Effect of exogenous steroids on sterol synthesis in L-cell mouse fibroblasts

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ABSTRACT A number of steroids have been tested in an L-cell tissue culture system to determine their effects on cellular sterol biosynthesis and cellular growth. Cholesterol, desmosterol, lathosterol, 7-dehydrocholesterol, and cholestanone reduce de novo synthesis and produce only limited toxicity at high concentrations of exogenous sterol. Considerable cellular toxicity is observed when cells are grown in the presence of coprostanol and Δ^4-cholestenone. No marked effect on either cell growth or sterol biosynthesis is produced by cholestanol, β-sitosterol, stigmasterol, campesterol, ergosterol, cholesteryl oleate, or cholestane.

SUPPLEMENTARY KEY WORDS cholesterol · desmosterol · feedback inhibition

In the absence of exogenous sterol, L-cell mouse fibroblasts synthesize all of the cellular sterol necessary for continued growth (1–4). Desmosterol is the major sterol recovered from these tissue culture cells when they are grown in sterol-free medium (5). When cholesterol is added to the culture medium, it is incorporated by these cells, and as the concentration of cholesterol in the medium is increased, there is a concomitant decrease in the synthesis of cellular desmosterol from acetate (1). The object of this investigation was to determine if sterols other than cholesterol could elicit a sterol feedback response and influence cell growth.

EXPERIMENTAL METHODS

The L-cell line of mouse fibroblasts used in these studies had been cultivated for approximately 2 yr in Eagle's medium containing double-strength amino acids and vitamins in Earle's balanced salt solution. This medium contained no whole serum but was supplemented with delipidized calf serum protein, which was added to the cultures at a level of 5 mg/ml. The delipidized protein was obtained by treating calf serum with ether, using the method of Scanu, Lewis, and Bumpus (6) as modified by Albutt (7). This delipidized protein served two functions in these experiments: (*a*) as a protein source, to allow attachment of the cells to glass and ensure rapid cell growth; and (*b*) together with added lecithin, to allow the solubilization of the various test sterols.

Methods for the relipidization of delipidized serum proteins have been described previously (1). In the present study, protein was added to the culture medium at a level of 5 mg/ml and lecithin at a level of 20 μg/ml. Sterols were added at levels ranging from 0.5 to 40 μg/ml. The lipids were added to the protein solution in ethanol to give a final ethanol concentration of 2.5% which was then reduced to a concentration of 0.5% by sterile dialysis procedures. This level of ethanol was present in all control media, which contained only lecithin.

Detailed procedures for the determination of cellular sterol synthesis from acetate-2-^{14}C have been described previously (1). Briefly, the following methods were used. Milk dilution bottles containing 20 ml of tissue culture medium supplemented with 5 mg/ml of delipidized calf serum protein were inoculated with 1×10^6 cells. After a 24-hr incubation period, the medium was removed

Systematic names of the sterols referred to in the text by their trivial names are as follows: cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; lathosterol, cholest-7-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; coprostanol, 5β-cholestan-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; ergosterol, cholest-5,7,22-trien-24-methyl-3β-ol; stigmasterol, cholest-5,22-dien-24-ethyl-3β-ol; sitosterol, cholest-5-en-24α-ethyl-3β-ol; campesterol, cholest-5-en-24β-ethyl-3β-ol; cholestanone, 5α-cholestan-3-one; Δ^4-cholestenone, cholest-4-en-3-one.

Abbreviations: GLC, gas–liquid chromatography.

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from the cell monolayers and replaced with fresh medium containing protein (5 mg/ml), lecithin (20 μg/ml), and sterol (0.5–40 μg/ml). Control cultures received the same medium minus the sterol. After a 24-hr incubation period in the presence of the sterol under study, a solution of sodium acetate-2-^{14}C of known specific activity (24.5 μCi/mmole) was added to each bottle at a level of 250 μg/ml of culture medium. The cell cultures containing the labeled acetate were incubated for 24 hr at 37°C. Preliminary experiments had indicated that increasing the level of labeled acetate twofold or decreasing it by one-half resulted in no significant alteration in the incorporation of ^{14}C into cellular sterol. Acetate incorporation into digitonin-precipitable sterol was continuous throughout the 24-hr exposure period. Thus, these experiments measured the amount of sterol synthesized from acetate by the cells during the last 24 hr of incubation.

After treatment with a 0.25% trypsin solution, the labeled sterol was recovered and the quantity estimated after removing the tissue culture medium and harvesting the cells. The cells and medium were pooled and then centrifuged at 3000 *g* for 10 min at 4°C. The cell pellets were washed two times by resuspension and recentrifugation in buffered salt solution (8), and the supernatant washes were pooled with the original culture medium. A portion of the pooled medium and wash solution was extracted with chloroform–methanol 2:1 (v/v), and after the lipids were saponified with alcoholic KOH, the free sterol was precipitated with digitonin. The washed cell pellets were suspended in saline and disrupted by sonication for 30 sec in a Branson Sonifier (37 watts). A sample of the sonicated cell suspension was removed and used for protein determination; the remainder was extracted with chloroform–methanol 2:1 (v/v) and the sterol was precipitated with digitonin after saponification. Since previous studies had demonstrated that under some growth conditions a portion of the synthesized sterol is lost to the culture medium (1), labeled sterol was assayed in both cells and medium so that a true estimate of sterol synthesis could be obtained.

The data obtained from these experiments are expressed as micrograms of ^{14}C-labeled acetic acid equivalents incorporated into the synthesized digitonin-precipitable sterol of the cells and medium per milligram of cell protein during the 24-hr period. The data are presented as percentages of the control cultures grown in medium; these were arbitrarily set equal to 100. Thus, any value less than 100% indicates inhibition.

Cell growth is expressed as a percentage of that of the control cultures, the medium of which contained only lecithin and no sterol. The cellular protein values obtained from these controls were set equal to 100%. As in the case of measurements for sterol synthesis, values less than 100% indicate an inhibition of cellular growth produced by the test sterols. Determinations were made in those cultures which contained intact cells; in some cases higher concentrations of the added sterol produced complete cellular toxicity and lysis.

Reagents and Analytical Procedures

All lipid extractions were made with chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (9). Lipids were saponified in 10% ethanolic KOH at 60°C for 30 min, the aqueous layer was extracted once with 3 vol of petroleum ether and twice with 3 vol of diethyl ether, and the extracts were washed with water. Sterols were precipitated with digitonin following the method of Sperry and Webb (10). Digitonin precipitates were assayed for radioactivity by dissolving the dried precipitates in methanol (11) and counting in a Packard liquid scintillation spectrometer, using 0.6% 2,5-diphenyloxazole and 0.02% dimethyl-1,4-bis-2-5(phenyloxazolyl)benzene in toluene as the scintillator.

Thin-layer chromatography of sterols was performed using the silver nitrate system described by Ditullio, Jacobs, and Holmes (12). GLC analyses were conducted using a Glowall model 310 equipped with an argon ionization detector. The columns used were: 80–100 mesh Supelcoport coated with 3% SE-30 (6-ft columns, 235°C), and 100–120 mesh Supelcoport coated with 3% XE-61 (6-ft column, 235°C); the inlet pressure was 25 psi.

The purity of the sterols was assayed by both thin-layer and gas–liquid chromatography. 7-Dehydrocholesterol, stigmasterol, cholestanol, lathosterol, and desmosterol were kindly supplied by Dr. Robert Conner, Department of Biology, Bryn Mawr College. The desmosterol, upon GLC analysis, exhibited a minor peak comprising less than 10% of the total sterol. Although this minor compound was not identified, Svoboda and Thompson (13) have shown that cholesta-5,25-dien-3β-ol is a common contaminant in many preparations of desmosterol. The other sterols produced a single peak.

The following compounds were obtained from Dr. David Kritchevsky, Wistar Institute: β-sitosterol, cholestan-3-one, and ergosterol. Analysis of the β-sitosterol following crystallization from ethanol demonstrated the presence of 10% contaminating campesterol. Analysis of the other sterols revealed no contaminating compounds. Cholest-4-en-3-one was purchased from Mann Research Laboratories, New York, and was purified by crystallization from ethanol. Pure campesterol was obtained from Applied Science Laboratories Inc., State College, Pa., and coprostanol from Steraloids, Inc., Pawling, N.Y. Cholestane and cholesteryl oleate were purchased from Supelco, Inc., Bellefonte, Pa.

Sodium acetate-2-^{14}C (2 mCi/mmole), purchased from New England Nuclear Corp., Boston, Mass., was mixed with carrier sodium acetate to give a final specific activity of 24.5 μCi/mmole. The solution was sterilized by filtration through a 0.45-μ Millipore filter. Egg lecithin (98% pure) was purchased from Supelco Inc. Protein was assayed by the method of Lowry et al. (14). All solvents were reagent grade, and all evaporation procedures were carried out under nitrogen.

RESULTS AND DISCUSSION

The data obtained from these studies of a number of different steroids are presented in Tables 1 and 2. Table 1 lists those steroids which, when added to the growth medium, significantly reduced de novo sterol biosynthesis from acetate-^{14}C in this L-cell system. The inhibition of synthesis caused by cholesterol in this series of experiments corresponds to the results obtained in previous studies (1). At the highest level of cholesterol tested, cellular growth, measured as cellular protein content, was depressed by approximately 40%.

The rate of influx of a sterol from the medium to the cell and its subsequent incorporation into subcellular membranes would be related to the ability of the given sterol to be solubilized in serum and cellular lipoproteins. The incorporation of a sterol into lipoproteins has been shown to require the interaction of the sterol with phospholipid (15). Differences exist in the abilities of the steroids used in this investigation to interact with, and be solubilized by, phospholipids. Strict correlations, however, do not exist between the degrees of incorporation of a given sterol into artificial dispersions, serum lipoproteins, and cellular lipoproteins (16). The extent to which the data obtained in this study are influenced by the solubility of the steroids in the "reconstituted serum lipoprotein," as opposed to a selectivity at the level of the cellular membranes, is unknown. In general, however, sterols such as the C_{28} and C_{29} phytosterols, which have little effect in this L-cell system, are not readily incorporated into membranes (16, 17), while sterols such as lathosterol, 7-dehydrocholesterol, Δ^4-cholesten-3-one, and coprostanol have been shown to be incorporated into cellular membranes (16, 17).

Desmosterol, at all concentrations, reduced synthesis more effectively than did cholesterol. Some reduction of cellular growth was observed with desmosterol at both 20 and 40 μg/ml. This inhibition was similar to that obtained with 40 μg/ml of cholesterol. Because the desmosterol used in these experiments contained a small amount (<10%) of an unidentified sterol which might be responsible for the enhanced inhibition, an additional experiment was conducted in which purified desmosterol (obtained by extraction of L cells, and exhibiting a single peak upon GLC analysis) was tested in this experimental system. L cells grown in medium containing 20 μg/ml of this sterol exhibited 4.8% synthesis, indicating that the very effective reduction observed in these experiments was due to desmosterol itself and not to contaminating sterols.

Lathosterol was less effective in inhibiting sterol synthesis than was cholesterol, while the response to 7-dehydrocholesterol was similar to that of cholesterol. As is the case with all sterols shown in Table 1, some inhibition of cellular growth was noted at the highest concentration of these sterols.

The saturated sterol, coprostanol, was less effective than cholesterol in reducing sterol biosynthesis; however, it produced greater inhibition of cell growth. It is possible that the reduction in sterol biosynthesis seen in the presence of coprostanol is a reflection of the cellular toxicity, rather than being a specific sterol feedback response.

The two ketosteroids used in this study produced markedly dissimilar results. Cholestanone elicited a significant reduction in acetate incorporation into cellular sterol, yet had no pronounced effect on cellular growth, whereas Δ^4-cholestenone demonstrated toxicity

TABLE 1 INFLUENCE OF STEROIDS ON CELLULAR GROWTH AND DESMOSTEROL SYNTHESIS IN L CELLS

Compound	5 μg		20 μg		40 μg	
	Synthesis	Growth	Synthesis	Growth	Synthesis	Growth
	*% of control values**					
Cholesterol, Δ^5	76 ± 3	94 ± 4	32 ± 3	92 ± 3	17 ± 2	60 ± 5
Desmosterol, $\Delta^{5,24}$	46 ± 6	98 ± 5	7 ± 1	69 ± 7	5 ± 1	63 ± 3
Lathosterol, Δ^7	104 ± 11	97 ± 9	66 ± 4	97 ± 6	30 ± 9	67 ± 4
7-Dehydrocholesterol, $\Delta^{5,7}$	87 ± 2	95 ± 3	30 ± 3	86 ± 5	9 ± 1	71 ± 8
Cholestanone, 3 keto, 5αH	47 ± 8	105 ± 9	29 ± 7	82 ± 9	22 ± 6	69 ± 13
Coprostanol, 5βH	90 ± 3	105 ± 2	75 ± 10	40 ± 4	41 ± 6	29 ± 2
Δ^4-Cholestenone	18 ± 7	26 ± 2				

Controls contained delipidized calf serum protein (5 mg/ml) plus lecithin (20 μg/ml). Values are averages of at least four experiments ± SE.

* Control value = 100%.

when present in the culture medium at a level of 5 μg/ml. Complete cellular lysis was obtained when this compound was added at a level of 20 μg/ml. In animals, high dose levels of cholestenone have been shown to be toxic (18). At concentrations of 1 μg/ml (not shown in Table 1) Δ^4-cholestenone had no effect on cellular growth; however, sterol synthesis was 60 ± 8% that of control cultures. Preliminary experiments indicated that both cholestanone and Δ^4-cholestenone are actively metabolized by L cells, although the end products of this metabolism have not yet been identified. Studies in other experimental systems would suggest that cholestanol could be an end product of the metabolism of Δ^4-cholestenone and cholestanone (19–21). Cholestanol is probably not the sole product in this L-cell system, since cholestanol produces only a very limited effect on sterol biosynthesis and L-cell growth (Table 2). Studies on progesterone metabolism in L cells (22) have demonstrated that these cells possess 2α- and 3β-hydroxysteroid dehydrogenase and Δ^4-5α-reductase. It is possible that these enzymes play a role in the active metabolism of the Δ^4-cholestenone in L cells.

Table 2 lists compounds which, when added to the culture medium, produced little or no inhibition of sterol biosynthesis in L cells. Cholestanol elicited only a slight response at the highest concentrations. This lack of activity of cholestanol in the tissue culture system contrasts with results from experiments in mice and rats which have demonstrated reduced synthesis of sterols in livers of animals fed high levels of cholestanol (21, 23). However, Kandutsch and Packie (21) failed to demonstrate an inhibition of liver sterol synthesis in mice injected with cholestanol. The differences observed may be related to the ability of various cells to convert this sterol to other products. It has been demonstrated that cholestanol is not actively metabolized in L cells (24).

The four phytosterols, β-sitosterol, campesterol, stigmasterol, and ergosterol (Table 2), have no pronounced effect on sterol biosynthesis. A common feature of these compounds is that they contain a methyl or ethyl group at the 24 carbon of the sterol side chain. A detailed study of the metabolism of sitosterol has indicated that this compound is incorporated into L cells at rates considerably lower than that of cholesterol (24). Probably other C_{28} and C_{29} sterols exhibit characteristics similar to sitosterol.

The lack of response noted when cholesteryl oleate was added to the culture medium can also be attributed to the relatively low rate of incorporation of cholesteryl esters in tissue culture cells (25, 26). Previous studies have shown that the L cell is capable of hydrolyzing a large percentage of the esters incorporated from the culture medium (27), so that if cholesteryl oleate were readily incorporated by these cells, sufficient quantities of free cholesterol should be available to produce inhibition of sterol biosynthesis.

Cholestane also did not have any effect on L cells. It is probable that this compound, as well, is incorporated only to a limited degree by the cells.

In an experimental system of the type used in this study, a number of different effects could be expected. If the exogenous sterol were capable of sparing biosynthesis while also being incorporated into functional cellular membranes, then de novo synthesis would be reduced, while cell growth could continue. An example of this type of result was obtained with exogenous cholesterol, which reduced the cellular synthesis of desmosterol but had only a limited effect on cell growth. Lathosterol and 7-dehydrocholesterol produced similar results; however, it has not been established that these compounds are not metabolized by L cells.

If a steroid had the ability to spare biosynthesis but could not be integrated into functional membranes, a pronounced reduction in cell growth could result. The data obtained with coprostanol might be attributed to its inability to function as an integral part of cell membranes,

TABLE 2 INFLUENCE OF STEROIDS ON CELLULAR GROWTH AND DESMOSTEROL SYNTHESIS IN L CELLS

Compound	5 μg		20 μg		40 μg	
	Synthesis	Growth	Synthesis	Growth	Synthesis	Growth
	*% of control values**					
Cholesterol, Δ^5	76 ± 3	94 ± 4	32 ± 3	92 ± 3	17 ± 2	60 ± 5
Cholestanol, 5αH	103 ± 1	103 ± 2	96 ± 7	102 ± 6	87 ± 4	86 ± 8
β-Sitosterol, Δ^5, 24 Et	96 ± 3	106 ± 3	95 ± 3	107 ± 6	77 ± 7	105 ± 9
Campesterol, Δ^5, 24 Et	109 ± 5	94 ± 4	89 ± 8	92 ± 8	72 ± 7	90 ± 10
Stigmasterol, $\Delta^{5,22}$, 24 Et	114 ± 3	100 ± 1	102 ± 2	92 ± 7	113 ± 9	84 ± 8
Ergosterol, $\Delta^{5,7,22}$, 24 Me	94 ± 2	106 ± 3	100 ± 4	97 ± 5	94 ± 6	76 ± 6
Cholesteryl oleate, Δ^5, 3 OR	107 ± 5	95 ± 10	104 ± 4	92 ± 4	98 ± 5	92 ± 4
Cholestane	116 ± 7	108 ± 6	104 ± 5	108 ± 9	96 ± 6	99 ± 3

Controls contained delipidized calf serum protein (5 mg/ml) plus lecithin (20 μg/ml). Values are averages of at least four experiments ± SE.

* Control value = 100%.

while still retaining the ability to reduce the synthesis of cellular sterol. Any system, however, that exhibits a reduction of sterol synthesis together with cellular toxicity must be regarded with caution, since the inhibition of synthesis may not result directly from a feedback response provoked by the steroid, but rather from a secondary response to the inhibitory action of the steroid at some other metabolic level. The results observed when Δ^4-cholestenone was incorporated into the medium may be of this type, since this steroid had a very pronounced inhibitory effect on cellular growth, and there is additional evidence that it is actively metabolized to other compounds.

Finally, it might be expected that with some steroids no effect on either synthesis or growth would be observed. Table 2 lists compounds of this type. A lack of cellular response to exogenous steroid could be explained by lack of incorporation or by metabolism to inactive compounds. Previous investigations have indicated that increasing the concentration of sterol in the culture medium while keeping phospholipid and protein levels constant results in increased incorporation (28). A recent study by Avigan, Williams, and Blass (29) has shown that a number of sterols, including stigmasterol, β-sitosterol, and cholestanol, when present in the incubation medium at concentrations 5–40 times as great as the present experiments, can reduce sterol biosynthesis in diploid human skin fibroblasts. However, even at sterol levels of 200–1000 μg/ml, differences in the extent of synthesis reduction were evident (29) and generally paralleled those shown in Tables 1 and 2. An accompanying publication (24) presents the results from studies on the metabolism of β-sitosterol and cholestanol, two compounds which do not greatly affect sterol synthesis when present at levels of up to 40 μg/ml.

The present study demonstrates the potential usefulness of tissue culture cell systems for the study of the cellular metabolism of sterols. Those sterols, such as desmosterol and cholestanol, which differ from cholesterol in the extent to which they initiate a feedback response may prove useful in elucidating the mechanisms involved in sterol feedback. In addition, the data derived from this investigation suggest that sterols, other than cholesterol, may be utilized for the formation of functional cellular membranes.

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