydroxy vitamin D_3 or 24(R),25-dihydroxy vitamin D_3. The drug also prevented the metabolism of [^{3}H]vitamin D_3 to 25-hydroxy vitamin D_3 and to other polar derivatives. These results strongly indicate that inhibition of HMG-CoA reductase activity by vitamin D_3 requires a hydroxylation step that is sensitive to ketoconazole. The observation of varying effects of vitamin D_3 and its hydroxylated derivatives on HMG-CoA reductase activity and sterol biosynthesis establishes new site(s) for the activity of vitamin D_3 derivatives in addition to already known biological functions.—**Gupta, A. K., R. C. Sexton, and H. Rudney.** Effect of vitamin D_3 derivatives on cholesterol synthesis and HMG-CoA reductase activity in cultured cells. *J. Lipid Res.* 1989. **30:** 379–386.

Supplementary key words IEC-6 cells • GM-43 cells • J-774 cells • Hep G2 cells • vitamin D_3 • 25-hydroxy vitamin D_3 • 1,25-dihydroxy vitamin D_3 • 24(R),25 dihydroxy vitamin D_3 • ketoconazole • lanosterol 14α-demethylase

Cholesterol is an essential component of mammalian cell membranes and is necessary for cell growth. It is widely accepted that the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the major rate-limiting step in the biosynthesis of cholesterol (1). Recent studies from several laboratories have suggested that oxygenated derivatives of cholesterol are physiological regulators of cholesterol synthesis by inhibiting the activity of HMG-CoA reductase in cultured cells (2–8). Such oxygenated sterols can be formed either by controlled enzymatic oxidation of cholesterol, or from lanosterol, or from diversion of squalene 2,3 epoxide to squalene 2,3:22,23 dioxide and subsequent cyclization (3, 8).

Cholecalciferol compounds (vitamin D_3 derivatives) are derived from 7-dehydrocholesterol, an intermediate in cholesterol biosynthesis. The metabolic formation of various vitamin D_3 derivatives and their biological action have been reviewed (9, 10). Side chain hydroxylation of the vitamin D_3 is a key step in the formation of the active forms of the vitamin. Since side chain hydroxylation of sterols generates potent inhibitors of reductase activity (2), it was of interest

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; vitamin D_3, cholecalciferol; 25-hydroxy vitamin D_3, 25-hydroxy cholecalciferol; 1,25-dihydroxy vitamin D_3, 1,25-dihydroxy cholecalciferol; 24(R),25-dihydroxy vitamin D_3, 24(R), 25-dihydroxy cholecalciferol; LPDS, lipoprotein-deficient fetal bovine serum ($d > 1.21$); TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; DMSO, dimethylsulfoxide.

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to study the effect of vitamin D_3 and its hydroxylated derivatives on reductase activity and sterol biosynthesis. There is an early observation that hydroxylated derivatives of vitamin D_3 inhibited cholesterol biosynthesis from acetate in normal and leukemic (L_2C) guinea pig lymphocytes (11). Defay et al. (12, 13) have shown that hydroxylated derivatives of vitamin D_3 inhibited HMG-CoA reductase activity in phytohemaglutinin-stimulated human lymphocytes. We report here our investigations on the site of action of vitamin D_3 and its hydroxylated derivatives on sterol biosynthesis in several cultured cell lines. Our results show that only the hydroxylated derivatives of vitamin D_3 decrease cholesterol biosynthesis by inhibiting the activity of HMG-CoA reductase and lanosterol 14α-demethylase. Unexpected results were obtained with the dihydroxy derivatives of D_3, 1,25-dihydroxy vitamin D_3, the biologically active form of the steroid hormone. This secosteroid inhibited lanosterol 14α-demethylase activity in all cell line tests and yet expressed a variable effect on HMG-CoA reductase activity. Overall, our results suggest that the hydroxylated derivatives of vitamin D_3 may have a role in the regulation of cholesterol biosynthesis.

EXPERIMENTAL PROCEDURES

Materials

(R,S)-[3-^{14}C]HMG-CoA (57 mCi/mmol), (R,S)-[5-^{3}H]mevalonolactone (24 Ci/mmol), [^{3}H]acetate (1.6 Ci/mmol), [4-^{14}C]cholesterol, and [1,2-^{3}H(N)]cholecalciferol (35 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Cholecalciferol (vitamin D_3) was purchased from Sigma Chemical Co. (St. Louis, MO). 25-Hydroxy vitamin D_3, 1,25-dihydroxy vitamin D_3, and 24(R),25-dihydroxy vitamin D_3 were obtained as generous gifts from Dr. M. Uskokovic of Hoffmann-LaRoche. Ketoconazole was supplied by Janssen Pharmaceutical, Inc., (New Brunswick, NJ). All other chemicals were obtained from local sources and were of highest purity grade available.

Cell cultures

Rat intestinal epithelial cells (IEC-6 cells, CRL-1592), obtained from American Type Culture Collections (ATCC), were grown as monolayers as described previously (4, 14). Human skin fibroblast cells (GM-43 cells) were obtained from Human Genetic Cell Repository (Rockville, MD) and grown as described previously (15). Briefly, on day 0, 2×10^5 cells were seeded in 60 × 15 mm plastic petri dishes in Eagle's minimal essential medium containing 10% (v/v) fetal bovine serum. On days 3 and 4, the monolayers were fed 2.0 ml of medium containing lipoprotein-deficient serum protein (4 mg/ml). On day 4, cells were treated with vitamin D_3 derivatives as described in appropriate legends of figures or table. Mouse peritoneal macrophages (J-774) and human hepatoma cells in culture (Hep G2) obtained from ATCC were grown as monolayers as described earlier (16, 17). Briefly, J-774 and Hep G2 cells were seeded in 60 × 15 mm petri dishes containing DMEM medium supplemented with 5% (v/v) and 10% (v/v) fetal bovine serum, respectively. On days 2 and 3, J-774 and Hep G2 cells were fed medium containing LPDS protein 2 mg/ml and 4 mg/ml, respectively.

HMG-CoA reductase activity and sterol synthesis

The activity of HMG-CoA reductase in logarithmically growing cultures was determined as previously described (4, 18). One unit of enzyme activity equals the formation of 1 pmol mevalonate/min. The results are presented as the average of triplicate determinations at each data point. For the measurement of enzyme activity as well as of lipid synthesis from acetate, 2×10^5 rat intestinal epithelial cells were seeded in 60 × 15 mm plastic petri dishes on day 0 in medium containing 5% (v/v) fetal bovine serum. On days 3 and 4, the monolayers were refed 2.0 ml of medium containing LPDS protein 2 mg/ml. The LPDS ($d > 1.21$ g/ml) was prepared from fetal bovine serum by ultracentrifugation using KBr for density adjustment (19). On day 4, cells were pretreated with the indicated concentration of vitamin D_3 derivatives in 20 μl of DMSO. Control cells received an equivalent volume of DMSO. After 5 hr of pretreatment, the monolayers were pulsed with [^{3}H]acetate (10 μCi/dish) for 1 hr. At the end of incubation, monolayers were rinsed three times with ice-cold saline, drained thoroughly, and the total lipids were extracted by the addition of 5 ml of hexane–isopropanol 60:40 containing carrier amounts of cholesterol, lanosterol, 24,25-epoxylanosterol, ubiquinone, squalene 2,3-epoxide, squalene 2,3:22,23 dioxide, squalene, and [4-^{14}C]cholesterol (20,000 dpm) as an internal standard (4, 20). After incubating at room temperature for 20–30 min (4, 20), the extract was removed and the cell residue remaining on the dishes was digested with 0.1 N NaOH for determination of protein content (4). Total lipids were saponified and nonsaponifiable lipids were extracted as described before (21). The nonsaponifiable lipids were separated by TLC on Whatman K5 silica gel plates using petroleum ether–acetone 90:10 as the developing solvent. Typical R_f values were as follows: cholesterol, 0.25; 24,25-epoxy lanosterol, 0.31; lanosterol, 0.38; ubiquinone, 0.52; squalene 2,3:22,23 dioxide, 0.57; squalene 2,3-epoxide, 0.68; squalene, 0.75. The lipids were visualized by spraying with *p*-anisaldehyde (19). The radioactivity of individual lipids was determined in a Beckman LS-3801 liquid scintillation spectrometer. The results were corrected for the recovery of [^{14}C]cholesterol internal standard.

HPLC separation of [^{3}H] vitamin D_3 metabolites

Metabolism of [^{3}H]vitamin D_3 by cells grown in the presence and absence of ketoconazole was followed by a modified HPLC method described previously (22). The

lipids isolated from monolayers as described earlier (22) were separated on a Zorbax ODS column (0.46 × 25 cm) column developed with 5% water in methanol at a flow rate of 1.5 ml/min. The column effluent was passed through a Flo-one Model HP radioactivity detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). The detector signal was integrated using a Gilson Datamaster system. The retention times of authentic standards of vitamin D_3 and 25-hydroxy vitamin D_3 were 24.2 min and 5.1 min, respectively.

RESULTS

Effect of vitamin D_3 derivatives on sterol synthesis in IEC-6 cells

Treatment of IEC-6 cells with vitamin D_3 and its hydroxylated derivatives, such as 25-hydroxy vitamin D_3, 24,25-dihydroxy vitamin D_3, and 1,25-dihydroxy vitamin D_3, inhibited the incorporation of [^{3}H]acetate into cholesterol (**Fig. 1**). The hydroxylated derivatives of vitamin D_3 showed greater inhibition of cholesterol synthesis compared to vitamin D_3. The distribution of radiolabel from [^{3}H]acetate into various nonsaponifiable lipid fractions was examined. In addition to the inhibition of incorporation of [^{3}H]acetate into cholesterol, the hydroxylated derivatives of vitamin D_3 at concentrations > 2 μg/ml also caused a concomitant accumulation of radiolabel into lanosterol (Fig. 1). Vitamin D_3 showed relatively minor effect on the incorporation of acetate into lanosterol. There was no change in the radioactivity associated with other nonsaponifiable fractions such as squalene, squalene 2,3-epoxide, squalene 2,3:22,23 dioxide, and ubiquinone (data not shown). These results indicated that hydroxylated derivatives of vitamin D_3 inhibited the conversion of lanosterol to cholesterol possibly by inhibiting the activity of 14α-lanosterol demethylase. The lack of effect of vitamin D_3 on conversion of lanosterol to cholesterol suggested that hydroxylation of vitamin D_3 is required to inhibit 14α-lanosterol demethylase. Vitamin D_3 and its derivatives had no inhibitory effect on the incorporation of [^{3}H]acetate into free fatty acid under the same experimental conditions, rather there appeared to be a small stimulation by 25-hydroxy vitamin D_3 and 1,25-dihydroxy vitamin D_3 (data not shown). Thus the inhibitory effects of vitamin D_3 derivatives appear to be specifically exerted on sterol biosynthesis.

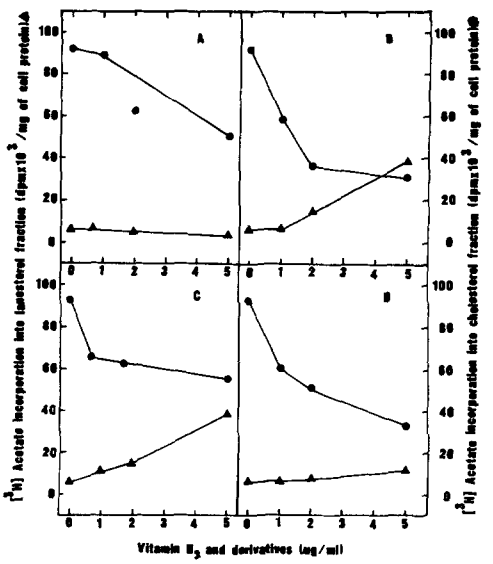

Fig. 1. Effect of vitamin D_3 and its hydroxylated derivatives on incorporation of [^{3}H]acetate into cholesterol and lanosterol in IEC-6 cells. The cells were grown for 4 days as described in Experimental Procedures. On day 4, vitamin D_3 compounds in 20-μl aliquots of DMSO were added to the culture medium at the indicated concentrations. An equivalent volume of DMSO was added to control dishes. After 5 hr, the cells were pulsed with [^{3}H]acetate (10 μCi/dish) for 1 hr. Triplicate dishes were harvested for determination of radioactivity incorporated into sterols as described in Experimental Procedures. The total nonsaponifiable lipids were separated by TLC as described in Experimental Procedures. A, Cholecalciferol (vitamin D_3); B, 25-hydroxycholecalciferol (25-hydroxy vitamin D_3); C, 1,25-dihydroxy cholecalciferol, (1,25-dihydroxy vitamin D_3); and D, 24(R),25-dihydroxy cholecalciferol (24,25-dihydroxy vitamin D_3). The total radioactivity in nonsaponifiable lipids of control cells was 1.2 × 10^5 dpm/mg cell protein. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean.

Effect of vitamin D_3 derivatives on HMG-CoA reductase activity

Since side chain hydroxylated derivatives of cholesterol are strong inhibitors of reductase activity, it was of interest to determine the effect of vitamin D_3 derivatives on HMG-CoA reductase activity, the rate limiting enzyme in the cholesterol biosynthetic pathway. Treatment of IEC-6 cells for 6 hr with varying concentrations of vitamin D_3 derivatives showed that all derivatives of vitamin D_3, except 1,25-dihydroxy vitamin D_3, inhibited the activity of HMG-CoA reductase (**Fig. 2**). The inhibition of reductase activity was concentration dependent.

The lack of effect of 1,25-dihydroxy D_3 on reductase activity was not due to impaired uptake, since under similar conditions this derivative inhibited the conversion of lanosterol to cholesterol (Fig. 1C). This observation was unexpected because 25-hydroxy vitamin D_3 inhibited both reductase activity and sterol synthesis and most of the biological activity of the vitamin D_3 series is attributed to 1,25-dihydroxy vitamin D_3. These data point to a unique effect resulting from the substitution of an hydroxyl group on the ring at the 1α position, since an additional hydroxyl group on the side chain at position 24 did not alter the inhibitory activity of the 25-hydroxy derivative on reductase activity. All vitamin D_3 derivatives when added to the in vitro assay system for reductase had no effect on the activity, indicating that intact cells are required for expression of the activity of vitamin D_3 and derivatives.

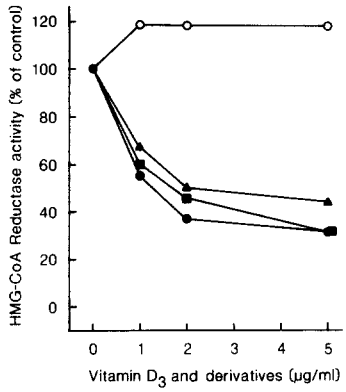

Fig. 2. Effect of vitamin D_3 derivatives on HMG-CoA reductase activity in IEC-6 cells. The cells were grown for 4 days as described in legend to Fig. 1. On day 4, varying concentrations of vitamin D_3 derivatives were added. After 6 hr of incubation, the cells from triplicate dishes were harvested for determination of reductase activity. Vitamin D_3 (●); 25-hydroxy vitamin D_3 (▲); 1,25-dihydroxy vitamin D_3 (○); and 24(R),25-dihydroxy vitamin D_3 (■). Control reductase activity was 144 pmol mevalonate/min per mg protein. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean.

The inhibitory effect of vitamin D_3 derivatives on HMG-CoA reductase activity was expressed in cells of different origin. The results presented in **Fig. 3** indicate that vitamin D_3 and 25-hydroxy vitamin D_3 inhibited HMG-CoA reductase activity in human skin fibroblasts (GM-43 cells), Hep G2 (transformed human liver cells), and J-774 (mouse peritoneal macrophages). 1,25-Dihydroxy vitamin D_3, however, showed a variable effect on reductase activity which was dependent on the cell line. For example, in human skin fibroblasts and J-774 macrophages, a biphasic response was observed such that at low concentrations (< 1 μg/ml) the derivative caused inhibition of reductase activity and, as the concentration of D_3 derivatives increased, the reductase activity returned to the control value. On the other hand, 1,25-dihydroxy vitamin D_3 caused a marked stimulation of reductase activity in Hep G2 cells. This stimulation by 1,25-dihydroxy vitamin D_3 was attenuated by the addition of 25-hydroxy vitamin D_3 (**Fig. 4**). The percent inhibition caused by 25-hydroxy vitamin D_3 was the same regardless of the presence of 1,25-dihydroxy vitamin D_3. Conversely, the percent stimulation caused by 1,25-dihydroxy vitamin D_3 was the same regardless of the presence of 25-hydroxy vitamin D_3. It is not clear whether these effects are linked through a common mechanism.

Hydroxylation of vitamin D_3 is required for inhibition of reductase activity

In view of the fact that hydroxylated derivatives of cholesterol are potent inhibitors of reductase activity and that hydroxylated derivatives of vitamin D_3 showed greater inhibition of sterol synthesis compared to vitamin D_3, we examined whether hydroxylation of vitamin D_3 was essential for expression of inhibitory action on HMG-CoA reductase activity. Ketoconazole, a known inhibitor of cytochrome P-450-dependent hydroxylation reactions and shown to inhibit the 25-hydroxylation of vitamin D_3 (23, 24), was used to test this point. Pretreatment of IEC-6 cells with ketoconazole (30 μM) attenuated the effect of vitamin D_3 on reductase activity, whereas the drug had no effect on the inhibition of reductase activity by either 25-hydroxy vitamin D_3 or 24(R),25-dihydroxy vitamin D_3 (**Fig. 5**). Similar results were obtained with Hep G2 cells. As shown in **Fig. 6**, ketoconazole pretreatment of Hep G2 cells attenuated the inhibition of reductase activity by vitamin D_3 but had no effect on the inhibition of reductase activity by 25- hydroxy vitamin D_3. These results indicated that ketoconazole attenuated the vitamin D_3 action on reductase activity by inhibiting the conversion of vitamin D_3 to hydroxylated vitamin D_3, since the drug had no effect on 25-hydroxy vitamin D_3 and 24,25-dihydroxy vitamin D_3. These results are analogous to our previous observations regarding the inability of ketoconazole to prevent the suppressive action of hydroxylated sterols on reductase activity in IEC-6 cells (4).

To determine whether ketoconazole affected the uptake of vitamin D_3 and thereby prevented vitamin D_3 action on reductase activity, IEC-6 cells were incubated with [^{3}H]vitamin D_3 in presence and absence of ketoconazole. There was 20–25% uptake of [^{3}H]vitamin D_3 in cells treated with or without ketoconazole indicating the drug had no effect on the uptake of vitamin D_3 (data not shown). When [^{3}H]vitamin D_3 was incubated in the medium minus cells, nearly 18% of the radiolabel was associated with polar vita-

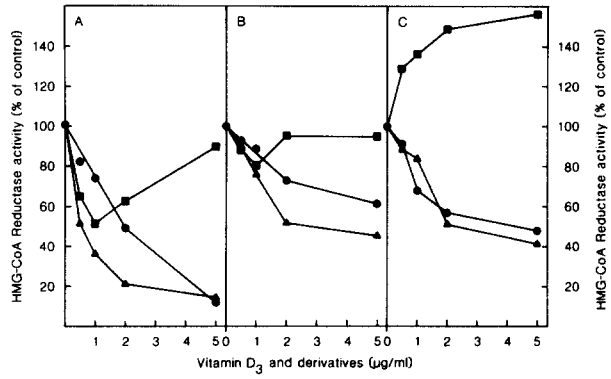

Fig. 3. Modulation of reductase activity by vitamin D_3 derivatives in GM-43 cells (A), J-774 cells (B), and Hep G2 cells (C). The cells from each cell type were grown for 4 days as described in Experimental Procedures. On day 4, D_3 derivatives were added at indicated concentrations. After 6 hr of incubation, cells were harvested from triplicate dishes for the determination of reductase activity. The control activity for GM-43 cells, J-774 cells, and Hep G2 cells was 189, 229, and 248 pmol mevalonate/min per mg protein, respectively. (●) Vitamin D_3; (▲) 25-hydroxy vitamin D_3; and (■) 1,25-dihydroxy vitamin D_3. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean.

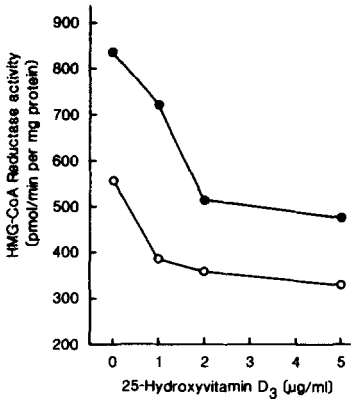

Fig. 4. Effect of 25-hydroxy vitamin D_3 on inhibition of reductase activity in presence (●) and absence (○) of 1,25-dihydroxy vitamin D_3. Hep G2 cells were seeded in 10% calf serum at 1×10^5 cells in 60-mm petri dishes. On days 1 and 2, cells were fed LPDS. On day 2, 1,25-dihydroxy vitamin D_3 (5 μg/ml) was added to a group of dishes. After 1 hr pretreatment, indicated concentrations of 25-hydroxy vitamin D_3 were added. The cells were harvested after 6 hr for the determination of reductase activity as described under Experimental Procedures. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean.

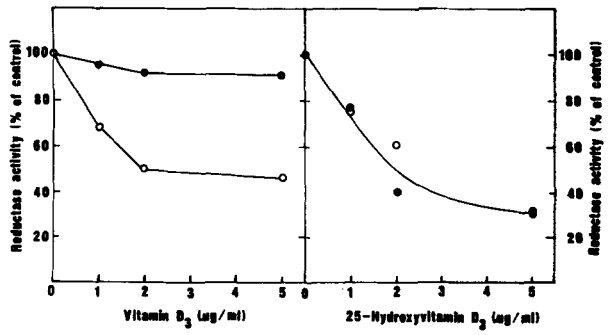

Fig. 6. Effects of ketoconazole on the inhibition of reductase activity by vitamin D_3 and 25-hydroxy vitamin D_3. The Hep G2 cells were grown for 4 days as described in Experimental Procedures. On day 4, cells were fed medium with (●) or without (○) ketoconazole (30 μM). In control cells equivalent amounts of ethanol were added. After 1 hr pretreatment, varying concentrations of vitamin D_3 derivatives were added. The cells were harvested from triplicate dishes for determination of reductase activity. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean. Reductase activity for control and ketoconazole-treated cells was 101 ± 4 and 344 ± 10, respectively.

min D_3 compounds as a result of autooxidation (**Table 1**). Ketoconazole had very little effect on this distribution, indicating that it did not prevent autooxidation. Incubation of [^{3}H]vitamin D_3 with IEC-6 cells caused a 92% increase in the radiolabel associated with polar vitamin D_3 fractions over the basal autooxidation level, indicating a cell-mediated metabolism of vitamin D_3 to move polar metabolites. Of further interest was the observation that a fraction coeluting with authentic 25-hydroxy vitamin D_3 (RT' 4–6) accounted for a majority of the increase in the radiolabel due to cellular metabolism. In this fraction alone

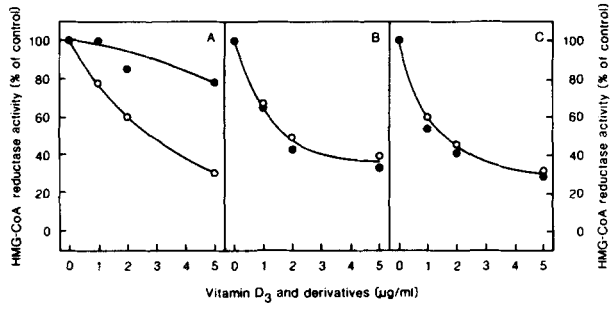

Fig. 5. Effect of ketoconazole on the inhibition of reductase activity by vitamin D_3 derivatives in IEC-6 cells. The cells were grown for 4 days as described in the legend to Fig. 1. On day 4, cells were fed fresh LPDS medium with (●) or without (○) ketoconazole (30 μM). In control cells, equivalent amounts of ethanol were added. After 1 hr of pretreatment, varying concentrations of vitamin D_3 derivatives were added. The cells were harvested from triplicate dishes for determination of reductase activity. A, Vitamin D_3; B, 25-hydroxy vitamin D_3; C, 24(R),25-hydroxy vitamin D_3. Reductase activity of control and ketoconazole-treated cells was 142 and 229 pmol mevalonate/min per mg protein, respectively. Data points represent the mean of triplicate determinations and the SE of each point in all treatment groups is ≤ 5% of the mean.

there was nearly a 256% increase in radioactivity, increasing from 5.8% (basal autooxidation) to 14.9% (basal autooxidation and cellular metabolism). Treatment of IEC-6 cells with ketoconazole prevented an increase in radioactivity associated with the total polar vitamin D_3 fraction as well as in the fraction coeluting with authentic 25-hydroxyl vitamin D_3. In the presence of Hep G2 cells, 46% of the radioactivity was associated with polar vitamin D_3 derivatives. Ketoconazole prevented this increase in polar vitamin D_3 derivatives by Hep G2 cells. These results support the conclusion that ketoconazole attenuated the inhibitory effect of vitamin D_3 on reductase activity by preventing its metabolism to hydroxylated derivatives. These results are analogous to the observation that in IEC-6 cells, ketoconazole prevented the inhibitory action of 24(S),25-epoxylanosterol on reductase activity by preventing the conversion of epoxylanosterol to an inhibitory oxysterol (4).

DISCUSSION

Recent investigations have verified the endogenous formation of hydroxylated derivatives of cholesterol in cultured cells (2–8). Side chain hydroxylation of sterols results in the production of powerful inhibitors of HMG-CoA reductase activity and cholesterol biosynthesis (2, 3). Vitamin D_3 also undergoes hydroxylation reactions leading to the formation of both biologically active as well as inactive metabolic end products (9, 10). In view of the structural similarities between hydroxylated sterols and hydroxylated secosterols, the possibility of hydroxylated vitamin D_3 derivatives as regulators of sterol biosynthesis was evaluated.

Treatment of IEC-6 cells with vitamin D_3 and its derivatives caused varied degrees of inhibition of the incorpora-

TABLE 1. Metabolism of [^{3}H]vitamin D_3 in cultured cells in cells grown in the presence and absence of ketoconazole

Cell Line	Retention Time	Assigned Identification[a]	% Distribution of Total Radioactivity			
			− Cells		+ Cells	
			− KC	+ KC (30 μM)	− KC	+ KC (30 μM)
	min					
IEC-6	2–3	Dihydroxy vitamin D_3	2.0 ± 0.7	1.9 ± 0.3	2.5 ± 0.3	1.6 ± 0.2
IEC-6	4–6	25-Hydroxy vitamin D_3	5.8 ± 0.4	4.8 ± 0.7	14.9 ± 0.3	5.6 ± 0.3
IEC-6	8–10	Unknown	5.3 ± 0.6	4.5 ± 0.8	9.4 ± 0.2	4.0 ± 0.2
IEC-6	12–16	Unknown	4.9 ± 2.1	3.5 ± 1.0	7.8 ± 0.7	2.3 ± 0.3
IEC-6	21–23	Vitamin D_2[b]	8.4 ± 1.7	14.7 ± 0.5	4.4 ± 0.8	8.0 ± 0.1
IEC-6	24–26	Vitamin D_3	61.6 ± 3.6	62.2 ± 3.7	42.6 ± 2.6	68.4 ± 1.3
IEC-6	27–29	Unknown	5.2 ± 1.1	4.7 ± 0.2	8.0 ± 0.2	2.0 ± 0.1
Hep G2	2–3	Dihydroxy vitamin D_3			11.1 ± 1.7	2.6 ± 1.5
Hep G2	4–6	25-Hydroxy vitamin D_3			11.0 ± 2.2	3.1 ± 1.1
Hep G2	8–10	Unknown			12.3 ± 1.0	3.9 ± 1.3
Hep G2	12–16	Unknown			12.0 ± 1.5	ND
Hep G2	21–23	Vitamin D_2[b]			6.2 ± 1.0	5.2 ± 0.6
Hep G2	24–26	Vitamin D_3			34.2 ± 5.8	78.3 ± 7.1
Hep G2	27–29	Unknown			4.0 ± 1.4	ND

Cells were grown for 4 days as described in the legend to Fig. 1. On day 4 cells were fed medium with or without ketoconazole (KC) (30 μM). A set of triplicate dishes was incubated with medium alone. After 1 hr pretreatment, 0.2 μCi of [^{3}H]vitamin D_3 was added. Cells were harvested after 6 hr and the lipid was extracted and separated by HPLC as described in Experimental Procedures; ND, nondetectable.

[a]Based on coelution with authentic standards.

[b]Based on retention time relative to vitamin D_3 as per reference 22.

tion of [^{3}H]acetate into cholesterol. The side chain hydroxylated derivatives were the most effective. Studies on the site of inhibition revealed variable effects of vitamin D_3 derivatives on HMG-CoA reductase activity as well as on post-mevalonate steps in the cholesterol biosynthetic pathway. Treatment of cultured cells of varied tissue origin, i.e., rat intestinal epithelium (IEC-6), human skin fibroblasts (GM-43), mouse peritoneal macrophages (J-774), and human hepatoma cells (Hep G2), with vitamin D_3, 25-hydroxy vitamin D_3, and 24(R),25-hydroxy vitamin D_3 resulted in inhibition of HMG-CoA reductase activity. In IEC-6 cells, 25-hydroxy vitamin D_3 and 24(R),25-dihydroxy vitamin D_3 also caused an accumulation of lanosterol, whereas vitamin D_3 did not. This result suggested that hydroxylation of vitamin D_3 was required for inhibition of lanosterol demethylation. Intermediates derived from lanosterol may also play a role as endogenous regulator of reductase activity and sterol biosynthesis (25, 26), but in these experiments it is doubtful that the accumulation of lanosterol or its derivatives plays a role in the inhibition of reductase activity, since ketoconazole, which prevents the formation of lanosterol derivatives (4, 25), does not affect the inhibitory action of 25-hydroxy vitamin D_3 on reductase.

Further support for a role of hydroxylation of vitamin D_3 in generating derivatives with inhibitory effects on reductase activity was provided by the experiments with IEC-6 and Hep G2 cells. Treatment of cells with ketoconazole, a known inhibitor of vitamin D_3 metabolism (23, 24), prevented the effect of vitamin D_3 on reductase activity, but had no effect on the inhibitory action of 25-hydroxy or 24,25-dihydroxy vitamin D_3. This was confirmed by showing that ketoconazole prevented the conversion of vitamin D_3 to polar derivatives including 25-hydroxy vitamin D_3. These results suggest that ketoconazole prevented inhibition of reductase by vitamin D_3 by inhibiting the hydroxylation of vitamin D_3. Although liver and kidney are the major sites of hydroxylation of vitamin D_3, the fact that cultured intestinal cells can apparently do the same is in agreement with reports that other tissues possess this activity (27).

The substitution of a hydroxyl group on the 25-position of the side chain of vitamin D_3 suggests several analogies to the action of 25-hydroxycholesterol. Both sterols are structurally similar with respect to side chain hydroxylation. Both are inhibitory to HMG-CoA reductase activity in concentrations within the same order of magnitude, and both display similar dose response inhibition curves (14). The parent compounds of both hydroxy sterols, i.e., cholesterol and vitamin D_3, are not active in inhibiting reductase activity. Ketoconazole was unable to prevent the inhibitory effect of 25-hydroxy vitamin D_3 (this report) and 25-hydroxycholesterol (4) on reductase activity. The action of both hydroxylated sterols may involve binding proteins in their action (13, 28) despite some differences, e.g., the 25-hydroxycholesterol binding protein does not bind 25-hydroxy vitamin D_3 (13). We have shown that 25-hydroxy vitamin D_3 inhibits an additional site in cholesterol biosynthesis, i.e., at lanosterol 14α-demethylase. Other sterols hydroxylated in the side chain and ring, e.g., 7α- and 7β-hydroxycholesterol and 22(R)-hydroxydesmosterol (29) and oxygenated lanosterol analogues (30) have a similar effect. We are unaware of similar observations for 25-hydroxycho-

lesterol. It has been suggested on the basis of indirect evidence that 25-hydroxycholesterol plays a role in the regulation of cholesterol biosynthesis (6). Based on the above similarities, such a role may also be suggested for 25-hydroxy vitamin D_3.

1,25-Dihydroxy vitamin D_3, which is the biologically active form of the secosteroid (9, 10), provided unexpected results with respect to its effects on HMG-CoA reductase activity. We had expected that in view of the hydroxyl group on the 25-position in the side chain that this molecule, like 25-hydroxycholesterol and 25-hydroxy vitamin D_3, would be a strong inhibitor of HMG-CoA reductase. Instead, treatment of cells with 1,25-dihydroxy vitamin D_3 resulted in a variable effect on reductase activity dependent on the cell line. Thus, in IEC-6 cells there was a minor stimulation (20%) in reductase activity. In GM-43 and J-774 cells a biphasic response was observed, i.e., inhibition at lower concentrations and activity approaching control values at higher concentrations. In Hep G2 cells, 1,25-dihydroxy vitamin D_3 caused a marked (50%) stimulation of reductase activity. The absence of inhibition of reductase activity in IEC-6 cells was not due to impaired uptake of the vitamin derivative, since under similar conditions it inhibited the conversion of lanosterol to cholesterol. Thus, it appears that the addition of a hydroxyl group in the 1α position profoundly changed the physiological effects of the parent 25OH-vitamin D_3 molecule from a consistent inhibitor to a variable stimulator or inhibitor of reductase activity depending on the cell line examined. This stimulation of reductase activity with 1,25-dihydroxy vitamin D_3 was inhibited by the presence of 25-hydroxy vitamin D_3 suggesting that these hydroxylated derivatives of vitamin D_3 may not be acting through similar mechanisms (see beyond).

In the present studies, it should be noted that the concentration of the vitamin D_3 analogues in the culture medium required for 50% inhibition of reductase activity and cholesterol biosynthesis approached serum concentrations when vitamin D_3 was supplied in pharmacological doses to normal and anephric pigs (31). In humans, the normal concentrations of vitamin D_3 analogues are increased signficantly in the anephric subject, or in normal subjects after long exposure to sunlight (32). When cellular actions of steroids are exerted at higher than physiological concentrations, a question arises concerning whether these effects are due to genomic sites of action or liponomic effects. The latter are the result of direct interactions of steroid molecules with membrane structures, leading to alterations in function (33). Liponomic effects are generally observed with much higher concentrations of steroid molecules than those required to elicit genomic effects, although the two are not mutually exclusive. Consideration of these two effects raises the same question with respect to the data we have reported on the inhibitory effects of 25-hydroxy vitamin D_3 as well as inhibitory and stimulatory effects of 1,25-dihydroxy vitamin D_3 on reductase activity in different cell lines. One reasonable assumption might be that the variable effects of 1,25-dihydroxy vitamin D_3 in different cell lines are due to membrane alterations, which vary with respect to distinct membrane lipid and protein profiles in the cell lines. On the other hand, if 25-hydroxy vitamin D_3 is also causing inhibition of the reductase via liponomic effects, then it suggests there are two specific liponomic (inhibitory or stimulatory or both) effects exerted by each vitamin D_3 analogue which differ from each other by the location of a single hydroxyl substitution. Alternately, one cannot rule out the possibility that these secosteroids may also be acting at the genomic level, as well as the membrane. The same question with respect to other biological responses of 1,25-dihydroxy vitamin D_3 has recently been reviewed (34). In progress are further studies of the role of vitamin D_3 analogues in the regulation of the biosynthetic pathway leading to the formation of the parent vitamin D_3 intermediate, 7-dehydrocholesterol.

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