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REGULATION OF STEROL BIOSYNTHESIS AND LYSIS OF CULTURED HEPATOMA CELLS: INHIBITION OF LANOSTEROL DEMETHYLATION BY HYDROXYSTEROLS

Paul R. Ortiz de Montellano,[*a,c] Jean Paul Beck[b], and Guy Ourisson[c]

[a]Department of Pharmaceutical Chemistry, School of Pharmacy, [b]University of California, San Francisco, California 94143 (USA); [b]Institut de Physiologie et de Chimie Biologique and [c]Institut de Chimie, Universite Louis Pasteur, 67008 Strasbourg (France)

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SUMMARY Demethylation of lanosterol by cultured HTC cells is impaired in the presence of 7α- and 7β-hydroxycholesterol, 22(R)-hydroxydesmosterol, and miconazole. The toxicity of these hydroxysterols does not correlate with their ability to inhibit lanosterol demethylation or to depress HMG-CoA reductase activity, although parallel changes in the latter two activities suggest that both are modulated by interaction of hydroxysterols with a single cellular target.

The biosynthesis of cholesterol is regulated in most cells by tight physiological control of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34), the rate-limiting enzyme in the biosynthetic pathway (1-5). However, although an inverse relationship between cellular cholesterol levels and HMG-CoA reductase activity has been established (1,2), recent work has shown that cholesterol derivatives bearing an additional oxygen atom are substantially more potent than cholesterol as regulators of HMG-CoA reductase. The activity of these hydroxysterols, some of which are physiological precursors or metabolites of cholesterol, has been demonstrated in cultured L-cells (6-9), mouse fetal liver cells (6-9), human fibroblasts (10), and hepatoma tissue culture (HTC) cells (11). In addition to their effect on HMG-CoA reductase, hydroxysterols stimulate cholesterol ester formation in normal human fibroblasts (12) but not in HTC cells (11).

As a consequence of an investigation by the Strasbourg group of Chinese medicinal plants, a number of hydroxysterols have been found to be preferentially cytotoxic to cultured tumor cells, causing lysis of HTC and Zajdela ascitic hepatoma cells at concentrations which have little effect on normal

*Author to whom correspondence should be addressed in San Francisco.

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mouse fibroblasts (15-17). An important characteristic of this cytotoxicity is that affected cells not only lose the ability to exclude trypan blue dye but in fact undergo rapid lysis. Strikingly, in all cases examined, hydroxysterols which were cytotoxic also depressed the activity of HMG-CoA reductase, although many sterols which suppress HMG-CoA reductase are not particularly toxic. The absence of an intimate link between these two activities is evident in the fact that, although all four compounds attenuate HMG-CoA reductase equally well (7,11,16,17), 7β-hydroxycholesterol and 22(R)-hydrodesmosterol are highly cytotoxic whereas the isomeric 7α-hydroxycholesterol and 22(S)-hydroxydesmosterol are relatively nontoxic (16,17).

Little is known about the mechanism by which the toxicity of 7β-hydroxycholesterol and related agents is expressed other than that it is antagonized by addition of cholesterol to the culture medium (17), an observation which argues that localized or general cholesterol deprivation is either a necessary precondition or the actual cause of cell death. In view of this, and the lack of correlation between cytotoxicity and HMG-CoA reductase activity, we have investigated the possibility that hydroxysterols also inhibit cholesterol synthesis at a stage beyond mevalonic acid. The occurrence of cell lysis in the presence of toxic hydroxysterols focused our attention on lanosterol demethylation, since existing evidence suggests that the 14-methyl group of lanosterol is particularly disruptive of membrane structure and function (18,19) and since mutant cells which accumulate lanosterol apparently undergo lysis (20). We report here that hydroxysterols do indeed inhibit lanosterol demethylation in cultured HTC cells, and that this inhibitory action correlates well with suppression of HMG-CoA reductase but not with cytotoxicity.

MATERIALS AND METHODS

Synthetic samples of 7α- and 7β-hydroxycholesterol, and of 22(R)-hydroxydesmosterol, were provided by Dr. Yoichi Nakatani. The purity of these samples was checked before each experiment by thin layer chromatography on silica gel (diethyl ether). Merck aluminum-backed F254 silica gel plates were used for all chromatography. Miconazole nitrate (batch F17/1) was kindly provided by Dr. Hugo Van den Bossche of Janssen Pharmaceutica. (RS)-5-(^{3}H)-mevalonic acid sodium salt (8.7 Ci/mmol) was obtained from CEA, Gif-sur-Yvette, France. Cell culture media were purchased from Gibco Bio-cult.

Toxicity assays The toxicity of hydroxysterols and other substances was evaluated in spinner cultures of HTC cells as previously described (15-17).

Inhibition of lanosterol demethylation Approximately 10^6 HTC cells, derived from Morris hepatoma 7288c (21), were transferred to Falcon flasks containing 25 ml of Swim's medium supplemented with 10% (by volume) of newborn calf serum. After incubation at 37°C for 4 days, the medium was replaced with 15 ml of fresh solution. Incubation for a further 24 hrs provided monolayer cultures in which the cells were at approximately 80% of confluency. Test compounds were added as ethanol solutions, each flask receiving 60 µl total of ethanol (0.3% by volume). The cells were incubated at 37°C for 3 hr with the test compounds before radiolabeled mevalonic acid (2.5×10^6 dpm) was added in 200 µl of water. One hour later, the medium was removed and the cells were quickly washed twice with 10 ml portions of phosphate buffered saline prewarmed to 37°C. The cells were then detached by incubation with 4 ml of 2N NaOH for 20 min, the cell suspensions were transferred to erlenmeyer flasks, and the Falcon flasks were rinsed with 2 ml of 2N NaOH and 3 ml of ethanol, the rinses being added to the initial cell suspensions. Duplicate incubations were usually combined at this point. The cell suspensions, after addition of 50 µg each of lanosterol and cholesterol, were saponified for 90 min at 80°C. Extraction with methylene chloride (4 x 8 ml), washing of the extracts with 10 ml water, drying over anhydrous sodium sulfate, and solvent removal yielded residues which were subjected to thin layer chromatography (3% ethyl acetate-methylene chloride). An aliquot of the residue was removed for liquid scintillation counting before chromatography. The plates, after examination on a Berthold radioscanner, were visualized by brief iodine exposure and by spraying authentic sterols spotted on the outside edges with a dilute ethanol solution of berberine hydrochloride. The sterol fractions were scraped into vials for liquid scintillation counting or, alternatively, were extracted with methylene chloride-methanol and the recovered sterols were recrystallized with authentic carriers from ethyl acetate-methanol.

RESULTS AND DISCUSSION

The proportional incorporation of radiolabeled mevalonic acid into lanosterol and cholesterol by HTC cells exposed to several concentrations of 7α- and 7β-hydroxycholesterol has been determined. Mevalonic acid was used as the labeled precursor in order to circumvent the effect of hydroxysterols on HMG-CoA reductase. The results of one such experiment, in which the absolute values but not the pattern vary slightly from one experiment to another, are given in Fig. 1. As shown, whereas little label appeared in lanosterol in the absence of 7α- or 7β-hydroxycholesterol, in the presence of either of these agents at a 30 µM concentration approximately 30% of the labeled sterol was lanosterol. Preliminary experiments showed that 90-95% of the radioactivity on the plates was in the sterol bands, insignificant amounts of label accumulating in squalene or other resolvable products. The formation of labeled lanosterol was confirmed by cocrystallization with authentic

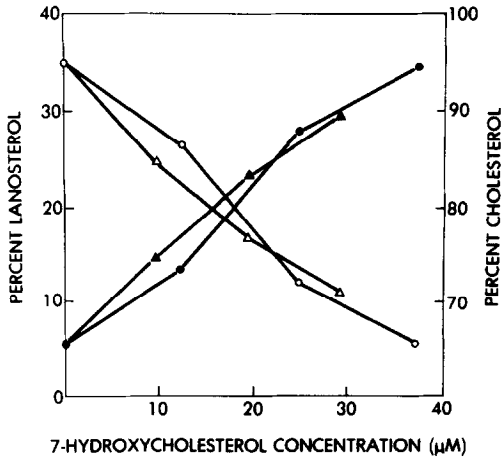

Fig. 1. Incorporation of (RS)-5-(^{3}H)mevalonic acid into lanosterol and cholesterol by cultured HTC cells exposed to 7α-hydroxycholesterol (○ and ●) and 7β-hydroxycholesterol (△ and ▲). The percent of the recovered label found in lanosterol (closed symbols) and cholesterol (open symbols) is plotted as a function of 7-hydroxycholesterol concentration.

carrier, although the state of unsaturation of the sidechain can not be specified since 24,25-dihydrolanosterol is not separated from lanosterol by the procedures used. Retention of the 4- and 14-methyl groups is firmly demonstrated, however. Although the total radioactivity recovered from treated cells was usually lower than that from untreated cells, the incorporation of label into lanosterol was enhanced in treated cells both in absolute terms and in relation to that incorporated into cholesterol. Lanosterol demethylation is also inhibited by 22(R)-hydroxydesmosterol (Table 1), the most toxic of the hydroxysterols examined in Strasbourg to date (16,17).

Miconazole is a clinically useful antimycotic agent which has been shown to inhibit the 14-demethylation of sterols in yeast cells (22). As shown in Table 1, a 30 μM concentration of this agent also virtually blocks the demethylation of lanosterol in HTC cells. In order to investigate the consequences of lanosterol accumulation in HTC cells, the toxicity of miconazole was evaluated. Miconazole at 16 μg/ml (34 μM) inhibited the growth of HTC cells (Fig. 2), while at higher concentrations it was highly toxic. However, microscopic examination of the cells after exposure to trypan blue dye revealed that cells killed by miconazole absorbed the dye but retained their

TABLE 1. Relationship between inhibition of lanosterol demethylation, suppression of HMG-CoA reductase activity, and toxicity of hydroxysterols in HTC cells.

Substance	HMG-CoA reductase[a] (% of control)	% of label in[b] lanosterol	toxicity[c]
none	100_{11}	5	600
7α-hydroxycholesterol	22_{17}	30	400
7β-hydroxycholesterol	35_{17}	30	10
22(R)-hydroxydesmosterol	30_{17}	25	0
miconazole		94	50

[a]Measured in HTC cells exposed to 5 μg/ml of the given sterol for 9 hrs (17) or 4 hrs (11).
[b]The percent of the total sterol label in lanosterol after 3 hrs preincubation of HTC cells with a 30 μM concentration of the inhibitor and subsequent incubation with labeled mevalonic acid for 1 hr.
[c]Toxicity given for HTC cells exposed to 25 μg/ml of the given agent (33 μg/ml of miconazole) for 72 hrs. Sterol data is from references 16 and 17. The number given is the number of viable cells divided by the initial number of cells times 100.

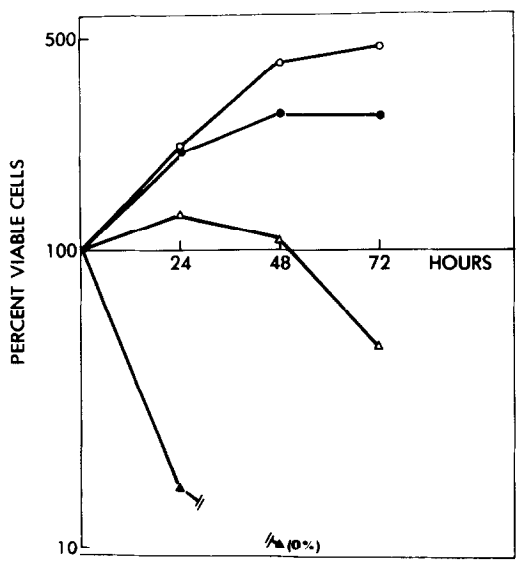

Fig. 2. Toxicity of miconazole for cultured HTC cells. The number of viable cells divided by the starting number of cells (110.000 cells/ml), times 10, is plotted against the time of incubation with different concentrations of miconazole. The miconazole concentrations are zero (0), 16 (●), 33 (Δ), and 66 μg/ml (▲).

cellular integrity. The rapid cell lysis characteristic of hydroxysterol toxicity was not observed, suggesting that miconazole toxicity, whether due to lanosterol accumulation (22) or oxidative processes (23), in any case differed from that of the hydroxysterols.

Inhibition of lanosterol demethylation by hydroxysterols is mediated by an unknown mechanism. There is no obvious connection, however, between inhibition of lanosterol demethylation and cytotoxicity due to hydroxysterols since both the toxic 7β-hydroxycholesterol and its non-toxic 7α-isomer inhibit demethylation equally well. This conclusion is supported by the fact that miconazole, a potent demethylation inhibitor, does not cause rapid cell lysis.

Despite the lack of correlation with toxicity, inhibition of lanosterol demethylation correlates surprisingly well with suppression of HMG-CoA reductase activity (Table 1). These two activities have also been correlated recently in hepatocytes by Watson and coworkers (24). The parallel changes in these two biosynthetic steps may be independent consequences of the interaction of hydroxysterols with a common receptor, or with a single cellular constituent such as a transport protein. On the other hand, the possibility exists that lanosterol accumulation is a signal by which oxygenated sterols regulate the activity of HMG-CoA reductase.

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