Regulation by thyrotropin of acyl-CoA: cholesterol acyltransferase in cultured thyroid cells

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ACAT activity measured in microsomes of thyroid cells cultured for 4 days in the presence of TSH (1 mU/ml) was two or three times lower than that of the control cells cultured for the same period in the absence of TSH. The pool of cellular cholesterol which served as the ACAT substrate, was not exchangeable with exogenous cholesterol provided in the form of liposomes. However, the incubation of microsomes with liposomes made it possible to increase the cholesterol content in the microsomes by 35%, and this resulted in the activation of ACAT activity. Nevertheless, maximum activity measured after activation in the microsomes of the control cells remained higher than that of the microsomes of cells cultured in the presence of TSH. These findings would suggest that TSH acted by diminishing the cellular content in ACAT enzyme, as well as modifying the distribution of cholesterol in the intracellular membranes.

Thyroid cell *Acyl - CoA : cholesterol acyltransferase* *Liposome* *Arachidonic acid*

1. INTRODUCTION

The metabolism of cholesterol esters has been the subject of numerous studies which have shown different means of hormonal regulation [1–6] in various tissues. Synthesis of cholesterol esters is achieved by means of a microcosmal enzyme, ACAT, using cholesterol and acyl-CoA. It would appear that this activity is chiefly regulated by the membrane cholesterol content in the vicinity of the enzyme [7], as cholesterol increases ACAT activity, by serving both as a substrate and as a non-substrate modulator [8].

In our previous studies, it was shown that the presence of TSH (1 mU/ml) in thyroid cells under culture, inhibited the capacity of these cells to incorporate arachidonic acid into the cholesterol ester fraction of the cellular lipidic pool. This led us to investigate the possible effect in this system, of a modification in the cholesterol concentration of the cellular membranes. As this could be carried out by cholesterol exchange between liposomes and cells under culture [7,10], it was decided to study the ways and means of this exchange as well as its effect on the ACAT activity of microsomes prepared from cultured thyroid cells using [^{14}C]arachidonate as substrate.

Abbreviations: TSH, thyrotropin; ACAT, acyl-CoA : cholesterol acyltransferase

2. MATERIALS AND METHODS

2.1. *Thyroid cells*

Thyroid cells were obtained as in [11], and incubated in polystyrene Petri dishes at 37°C in a 5% CO_2–95% air, water-saturated atmosphere in the absence (control cells) or in the presence (TSH cells) of 1 mU/ml TSH. The cells were cultured in Eagle's minimum essential medium supplemented by 10% newborn calf serum (Gibco). Since the polystyrene of the dishes was not treated for tissue culture, the cells were cultured for a period of 4 days as an unstirred suspension.

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2.2. *Liposomes*

Phosphatidylcholine/cholesterol (molar ratio 1:1) small unilamellar liposomes were prepared as follows. Egg yolk phosphatidyl choline (8 mg) and cholesterol (4 mg) were co-freeze-dried from chloroform in the presence of 20 μCi of ^{3}H-labeled cholesterol (NEN, 50 mCi/mmol), and the 4 ml sterilized Earle-Hepes buffer (NaCl, 137 mM; KCl, 5.36 mM; Na_2HPO_4, 0.4 mM; $MgSO_4$, 0.8 mM; glucose, 5.5 mM; $CaCl_2$, 1.8 mM; Hepes, 20 mM; pH 7.4) or buffer H (Tris, 10 mM, pH 7.4; sucrose, 0.25 M; DTT, 1 mM) were added to disperse the lipids. The dispersion was sonicated for 1 h in a nitrogen atmosphere at +4°C by using a Branson B12 sonicator with a 'micro' titanium probe and a power output of 50 W. The sonicate was centrifuged for 1 h at 100000 × *g* to remove titanium particles as well as larger multilamellar liposomes. When necessary, labeled cholesterol was omitted or replaced by a tracer amount of [^{14}C]cholesteryl oleate (NEN).

2.3. *Incubation of liposomes with cells*

Optimum conditions were studied in preliminary experiments by varying the liposome/cell ratio and by each time measuring the exchange kinetics of ^{3}H-labeled cholesterol. For this purpose, when the selected incubation time had elapsed, the cells were collected and washed 3 times by centrifugation in a liposome-free medium. The pellet of the cells was then dispersed by sonication in 0.5 ml buffer, and 10 ml scintillant mixture were added (NEN, Aquasol-2). Radioactivity was then measured in a liquid scintillation counter. When 4×10^6 cells were suspended in 1.5 ml medium containing 300 μg cholesterol in the form of liposomes, maximum speed of exchange was achieved. This equalled 1 μg cholesterol exchanged/mg cellular proteins per 4 h. This figure was close to that obtained under comparable experimental conditions by other authors [10] who used rat arterial smooth muscle cells.

2.4. *Preparation of microsomes and incubation with liposomes*

About 10^8 cells were collected on day 4, washed 3 times by centrifugation in buffer H, and homogenized in 4 ml cold buffer H containing 2 mM EDTA in a Dounce homogenizer, by 40 strokes of the tight-fitting pestle. The homogenate was centrifuged for 10 min at 600 × *g* and the resulting supernatant was centrifuged for 1 h at 100000 × *g* in a Beckman L75 centrifuge at 4°C. The pellet was resuspended in 0.4 ml buffer H, aliquoted, and stored at −80°C until utilization. The final suspension contained 0.6–1 mg microsomal protein per ml.

The measurement of cholesterol exchange between liposomes and microsomes was done in tubes containing 400 μg microsomal protein (i.e., approx. 18 μg cholesterol) and 100 μg cholesterol in the form of liposomes prepared in buffer H. After 1 h incubation at 37°C, the incubate was diluted using 5 ml cold buffer and centrifuged at 100000 × *g*. Five ml buffer were added to the pellet which was once more centrifuged at 100000 × *g*. The final pellet obtained was resuspended in 0.2 ml buffer H and the cholesterol was assayed using the enzyme procedure described in [9].

2.5. *Measurement of ACAT activity*

For each measurement point, approx. 80 μg microsomal protein (about 4 μg cholesterol) were preincubated at 37°C for 1 h in 0.2 ml buffer H containing, or not, 20 μg cholesterol in the form of liposomes prepared in the same buffer. The reaction was initiated by adding 0.4 μCi [^{14}C]arachidonyl-CoA (NEN 45 mCi/mmol), which corresponded to a final concentration of 10 μM. The reaction was stopped by adding 1 ml cold buffer, and the totality was extracted in the presence of 10 μg cold cholesteryl arachidonate with 5 ml chloroform/methanol (2:1). The extracted lipids were then separated by thin-layer chromatography as in [9], and the spot corresponding to cholesteryl-arachidonate was scraped and its radioactivity measured. Under these conditions the product apparition kinetic (cholesteryl-arachidonate) was linear for a period of 15 min (fig.1A), and the activity measured was proportional to the amount of protein added in the test, at least in the region 50–200 μg (fig.1B). The proteins were measured using a commercialized kit known as 'Bio-Rad Protein Assay'.

3. RESULTS

3.1. *Exchange of ^{3}H-labeled cholesterol between liposomes and whole cells*

Fig.2 shows that there was indeed an exchange

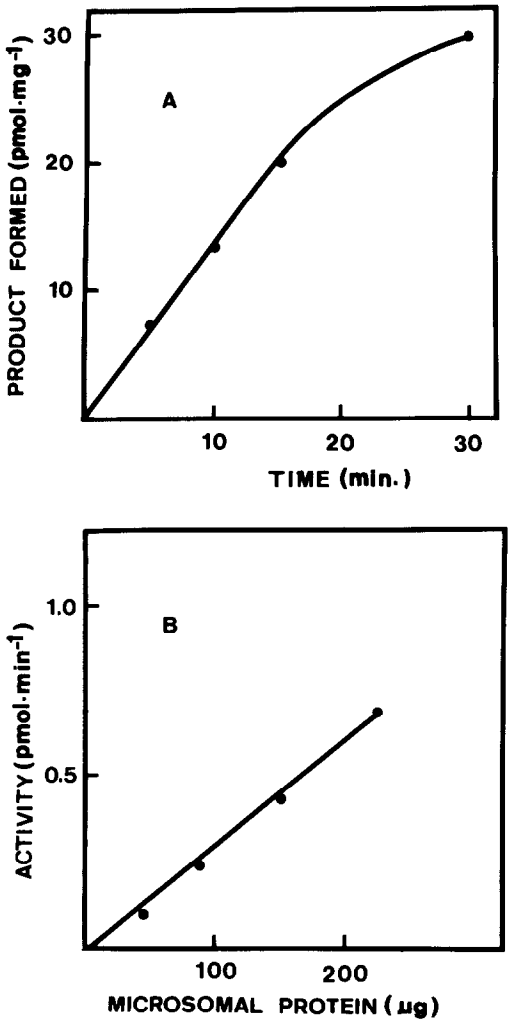

Fig.1. Characteristics of the assay for ACAT activity in microsomes from cultured thyroid cells. Assays were performed under standardized conditions described in section 2 except for the incubation time (A) and microcosmal proteins (B). Each point represents the mean of 3 assays.

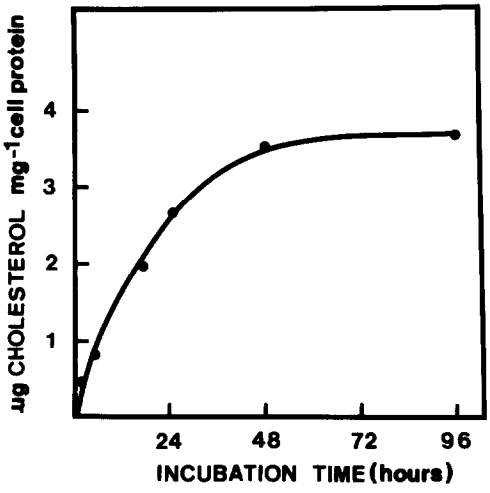

Fig.2. Exchange kinetics of ^{3}H-labeled cholesterol between liposomes and cultured thyroid cells. TSH cells were incubated in the presence of liposomes under pre-established conditions where the speed of cholesterol exchange is maximal. Each point represents the mean of 3 assays.

of ^{3}H-labeled cholesterol between the liposomes and the TSH cells, the kinetics of which presented a plateau after 48 h. The radioactivity associated with the cells was not due to fusion of the liposomes with the cellular membranes, since cholesteryl-[^{14}C]oleate, when used as a non-exchangeable marker, was not found in the cellular lipid pool even after 4 days incubation. Nonetheless, this was only an exchange and not a net transfer since the pre- and post-incubation assay of cellular cholesterol did not show any significant difference. The exchanged tritiated cholesterol was not the ACAT substrate, as the separation on a thin layer, of the cellular lipids extracted after 4 days incubation in the presence of liposomes revealed that no radioactivity whatsoever was associated with the cholesterol ester spot. By using the measured specific radioactivity of cholesterol in the liposomes, and by taking into account the cellular cholesterol pool, it can be calculated that approximately only 25% cellular cholesterol was exchanged. Moreover, after 4 days incubation in the presence of liposomes followed by washing the cells, the capacity to incorporate the arachidonate in the cholesterol ester fraction, remained at a low level in TSH cells (not shown).

3.2. *Measurement of ACAT activity of the microsomes*

As the initial results obtained varied from one batch of cells to another, two microsome pools, christened A and B, were prepared for each type of cell (control and TSH) from two successive primo-explantations, in sufficient quantity to be able to conduct, and as a result compare, on each pool, all the experiments described in this paper (cf. table 1 for results). For each preparation (A and B) ACAT

Table 1

ACAT activity of the microsomes of thyroid cells

Preincubation	TSH microsomes		Control microsomes	
	A	B	A	B
Buffer	0.34 ± 0.10	0.66 ± 0.15	1.00 ± 0.10	1.36 ± 0.20
Liposomes	2.70 ± 0.35	4.33 ± 0.28	3.7 ± 0.22	5.34 ± 0.28

The cholesteryl-arachidonate formed was measured after 15 min incubation under standard conditions described in section 2. Each result is the mean of 3 assays and is expressed in pmol/min per mg microsomal protein ± SE. A and B refer to the A and B pools of microsomes

activity of the microsomes originating from the control cells (control microsomes) was significantly higher than that of the microsomes originating from the TSH cells (TSH microsomes) after 4 days in culture. As this could reflect an increase in the amount of enzyme and/or cholesterol in the microsomal membranes, the microsomes were therefore preincubated in the presence of liposomes. Given that in general, the cholesterol content of the intracellular membranes is much lower than that of the plasma membranes [12], we hoped to observe a net transfer of the cholesterol of the liposomes to the microsomes, and subsequently an activation of ACAT activity. The findings in table 2 show that under these conditions the cholesterol content in the microsomes was subject to a marked increase. Moreover, this was accompanied by a very large increase in ACAT activity, both in the microsomes originating from the control cells and those originating from the TSH cells. Nevertheless, for each of the preparations, ACAT activity of the control microsomes remained significantly higher (table 1).

Table 2

Cholesterol content of microsomes after incubation in the presence of liposomes

	TSH microsomes	Control microsomes
Incubated 1 h without liposomes	47.0	48.8
Incubated 1 h with liposomes	64.5	66.6
Relative increase in cholesterol content	34%	36%

The figures represent the amount of cholesterol expressed in ng/μg protein. These are the results of a standard experiment performed on microsome pool A. Other experiments conducted on either pool A or pool B gave very similar results

When measured at the onset of culturing, ACAT activity of the cells' microsomes was low (0.32 ± 0.12 pmol/min per mg and 2.5 ± 0.35 pmol/min per mg after preincubation with liposomes), and comparable to the activity measured in TSH microsomes on day four. It was therefore concluded that the spontaneous rise in arachidonic acid incorporation in the cholesterol ester fraction of the control cells described in [9], is paralleled by the spontaneous increase in the ACAT activity described here. The chronic treatment by 1 mU/ml TSH, which was shown to completely inhibit the prostaglandin E_2 synthesis [17], also inhibits these two phenomena.

4. DISCUSSION

The regulation by TSH of the incorporation of labeled arachidonic acid in the cholesterol ester fraction of cultured thyroid cells led us to envisage the following two hypotheses: (a) either the amount of ACAT molecule per cell had increased during the first four days in the absence of TSH, and TSH had an inhibitory effect on this increase; or (b) although the total amount of cholesterol per cell had not increased in the control cells as compared to the TSH cells [9], the distribution of cholesterol in the cellular membranes was modified in such a way as to make ACAT inactive. The

possible increased hydrolysis of cholesterol esters was not investigated since it seems to be dependent only on the increase in the concentration of free cholesterol acceptors (HDL or other) in the medium [1]. The hypothesis of a mechanism of phosphorylation/dephosphorylation of ACAT which has been put forward by Gavey et al. [13] was set aside as this controversial proposal [14] only takes acute regulation phenomena into account.

To measure the relative importance of the two suggested mechanisms, an indirect method allowing the cholesterol content of the cellular membranes to be modified under certain conditions was used. In our system, the cholesterol-rich liposomes indeed exchanged cholesterol with whole cell membranes but this was simply an exchange and not a net transfer. The fact that the exchanged cholesterol was not found in the cholesterol ester fraction even after 4 days would suggest that only the cholesterol of the plasma membrane of the cells is exchangeable, as is proposed by Poznansky and Czekanski in another system [15]. On the other hand, using microsomes, the same method makes it possible to increase the cholesterol content of the intracellular membranes. This net transfer of cholesterol thus results in a large increase in ACAT activity of the microsomes and confirms the regulating role played by cholesterol. However, the difference in the rate of ACAT activity between microsomes originating from the control cells and the TSH cells is not entirely overcome.

For the time being, it can be concluded that the two working hypotheses suggested above are both partially valid: i.e., during culture in the absence of TSH, it is at the same time the cellular content in ACAT enzyme and the membrane content in cholesterol in the vicinity of this enzyme that are increased. This hypothesis can only be confirmed by directly assuming the amount of ACAT enzyme molecules and by studying the distribution of cholesterol in the vicinity of the enzyme molecules. This aspect has been approached by other authors who have shown that this distribution is not homogenous in thyroid cells [16]. In conclusion, it should be observed that the cholesterol esters represent one of the means of storing arachidonic acid, and they can therefore participate in the regulation of the metabolism of this eicosanoid precursor. This remark is consistent with the fact that TSH has an inhibitory effect on the synthesis of prostaglandin E_2 in cultured thyroid cells [17].

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