25-HYDROXYCHOLECALCIFEROL AND 1, 25-DIHYDROXYCHOLECALCIFEROL ARE POTENT INHIBITORS OF CHOLESTEROL BIOSYNTHESIS BY NORMAL AND LEUKEMIC (L_2C) GUINEA PIG LYMPHOCYTES

by

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SUMMARY

We have measured cholesterol biosynthesis in vitro by normal and leukemic (L_2C) guinea pig lymphocytes. As shown before (Philippot, J. R., Cooper, A. G. and Wallach, D. F. H. [1975] Biochim. Biophys. Acta 406: 161-166) L_2C cells produce cholesterol at 25-60 times the rate found with normal cells. 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol, both biologically active derivatives of vitamin D_3, at submicromolar concentrations inhibit cholesterol biosynthesis by both normal and neoplastic lymphocytes. Unoxygenated vitamin D_3 was not inhibitory. The rate of inhibition due to 25-hydroxycholecalciferol is considerably greater than that of the oxygenated cholesterol analogs.

One mechanism involved in the regulation of cholesterol biosynthesis has been clarified by Kandutsch and Chen (1, 2) whose in vitro experiments with L cells show that certain oxidation products of cholesterol, e. g. 25-hydroxycholesterol, 22-hydroxycholesterol, 20-hydroxycholesterol, 7-hydroxycholesterol and 7-ketocholesterol, rather than cholesterol itself, regulated sterol biosynthesis at the hydroxymethylglutaryl-CoA reductase level. The specific effect of these derivatives has also been documented for cells from individuals with homozygous familial hypercholesterolemia (3,4) and neoplastic (L_2C) guinea pig lymphocytes (5). Both of these cell types synthesize cholesterol at much greater rates than normal and exhibit deficient suppression of sterol synthesis upon cholesterol feeding or cholesterol addition in vitro.

The specific action of the oxidized cholesterol derivatives is rapidly

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reversible (6) and occurs between 5 x 10^{-8} and 5 x 10^{-7} M, levels low enough to be of biological interest. However, it has not yet been possible to show that these agents are physiologically important regulators of cholesterol biosynthesis.

We have therefore explored the possibility that 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol, two oxygenated vitamin D derivatives with some structural resemblance to inhibitory cholesterol analogs, might also function to suppress sterol biosynthesis. We find that these substances at submicromolar levels are highly specific inhibitors of cholesterol biosynthesis by normal and leukemic lymphocytes in vitro and that this inhibitory action proceeds much more rapidly than that of oxidized cholesterol derivatives.

MATERIALS AND METHODS

25-hydroxycholesterol was purchased from Steraloids Inc. (Wilton, N. H.), 25-hydroxy-7-dehydrocholesterol, cholecalciferol, 25-hydroxycholecalciferol hydrate and 1, 25-dihydroxycholecalciferol were a generous gift of Dr. John C. Babcock from the UpJohn Co. (Kalamazoo, Mich.). [^{14}C]-acetate, [^{3}H]-cholesterol, [^{3}H]-leucine and Aquasol 2 were purchased from New England Nuclear (Cambridge, Mass.). Hanks' balanced salt solution, RPMI 1640 medium and phosphate buffered saline were obtained from GIBCO (Grand Island, N. Y.). All other reagents were of analytical grade.

Stock solutions of vitamin D_3 and cholesterol derivatives (10^{-2} M) in ethanol were diluted so as to maintain the final concentration of ethanol below 0.5% (V/V). To measure incorporation of [1-^{14}C]-acetate into cellular lipids, we used 18 µCi per sample (specific activity of 32 Ci/mole). To measure protein synthesis our stock of [^{3}H]-leucine (60 Ci/mole) was diluted 5-fold with RPMI 1640 before addition to the sample.

Normal and L_2C leukemia were harvested as previously described (5). The cells, washed in Hanks' balanced salt solution, were washed once in RPMI 1640 with 15 mM HEPES, pH 7.4, 100 units penicillin and 0.1 mg streptomycin/ml, then resuspended in this medium and used either immediately or after overnight storage at 4°C in the incubation medium as stated.

To study the effects of various inhibitor concentrations on cholesterol, fatty acid or protein synthesis, 4 to 6 x 10^{6} cells were incubated for 2 hr at 37°C with different concentrations of inhibitor, before addition of [1-^{14}C]-acetate or [^{3}H]-leucine (10 µl) in a total volume of 2 ml. For kinetic experiments [^{14}C]-acetate was added at the same time as the inhibitors. Incubation was then continued for up to 4 hr at 37°C. After incubation the viable cells were counted microscopically by Trypan Blue exclusion. To determine incorporation of labeled acetate, cells were harvested using glass filters (93 LAH; Reeve Angel, Clifton, N. J.) and washed four times with 10 ml iced phosphate buffered saline, pH 7.4. Then about 10^{5} dpm of [^{3}H]-cholesterol were added as internal standard. Cholesterol and fatty acids were measured as in (8).

After saponification and extraction with petroleum ether, cholesterol was collected as the digitonin-precipitable fraction. The aqueous phase was brought to pH < 3 with concentrated HCl and extracted again with petroleum ether. The second ether extract, containing the fatty acids was dried before counting.

For assay of [^{3}H]-leucine incorporation, we precipitated the protein in an aliquot of cell suspension with iced trichloroacetic acid (5% final concentration) washed at 3000 rpm (20 min; 4°C) with iced 5% trichloroacetic acid before counting.

Counts were measured with a Packard Tricarb (3320) scintillation spectrometer, using 10 ml Aquasol 2 as scintillation mixture.

RESULTS

As shown before (5), L_2C cells synthesize cholesterol at 25 - 60 times the rate characteristic for normal lymphocytes (94 - 181 n moles/10^9 cells/h [1-^{14}C]-acetate incorporated vs 2.7 - 7.5 n moles/10^9 cells/h). The rate of fatty acid synthesis by the leukemic cells is also much greater (223 n moles/10^9 cells/h [1-^{14}C]-acetate incorporated vs 13 n moles/10^9 cells/h). Both biosynthetic

Table I: Effects of various sterols on the biosynthetic activities of normal and L_2C lymphocytes

Cells		Concentration (μM) required to 1/2-maximally inhibit biosynthesis*		
		Cholesterol	Fatty Acids	Proteins
L_2C	Cholecalciferol	[12.5 %]	[25 %]	[19.5 %]
	25-hydroxycholecalciferol	0.15	1.4	4.0
	1, 25-dihydroxycholecalciferol	0.6	2.0	[20 %]
	25-hydroxycholesterol	0.1	3.0	2.0
	25-hydroxy-7-dehydrocholesterol	0.08	[32.5 %]	[9.5 %]
Normal	Cholecalciferol	[25 %]	[13 %]	[12 %]
	25-hydroxycholecalciferol	0.26	0.75	1.25
	1, 25-dihydroxycholecalciferol	0.35	2.4	[40 %]
	25-hydroxycholesterol	0.08	0.8	1.25
	25-hydroxy-7-dehydrocholesterol	0.08	[47.5 %]	[45 %]

* Figures in brackets give percent of inhibition at the highest concentration [5 μM] of inhibitor employed.

processes can be inhibited by certain oxygenated derivatives of cholesterol and cholecalciferol (vitamin D_3).

Table I shows the extent to which these compounds suppress the biosynthesis of cholesterol, fatty acids and proteins by normal and (L_2C) guinea pig lymphocytes. Each potential inhibitor was tested at three concentrations, 5. 0.5, and 0.05 μM. The values in the table represent duplicate experiments carried out on three different cell populations.

In both cell types, 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol at low concentration inhibit cholesterol biosynthesis, in analogy to 25-hydroxycholesterol and 25-hydroxy-7-dehydrocholesterol. In contrast, the parental compound cholecalciferol does not significantly influence sterol biosynthesis. Both derivatives of cholecalciferol simulate the oxygenated cholesterol analogues, in that, at higher concentrations, they also inhibit fatty acid bio-

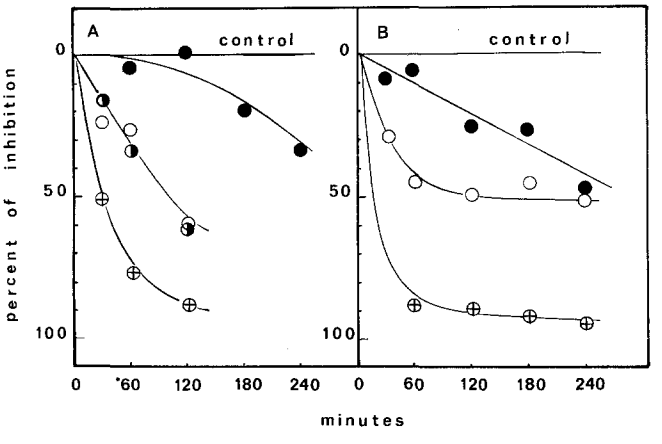

Figure 1: Time course of the inhibition of cholesterol biosynthesis by derivatives of cholesterol and cholecalciferol. Cholesterol biosynthesis assayed by incorporation of [1-^{14}C]-acetate into the digitonin-precipitable fraction of cellular lipids, is presented as % of controls not exposed to inhibitors (Control syntheses were in the range of 5 to 7.5 n moles [1-^{14}C]-acetate incorporated/10^9 cell/h for normal cells and 106 to 181 n moles for L_2C cells).

A. Normal lymphocytes. B. L_2C cells. ● Cycloheximide (10 μM ml). ○ 25-hydroxycholesterol (5 μM). ◑ 25-hydroxy-7-dehydrocholesterol (5 μM). ⊕ 25-hydroxycholecalciferol (5 μM).

synthesis (Table I). In terms of the inhibition of cholesterol biosynthesis relative to fatty acid and protein biosynthesis, 25-hydroxy-7-dehydrocholesterol is most "specific" for sterol metabolism, followed in close order by 25-hydroxycholesterol and 25-hydroxycholecalciferol.

Figure 1 shows how cholesterol biosynthesis by normal and L_2C lymphocytes declines with time following addition of several inhibitory sterols and for comparison, an inhibitor of protein synthesis, cycloheximide (10 μg/ml). The highest concentration (5 μM) of the sterols was used, to minimize the time of transit of the inhibitors to their action sites. We find that the cholesterol derivatives suppress cholesterol biosynthesis of L_2C cells, as well as that of normal lymphocytes. However, the rapid action of 25-hydroxycholecalciferol stands out in both cell types. This derivative of vitamin D_3 produced 1/2 maximal inhibition of cholesterol within 15 and 30 min, respectively, in L_2C and normal lymphocytes, compared with values near 60 or 90 min, in the case of 25-hydroxycholesterol or 25-hydroxy-7-dehydrocholesterol (cf. also 2,8).

DISCUSSION

The effect of vitamin D_3 on intestinal calcium transport and upon the calcium mobilization of bone is well recognized. Indeed, the potent action of 1, 25-dihydroxycholecalciferol has led to the suggestion that this vitamin behaves as a steroid hormone (9). Whether this is the case or not, an abundance of experimentation (cf. 10-12) has established that 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol are active forms of vitamin D_3.

Our present data add further to this field in documenting the hitherto unrecognized fact that 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol, two oxygenated derivatives of vitamin D_3, act as potent inhibitors of cholesterol biosynthesis. Moreover, this inhibition also extends to neoplastic L_2C lymphocytes, whose cholesterol production is extravagant and totally unresponsive to cholesterol administration *in vivo* (13) or *in vitro* (5).

In many respects 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol behave as do 25-hydroxycholesterol and 25-hydroxy-7-dehydrocholesterol (Table I).

However, the cholesterol derivatives tend to suppress protein synthesis somewhat less than the vitamin D_3 analogues. The major difference between the two classes of compounds is the very rapid inhibitory action of 25-hydroxycholecalciferol on cholesterol biosynthesis. Rate studies were not performed with 1, 25-dihydroxycholecalciferol because too little of this compound was available.

In view of the many actions of vitamin D_3 (e.g. 9-12,14), we would be premature in attempting to assign a fixed significance to our discovery. However, several points of speculation arise from our findings:

1. Are oxygenated derivatives of vitamin D_3 physiologic regulators of cholesterol biosynthesis?

2. Does regulation of cholesterol synthesis play a role in Ca^{++} transport or function in the cell? It is appropriate, in this context, to point to the fact that membrane cholesterol can profoundly inhibit the ATPase responsible for the transport of calcium into sarcoplasmic reticulum (15). One should therefore consider the possibility that vitamin D_3 might regulate calcium metabolism by modifying membrane cholesterol.

3. Is the high rate of cholesterol biosynthesis of certain neoplastic cells a requirement for growth? If so, can oxygenated derivatives of vitamin D_3 influence tumor growth?

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