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Cholesterol synthesis
cultured glia

HMG-CoA reductase
methylxanthines

Reduction of Cholesterol Synthesis by Methylxanthines in Cultured Glial Cells

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Summary

Methylxanthines are shown in this study to decrease cholesterol synthesis in cultured C-6 glial cells. Aminophylline (10^{-3}M) produced a rapid decline in cholesterol synthesis so that by 6 hr, synthesis in treated cells was less than 20% of that in untreated cells, and by 24 hr, less than 10%. Aminophylline induced parallel changes in 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, the rate-limiting enzyme in cholesterol synthesis. Theophylline and caffeine produced similar effects. Thus, methyxanthines caused a specific, enzyme-mediated reduction in cholesterol synthesis in C-6 glia. In an attempt to relate the methylxanthines' effect on cultured glial cells to the developing nervous system, cholesterol synthesis was studied in the presence of 10^{-4}M aminophylline. This represents a theophylline concentration in the culture medium that is of the same order of magnitude as that produced in whole brain of animals administered therapeutic doses of aminophylline. Under these conditions, cholesterol synthesis was reduced to approximately 60% of control after 48 and 72 hr.

Speculation

Cholesterol is the major lipid constituent of myelin and other cell membranes and reduction in synthesis of this lipid could have deleterious effects. C-6 glia are models of the glial stem cell found in developing brain before myelination, *e.g.*, human brain in the perinatal period. This study raises the question whether serious reductions in brain cholesterol synthesis occur *in vivo* during the treatment of neonatal apnea.

Methylxanthines, such as theophylline, aminophylline, and caffeine, currently are used extensively in the treatment of apnea in the neonatal period (1, 6, 7, 10, 15, 17). However, little is known about potential long and short-term effects of this class of drugs on the developing brain. The methylxanthines are known central nervous system stimulants (13) and have effects on enzyme systems and hormone release in organs other than the brain (14). These extraneural effects are produced primarily by inhibition of adenosine 3':5'-monophosphate (cAMP) phosphodiesterase and potentiation of the action of cAMP (14). The major biochemical effects of methylxanthines on the central nervous system remain to be elucidated.

The neonatal period is a time of active membrane proliferation and the onset of myelination in the human brain (5). A major lipid constituent of all cellular membranes, including myelin, is cholesterol. It is known that cholesterol of the developing brain is derived primarily from *de novo* synthesis, primarily in the glial elements of the brain (5).

Because of these facts, the present study of C-6 glial cells was undertaken with the following objectives: 1) to determine whether methylxanthines regulate cholesterol synthesis; 2) to define the enzymatic site of any regulatory effects; and 3) to determine whether regulatory effects occur at known tissue concentrations of the drugs. C-6 glial cells in culture were utilized to avoid the problems of cellular heterogeneity encountered in studies of whole brain or brain slices. Moreover, because C-6 glial cells exhibit properties of both oligodendroglia and astrocytes (3, 18, 23), they are considered to be good models of the stem cell that gives rise to these glial types and that is found in developing brain.

MATERIALS AND METHODS

MATERIALS

DL-3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), NADPH, dithiothreitol, theophylline, caffeine, digitonin, and cholesterol were purchased from Sigma Chemical Co., St. Louis, MO. Aminophylline Injection United States Pharmacopeia (25 mg/ml) was purchased from Abbott Laboratories, North Chicago, IL. The detergent, Kyro-EOB, was the generous gift of Proctor and Gamble, Cincinnati, OH. Dowex AG 1-X8 (200–400 mesh, formate) was purchased from BioRad, Richmond, CA. DL-3-Hydroxy-3-methyl-3-[^{14}C]glutaryl coenzyme A (18.0–26.2 Ci/mmole), DL-[5-^{3}H]mevalonic acid (dibenzylethylene-diamine salt) (6.7 Ci/mole), [1,2-^{3}H]cholesterol (60 Ci/mmole) and $^{3}H_2O$ (18 Ci/mole) were purchased from New England Nuclear, Boston, MA. [1-^{14}C]Acetate (59.1 Ci/mole) was purchased from Amersham-Searle, Arlington Heighhts, IL. Radioactive products were counted in a preblended scintillation fluid (3a70 scintillation fluid, Research Products International, Elk Grove, IL). All sera, antibiotics, and other reagents for cell culture were obtained from Grand Island Biological Co., Grand Island, NY. All tissue culture flasks were plastic and purchased from Falcon, Cockeysville, MD.

CELL CULTURE

C-6 glial cells were purchased from American Type Culture Collection, Rockville, MD. The methods of cell culture have been described (21, 22). C-6 glial cells have been adapted to growth in a basal medium free of serum. The medium is composed of glucose, gluconolactone, amino acids, vitamins, inositol, pyruvate, and various inorganic salts.

For each experiment, cells were derived from a single flask. The size of each inoculum was identical and adjusted to give a final concentration of 0.5×10^6 cells/ml in either 25- or 75-cm^2 flasks. The former were used for all measurements of cholesterol and protein synthesis and the latter for all determinations of HMg-CoA reductase activity.

PREPARATION OF CELL EXTRACTS FOR ENZYME ASSAYS

Cells were harvested with EDTA (0.02% w/v) as described (21) and centrifuged at 1500 rpm for 10 min. The cell pellet was frozen in liquid nitrogen, thawed, and resuspended in 200 μl of buffer (50 mM potassium phosphate, pH 7.5, 5 mM dithiothreitol, 1 mM EDTA, and 0.25% Kyro-EOB) as described by Brown *et al.* (4).

HMG-CoA REDUCTASE ASSAY

The enzyme was assayed in cell extracts by the method of Brown *et al.* (4) as modified in the authors' laboratory (19). The radioactive product ([^{14}C]mevalonic acid) was separated from the radioactive substrate ([^{14}C]HMG-CoA) by a modification (9) of the ion-exchange procedure of Avigan *et al.* (2). Recovery of the reaction product was adjusted to 100% by use of the internal standard ([^{3}H]mevalonate).

PROTEIN DETERMINATION

Protein in cellular extracts was determined by the method of Lowry *et al.* (8).

CHOLESTEROL SYNTHESIS

The synthesis of cholesterol in C-6 glia was determined by measuring the incorporation of [1-^{14}C]acetate or $^{3}H_2O$ into digitonin precipitable sterols after pulsing the cells for 2 or 3 hr, respectively. The cells were harvested by the addition of 2 ml of 2N NaOH to the flasks. Digitonin precipitation was performed by the method of Popjak (12), as modified (16).

PROTEIN SYNTHESIS

The synthesis of protein was determined by measuring incorporation of $^{3}H_2O$ into trichloroacetic acid precipitable material (20).

STATISTICAL PROCEDURES

Statistical significance was determined by Student's *t* test. The data presented in the tables and figures were subjected to this analysis and all differences discussed in the text are significant at the $P < 0.01$ level or better.

RESULTS

Initially, the effect of 10^{-3}M aminophylline on cholesterol synthesis in C-6 glial cells as a function of time of exposure to the drug was determined (Fig. 1). A rapid decline in cholesterol synthesis occurred in the treated cells. By 6 hr synthesis was reduced to less than 20% of control values, and by 24 hr, to less than 10%.

Next, the possibility was considered that such dramatic and rapid effects were related to a nonspecific alteration in cellular uptake of [^{14}C]acetate, its activation to acetyl-CoA, or to changes in intracellular pool sizes. Thus, the effect of aminophylline on cholesterol synthesis was examined at 6 hr with $^{3}H_2O$ as precursor. At the same time, in separate flasks, incorporation of $^{3}H_2O$ into protein was measured to determine the specificity of aminophylline's effect on cholesterol synthesis (Table 1). Cholesterol synthesis from $^{3}H_2O$ was reduced more than 2-fold after 6 hr of exposure to aminophylline. This effect is similar to that observed with [^{14}C] acetate. Moreover, the inhibitory effect on cholesterol synthesis was not accompanied by any alteration in total protein synthesis.

To establish further that the reduction of cholesterol synthesis induced by methylxanthines is a specific regulatory effect and not a nonspecific toxic effect, the reversibility of the changes was evaluated (Fig. 2). Thus, the cells were exposed to 10^{-3}M aminophylline for 6 hr. This caused a reduction in cholesterol synthesis to 20% of control values. The glia were then washed with buffer and placed in medium free of aminophylline. Cholesterol synthesis recovered dramatically within 18 hr of removal of the drug and was complete by 72 hr.

To define the enzymatic site of aminophylline's action, its effect on the rate-limiting enzyme, HMG-CoA reductase was evaluated

Table 1. *Effect of 10^{-3} M aminophylline on incorporation of $^{3}H_2O$ into cholesterol and protein after 6 hr*[1]

Addition	Cholesterol synthesis, cpm/mg protein	Protein synthesis, cpm/mg protein
None	897 ± 88	2546 ± 265
Aminophylline	357 ± 24	2525 ± 35

[1] C-6 glial cells were grown in 10^{-3}M aminophylline as described in the legend to Figure 1. At 6 hr, the cells were pulsed with $^{3}H_2O$ and harvested. Cholesterol was isolated by digitonin precipitation and total protein by trichloroacetic acid precipitation. Values are means ± SD obtained from separate determinations on each of 3 flasks and expressed as cpm of ^{3}H incorporated/mg protein.

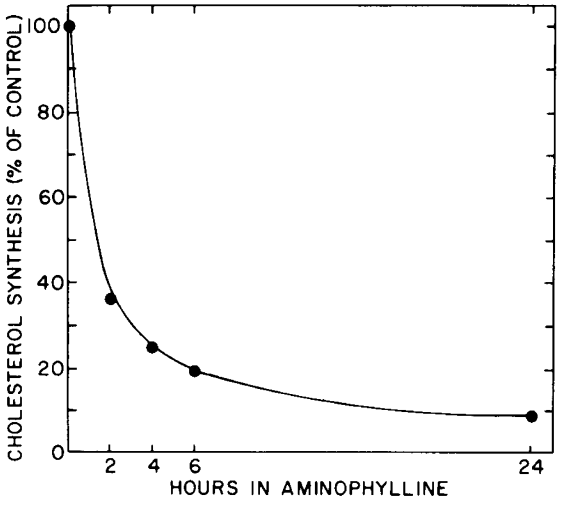

Fig. 1. Effect of aminophylline on cholesterol synthesis. C-6 glial cells were transferred to a series of flasks as described in *Methods*. After 48 hr, the medium was changed so that all flasks were free of serum. Aminophylline was added to one-half of the flasks in a final concentration of 10^{-3}M. At the indicated times, the cells were pulsed with [^{14}C]acetate, harvested, and cholesterol isolated by digitonin precipitation. Values are means obtained from separate determinations on each of 3 flasks, expressed as percent of control, *i.e.*, flasks without aminophylline, and did not vary more than 5–10%.

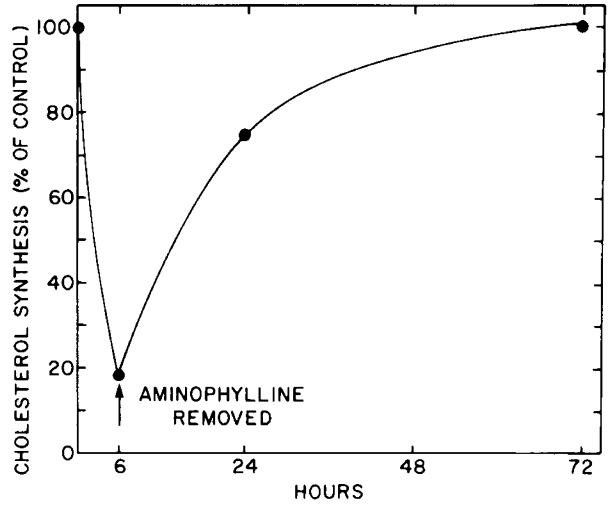

Fig. 2. Recovery of cholesterol synthesis after exposure to aminophylline for 6 hr. C-6 glial cells were grown in the presence of 10^{-3}M aminophylline for 6 hr as described in the legend to Figure 1. At 6 hr, flasks with and without aminophylline were pulsed with [^{14}C]acetate, harvested, and cholesterol isolated by digitonin precipitation. The remainder of the flasks were washed with 50 mM Tris buffer, pH 7.3, and fresh media added. At 24 and 72 hr, cholesterol synthesis was determined in the remaining cells. The values are means obtained from separate determinations on 3 flasks, expressed as percent of control, *i.e.*, flasks without aminophylline, and did not vary more than 5–10%.

(Table 2). After exposure of the cells to the drug for 2, 6, and 24 hr, reductase activity was observed to be 102, 30, and 9% of control values, respectively. The decreases in reductase activity seen at 6 and 24 hr correlate closely with the decreases in cholesterol synthesis described previously (see Fig. 1).

The other clinically utilized methylxanthines, theophylline, and caffeine, were also shown to cause a decrease in reductase activity after 24 hr of exposure to C-6 glia (Table 3). Thus, 10^{-3}M theophylline resulted in a decrease of reductase activity to 21% of control values, and 10^{-3}M caffeine, to 17%.

Finally, to approximate more closely the *in vivo* situation, cholesterol synthesis in glia exposed to a concentration of aminophylline (10^{-4}M) that is more nearly similar to that found in blood and brain of animals treated with therapeuutic doses of the drug was measured (11) (Table 4). Despite the 10-fold lower concentration of the drug, a distinct reduction of cholesterol synthesis occurred. Thus, after 48 and 72 hr, values in treated cells were only approximately 60% of those in control cells.

Table 2. *Effect of 10^{-3}M aminophylline on HMG-CoA reductase activity*[1]

Addition	Time, hr	HMG-CoA reductase activity, pmole/mg/min	% of control
None	2	8.7 ± 2.0	
Aminophylline		8.9 ± 2.0	102
None	6	35.4 ± 4.8	
Aminophylline		10.7 ± 1.8	30
None	24	25.8 ± 2.5	
Aminophylline		2.4 ± 0.3	9

[1] C-6 glial cells were grown in 10^{-3}M aminophylline as described in the legend to Figure 1. At the times indicated, the cells were harvested for assay of HMG-CoA reductase as described in *Methods*. Values for HMG-CoA reductase activity are means ± SD obtained from separate determinations on each of 3 flasks. The data are also expressed as percent of control, *i.e.*, flasks without aminophylline.

Table 3. *Effect of 10^{-3}M theophylline and caffeine on HMG-CoA reductase activity at 24 hr*[1]

Addition	HMG-CoA reductase activity, pmole/mg/min	% of control
None	29.9 ± 2.3	
Theophylline	5.7 ± 0.6	21
Caffeine	4.8 ± 0.5	17

[1] C-6 glial cells were grown in 10^{-3}M theophylline, 10^{-3}M caffeine or media free of drug as described in legend to Figure 1. At 24 hr, the cells were harvested for assay of HMG-CoA reductase as described in *Methods*. Values for HMG-CoA reductase are means ± SD obtained from separate determinations on 3 flasks. The data are also expressed as percent of controls, *i.e.*, flasks without added drug.

Table 4. *Effect of 10^{-4}M aminophylline (38 μg/ml theophylline) on cholesterol synthesis*[1]

Addition	Time hr	Cholesterol synthesis, cpm/mg protein	% of control
None	48	32,300 ± 2600	
Aminophylline		20,080 ± 1300	62
None	72	37,450 ± 1600	
Aminophylline		21,280 ± 1000	57

[1] C-6 glial cells were grown in 10^{-4}M aminophylline as described in the legend to Figure 1. At the times indicated, the cells were pulsed with [^{14}C] acetate, harvested, and cholesterol isolated by digitonin precipitation. The values are means ± SD obtained from separate determinations on 3 flasks and expressed as cpm of ^{14}C incorporated/mg protein. The values are also expressed as percent of controls, *i.e.*, flasks without aminophylline.

DISCUSSION

This study has dealt with regulation by methylxanthines of cholesterol synthesis in cultured C-6 glial cells. Cells exposed to these drugs at 10^{-3}M for only 6 hr exhibit a marked reduction in cholesterol synthesis. This effect was shown to be accompanied by a comparable change in the activity of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway. The effect was reproduced with $^{3}H_2O$ as precursor for measurements of cholesterol synthesis. In contrast, no effect on protein synthesis occurred under similar conditions. In addition, cholesterol synthesis in the treated cells was shown to recover completely after removal of the drug. These data suggest that methylxanthines have a specific regulatory effect on cholesterol biosynthesis in C-6 glial cells and that the site of their action is HMG-CoA reductase.

Cholesterol is a major lipid constituent of myelin and all cellular membranes. Thus, a reduction in cholesterol synthesis could have significant deleterious effects on membrane production and myelination. C-6 glial cells exhibit many of the characteristics of the glial stem cell found in the developing nervous system just before myelination. Thus, inhibition of cholesterol synthesis in C-6 glial raises the question: do such effects occur *in vivo* and, more specifically, in newborns administered methylxanthines for the treatment of apnea?

Because of this latter question, aminophylline's effect on glial cholesterol synthesis at a concentration of 10^{-4}M was investigated. Intracellular concentrations of methylxanthines *in vivo* under therapeutic conditions are unknown. However, Neese and Soyka (11) have determined concentrations of aminophylline in whole brain of 10-day-old mice administered a dose of 4 mg/kg rectally and found the levels to be 18.8 μg/g tissue wt at 15 min and 14.3 μg/g at 120 min after drug administration. Brain:blood ratios of the drug were found generally to be slightly greater than unity. This suggests that blood levels are a good approximation of brain levels. Currently, it is suggested that theophylline blood levels be maintained in the range of 10–20 μg/ml in treating neonatal apnea (16). Thus, 10^{-4}M aminophylline (38 μg/ml of theophylline) is of a similar order of magnitude. The fact that 10^{-4}M aminophylline decreases cholesterol synthesis significantly in cultured glial cells raises the possibility that therapeutic levels of methylxanthines may have similar effects *in vivo*.

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