Theophylline Reduces the Activity of Cerebroside-Sulfotransferase, a Key Enzyme in Myelination, in Cell Cultures from Newborn Mouse Brain¹

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Summary

Theophylline, a drug used in neonatology for the treatment of apnea, affects cholesterol synthesis if administered in concentrations of 10^{-4} M (a concentration found in serum of treated patients) for 24 hr to dissociated brain cell cultures. The rate-limiting enzyme of cholesterol synthesis, β -hydroxy- β -methylglutaryl-coenzyme A reductase (EC 1.1.1.34), is lowered to 45% 48 hr after removal of theophylline. At the same time, cholesterol content of the cells is lowered to 73%. Inasmuch as the phospholipid content of the cells remains stable, the treatment changes the cholesterol phospholipid ratio. Concomitant to this effect, the activity of cerebroside-sulfotransferase (EC 2.8.2.11) is lowered to 60% of control values. We postulate that these two effects are linked to each other by means of modulation of the cerebroside-sulfotransferase activity by membrane lipids.

Speculation

Theophylline reduces cholesterol synthesis and content and by that cerebroside-sulfotransferase activity in dissociated brain cell cultures. This raises the question of whether theophylline could have similar effects *in vivo*.

Theophylline and its derivatives have been used in perinatal medicine for the treatment of asphyxia in premature newborns ever since Uañy *et al.* (19) could demonstrate in 1975 the beneficial effects of theophylline on severe primary apnea in premature infants. Recently, Allan and Volpe (1) reported that theophylline and aminophylline reversibly inhibit the cholesterol synthesis in rat glioblastoma cells (C₆). The inhibition of cholesterol synthesis in brain cells by theophylline would be of special importance because the brain has to synthesize its cholesterol *de novo* (9). Dietary cholesterol does not penetrate the blood barrier. Cholesterol is a major lipid component of all brain cell membranes and especially of the myelin sheath (3, 18). In addition, the cholesterol and the phospholipid content in brain microsomes are involved in the regulation of enzyme activities important for the synthesis of myelin lipids during myelination (14, 17). In mouse brain microsomes, the developmental change of the cholesterol:phospholipid ratio modulates the activity of cerebroside-sulfotransferase [(CST) EC 2.8.2.11] so that it shows an age-dependent developmental pattern (17). CST catalyses the transfer of a sulfate group to cerebroside forming sulfatide, an acidic sphingolipid which is an important component of myelin (5, 13).

Changing the cholesterol content of glioblastoma C₆ cells in culture by estradiol results in a modulation of CST activity by changing the cholesterol:phospholipid ratio in the Golgi apparatus (15, 16).

Because the influence of theophylline on the brain cholesterol metabolism and its secondary effects on myelin lipid synthesis is

difficult to study in whole animal experiments, we have studied the effect of theophylline in dissociated brain cell cultures as a first step. This culture model is closer to the *in vivo* situation than the C₆ tumor cells. Brain cell cultures have previously been used to study drug effects (2, 7).

In the present study, we investigated whether theophylline inhibits β -hydroxy- β -methylglutaryl-coenzyme A reductase [(HMG CoA reductase) EC 1.1.1.34], which is a key enzyme in cholesterol synthesis, whether this inhibition is followed by a reduction of cellular cholesterol content, and whether we can observe an effect on CST activity.

MATERIALS AND METHODS

Dissociated brain cells were prepared from newborn mice (Jack River Albino) after a method described previously (2, 21). Mouse brains were removed within 2 hr after birth, dissected in Dulbecco's modified Eagle medium at room temperature, minced with razor blades, dissociated by trituration, and incubated in Petri dishes (diameter, 10 cm) with 10 ml of Eagle's modified Dulbecco medium at pH 7.4, 37°C, 80% relative humidity. Cell cultures were gassed with 5% CO₂, 40% O₂, and 55% N₂. Ten percent fetal calf serum (v/v) was present in the media. After 4, 7, and 10 days in culture, the medium was changed. At day 13, 10^{-4} M theophylline (Sigma Chemical Co., St. Louis, MO) were added in 10 ml of medium and left for 24 hr. Then the theophylline was removed by changing the medium, and the cells were incubated for another 24 hr in medium which again contained 10% fetal calf serum. Plates were analyzed immediately before theophylline administration (0 hr, after removal of theophylline (24 hr), and 24 hr after removal of the drug (48 hr). The cells were harvested with a rubber policeman after washing twice *in situ* with 10 ml cold 0.9% NaCl solution. The cells were centrifuged and resuspended in saline three times, finally suspended in 1 ml bidistilled water, and homogenized by sonication for 30 sec at 0°C with a Branson sonifier.

HMG CoA REDUCTASE ASSAY (10, 14)

One hundred μ l of the homogenate containing 200 to 300 μ g protein were preincubated with 2 μ moles NADPH (Boehringer Mannheim) in 30 μ l H₂O and 100 μ l phosphate buffer, 250 mM (pH 7.2) for 10 min at 37°C. After 10 min 1 to 2 (100,000 dpm) nmoles of β -hydroxy- β -[¹⁴C]methylglutaryl-coenzyme A (51.3 mCi/mmole) in 30 μ l H₂O were added, and the incubation was carried out for 1 hr at 37°C in a total volume of 260 μ l. To stop the reaction and to accomplish the formation of mevalonolactone from the product, 40 μ l of 6 N HCl were added, and the mixture was shaken for another 30 min. Then, 1 mg of mevalonolactone (Fluka, Buchs, Switzerland) was added as a carrier, and the incubates were washed with 1 ml of cold benzene. After centrifugation, 50 μ l of the aqueous layer were spotted on a thin-layer chromatography plate (Merck, Darmstadt Germany) and devel-

¹ Dedicated to Andrea Prader on the occasion of his 60th birthday.

oped in benzene:acetone (1:1). The chromatography was stopped after the solvent front was 10 cm above the start, and the spots with an R_F value of 0.48 to 0.58 were scraped out, transferred to a counting vial, and counted after addition of 10 ml Aquassure (New England Nuclear, Dreieich, Germany) in a Packard liquid scintillation counter. The results are expressed as pmoles mevalonolactone formed per hr and mg protein.

CST ASSAY

CST was assayed as described elsewhere with the following modifications (17): 100 μ l of protein suspension (200 to 300 μ g protein), 200 μ l of imidazole buffer (100 mM, containing 20 mM $MgCl_2$ and 200 mM NaCl), 450,000 dpm 3',5' diphosphoadenosyl 5'-sulfate (specific activity, 1 mCi/mole) in 100 μ l 0.9% NaCl solution, and 0.1% Triton X-100 (total volume 500 μ l) were incubated for 20 min at 30°C at pH 6.8. The incubation was stopped by addition of 4 ml chloroform:methanol (2:1) separated in two phases with 1.2 ml of 0.74% KCl. The upper phase was discarded, and the lower phase was washed twice with theoretical upper phase (chloroform:methanol:water, 4:48:47), transferred to a counting vial, dried with a hair dryer, and counted after addition of 10 ml Aquassure in a liquid scintillation counter. The addition of Triton instead of deoxycholate (17) did not solubilize the enzyme protein from the membrane, as checked by centrifugation for 90 min at $100,000 \times g$, but allowed higher protein concentrations in the assay.

Both of the enzyme reactions were linear with protein up to 500 μ g/ml and with time up to 90 min.

LIPIDS

Lipids were extracted from the cell cultures after the method of Folch-Pi *et al.* (6). Cholesterol content was determined after the

method of Zlatkis *et al.* (22). Phospholipids were analyzed as total lipid phosphorus after Donaldson *et al.* (4) and protein after Lowry *et al.* (11).

Sulfatide was checked by thin-layer chromatography as the only lipid containing radioactive sulfate in the CST assay.

RESULTS

The standard experiments were performed as described in "Materials and Methods." The effect of theophylline on HMG CoA reductase activities is shown in Table 1.

The enzyme activities of the control cultures increased up to the end of the experiment whereas in the test cultures, the activity remained constant. Thus, a significant progressive decrease of the relative activity occurs in theophylline-treated cultures even after removal of the drug.

Table 2 summarizes the results of the protein, cholesterol, and phospholipids content in treated and untreated cultures. Throughout the observation period, cholesterol content of the cultures decreases. However, in theophylline-treated cultures, the lowering of cholesterol content is more rapid; therefore, a significantly lower cholesterol content is found after the 24 hr of drug exposure and during the 24-hr recovery period. There is no consistent change in phospholipid and protein content. Because of the changes of the cholesterol content, the molar cholesterol:phospholipid ratio is significantly reduced at the end of the drug exposure and 24 hr thereafter.

The results of the determination of the CST activity in test and control cultures are shown in Table 3. Whereas CST activity in control cultures remains stable during the observation period, in theophylline-treated cultures the CST activity falls after the 24 hr incubation and reaches a value of 58% that of controls after the recovery period.

Table 1. Activity of β HMG CoA reductase (EC 1.1.1.34) in dissociated mouse brain cell cultures treated with 10^{-4} M theophylline for 24 hr¹

Days in culture	Hr after inoculation		HMG CoA reductase activity (pmoles mevalonate/mg protein $\times$ hr)	P^2	% of controls
13	0	Theophylline added (control)	36.6 ± 7.93^1		100
14	24	Control	35.38 ± 2.89		
		Theophylline with- drawn	22.33 ± 2.52	<0.05	63 ± 4
15	48	Control	42.67 ± 6.50		
		Recovery period	19.17 ± 3.32	<0.001	45 ± 8

¹ For details of incubation and experimental design, see "Materials and Methods."

² P 's were calculated as Student's t tests of normal and control plates.

³ Means $\pm$ S.D. of 3 determinations with 2 plates.

Table 2. Cholesterol, phospholipid, and protein content of dissociated brain cell cultures treated with 10^{-4} M theophylline for 24 hr¹

Days in culture	Hr after inoculation		Protein (mg/plate)	Cholesterol (μ g/mg protein)	Phospholipids (μ g/mg protein)	Molar chole- sterol:phospho- lipid ratio
13	0	Theophylline added (control)	3.15 ± 0.0^2	39.97 ± 3.4	256.88 ± 4.62	0.29 ± 0.02
14	24	Control	2.78 ± 0.62	43.77 ± 2.76	266.33 ± 15.37	0.33 ± 0.02
		Theophylline with- drawn	3.08 ± 0.06^3	34.18 ± 3.03^4	303.61 ± 17.21^1	0.23 ± 0.04^4
15	48	Control	3.46 ± 0.47	33.51 ± 1.59	244.33 ± 30.37	0.27 ± 0.02
		Recovery period	3.76 ± 0.55^1	26.83 ± 1.73^4	232.33 ± 22.50^3	0.22 ± 0.01^5

¹ Incubation conditions and determination of the lipids were as described in "Materials and Methods." The cholesterol:phospholipid ratio was calculated from each of two single plates. As a basis for the calculation of the molar ratios, the molecular weight of cholesterol was taken as 387, and for the phospholipids, an average value of 775 was assumed.

² Mean $\pm$ S.D. of 3 experiments of two plates each.

³ Not significant according to Student's t test.

⁴ $P < 0.05$ according to Student's t test.

⁵ $P < 0.001$ according to Student's t test.

Table 3. Activity of CST (EC 2.8.2.11) in dissociated mouse brain cell cultures treated with 10^{-4} M theophylline for 24 hr¹

Days in culture	Hr after inoculation		CST activity (pmoles sulfatide mg protein × hr)	P ²	CST activity in % of controls
13	0	Theophylline added (control)	201 ± 37 ³		
14	24	Control	176 ± 14	<0.001	68
		Theophylline with- drawn	120 ± 6		
15	48	Control	172 ± 3	<0.001	58
		Recovery period	99 ± 9		

¹ For details of incubation and experimental design, see "Materials and Methods."

² P's are calculated as Student's *t* tests of normal and control plates.

³ Mean ± S.D. of 3 determinations. For the calculations of pmoles sulfatide, the specific activity of the added 3',5' diphosphoadenosyl-5'-sulfate was used, assuming that it was not diluted by endogenous precursor. The values are corrected for a lipid-protein ratio of 0.35, as indicated elsewhere (15). This ratio was found in control plates if the medium is changed daily.

Fig. 1. Comparison of the cholesterol:phospholipid ratio with the CST activity as extracted from Tables 2 and 3. ○, values after treatment with 10^{-4} M theophylline for 24 hr; ●, control values. For details, refer to Tables 2 and 3.

DISCUSSION

Theophylline in concentrations of 10^{-4} M added to the medium in brain cell cultures for 24 hr reduces HMG CoA reductase activity to 63%. No recovery was observed within 24 hr after removal of theophylline. Control experiments adding 10^{-4} M theophylline directly to the enzyme assay had no effect on the HMG CoA reductase activity. These results are in accordance with the observation of Allan and Volpe (1), who studied the effect of theophylline on the activity of C₆ cells in culture. Concomitant to the reduced enzyme activity, the cholesterol content of the membranes decreases to 78% during the 24-hr incubation. This decreased cholesterol content in the treated cells remains constant for the 24 hr recovery period compared to the controls. The decreased cholesterol content leads to a significant decrease in the cholesterol:phospholipid ratio 24 hr after the incubation and does not reach control values again during the recovery period.

During the period of decreased cholesterol:phospholipid ratio, CST activity drops to 68% and 58%, respectively. Again, control experiments adding 10^{-4} M theophylline directly to the enzyme assay had no effect on the enzyme activity. Plotting CST activity against cholesterol:phospholipid ratio (Fig. 1) in normal and treated cells suggests that CST activity is modulated by the changed cholesterol:phospholipid ratio. A dependence of CST activity on the cholesterol:phospholipid ratio has been previously

shown in microsomes of mouse brain during development (17), in correction experiments of the decreased CST activity in jimpy (12) brain microsomes (8), and in C₆ cells in culture treated with estradiol (15).

Theophylline is known to affect fatty acid and cholesterol synthesis (20). We have now shown that CST activity is also affected. We have to stress, however, the fact that the system used does not allow us to answer the question of whether the decreased CST activity has an effect on myelination. It will, therefore, be of importance to expand the brain cell culture experiments to the *in vivo* development because in brain cell cultures only few myelination occurs.

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