Dual modulation of hepatic and intestinal acyl-CoA: cholesterol acyltransferase activity by (de-)phosphorylation and substrate supply in vitro

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Acyl-CoA:cholesterol acyltransferase (ACAT) activity in microsomes from rat liver and rat intestinal epithelial cells was increased by incubation of the microsomes with the 100000 × *g* supernatant fraction in the presence of ATP/$MgCl_2$ and NaF. The measured activity was further increased by including cholesterol-rich liposomes in the preincubation. The ACAT activity in rat liver microsomes could be inhibited by preincubation in the presence of 100000 × *g* supernatant and $MgCl_2$ and microsomes preactivated by ATP/$MgCl_2$ could also be inhibited in this way. The results suggest that ACAT activity in vitro is modulated by substrate supply and also reversibly by an ATP-dependent process which may be protein phosphorylation.

Acyl-CoA:cholesterol acyltransferase *Regulation* *Rat liver* *Rat intestine* *Phosphorylation* *Liposomes*

1. INTRODUCTION

Intracellular cholesterol metabolism is largely governed by 3 microsomal regulatory enzymes: HMG-CoA reductase (EC 1.1.1.34), as the key enzyme regulating synthesis [1], cholesterol 7α-hydroxylase (EC 1.14) which initiates the hepatic degradation of cholesterol to bile acids [2] and acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26) which promotes cholesteryl ester formation [3]. The latter enzyme appears to be regulated both by protein synthesis and by the supply of substrate cholesterol available to the enzyme in microsomal and reconstituted preparations [4,5]. During a study of ACAT activity in homogenates of rat intestinal mucosal cells we observed an increase in the rates of cholesteryl ester formation when the homogenates were preincubated at 37°C for 30 min. The yield of product could be raised still further by incubating the homogenates in the presence of cholesterol-rich liposomes. This stimulation of measured enzyme activity by preincubation in vitro is similar to that previously reported for intestinal HMG-CoA reductase, which was shown to be due to a dephosphorylation process [6]. HMG-CoA reductase is known to be activated in vitro by dephosphorylation and inhibited by a protein kinase-dependent phosphorylation [1]. It has recently been reported that rat liver cholesterol 7α-hydroxylase can be activated in vitro by an ATP-dependent process and inhibited by dephosphorylation [7,8]. These findings raise the possibility that ACAT and these major enzymes of cholesterol metabolism may be regulated in a coordinated way by the same mechanism.

We here report studies designed to investigate the effects of treatments similar to those used to

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Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; ACAT, acyl-CoA:cholesterol acyltransferase

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modulate HMG-CoA reductase activity on the rates of cholesteryl ester formation in the microsomal fractions of both rat liver and intestinal mucosal cells. The results show that the amounts of cholesteryl ester formed in both tissue preparations can be increased by two mechanisms which are additive in effect:

(1) The supply of additional substrate cholesterol from liposomes;
(2) Preincubation of microsomes with ATP and Mg^{2+} in the presence of fluoride and cytoplasmic proteins.

A preliminary report which reaches similar conclusions to some of the present work was published in [9].

2. MATERIALS AND METHODS

Microsomes were prepared from rat liver as in [10], the homogenate being buffered in 10 mM imidazole–HCl or in 50 mM potassium phosphate (pH 7.4). EDTA was not included in the buffers. Both buffers gave similar results. Intestinal cells were prepared by the chelation technique in [11]. The cells were washed twice in buffer containing 0.1 M sucrose, 50 mM KCl, 3 mM dithiothreitol and 50 mM potassium phosphate (pH 7.2) and were then disrupted by sonication for 30 s at 100 W. The homogenate was fractionated by standard methods. The microsomal fractions were washed once with buffer before assay. Potassium phosphate buffer was used in the experiments with intestinal cells because the imidazole buffer inhibited ACAT activity in this tissue.

Fractionation of the 100000 × *g* supernatant was carried out as in [12] to give a protein fraction which is capable of reactivating HMG-CoA reductase which has been inhibited by phosphorylation.

Modifications of activity were achieved by preincubating the microsomes for various times at 37°C with the additions, such as ATP and 100000 × *g* supernatant, given in the results section. In the intestine, the whole 8500 × *g* supernatant fraction was used for the preincubations and the microsomes were isolated by centrifugation and used in the assays.

ACAT activity was measured as in [5] using [1-^{14}C]oleoyl co-enzyme A. Liver microsomal protein, 500 μg, or intestinal microsomal protein, 250 μg, were used in the assays which also contained 0.5 mg defatted bovine serum albumin and 0.5 mg glutathione in 0.5 ml 20 mM imidazole–HCl buffer or 0.1 M potassium phosphate buffer (pH 7.4). The assays were initiated after preincubation by the addition of the radioactive oleoyl-CoA (spec. act. 2.75 dpm/pmol, 40 nmol added for the liver and 5.5 dpm/pmol, 20 nmol added for the intestine). The assays were continued for 2 min, during which time the rate of ester formation was linear with respect to time and to microsomal protein in the range used.

3. RESULTS AND DISCUSSION

We first investigated the effect of preincubating rat liver microsomes with various additions. Table 1 shows the effect of stepwise additions of these components. Addition of ATP (4 mM) caused a small increase in the amount of cholesteryl ester formed during the 2-min assay, as in [13]. The presence of the 100000 × *g* supernatant in an amount proportional to the microsomal protein in the total homogenate caused a further increase. The activation was more dramatically stimulated by the addition of NaF (40 mM) and $MgCl_2$ (4 mM).

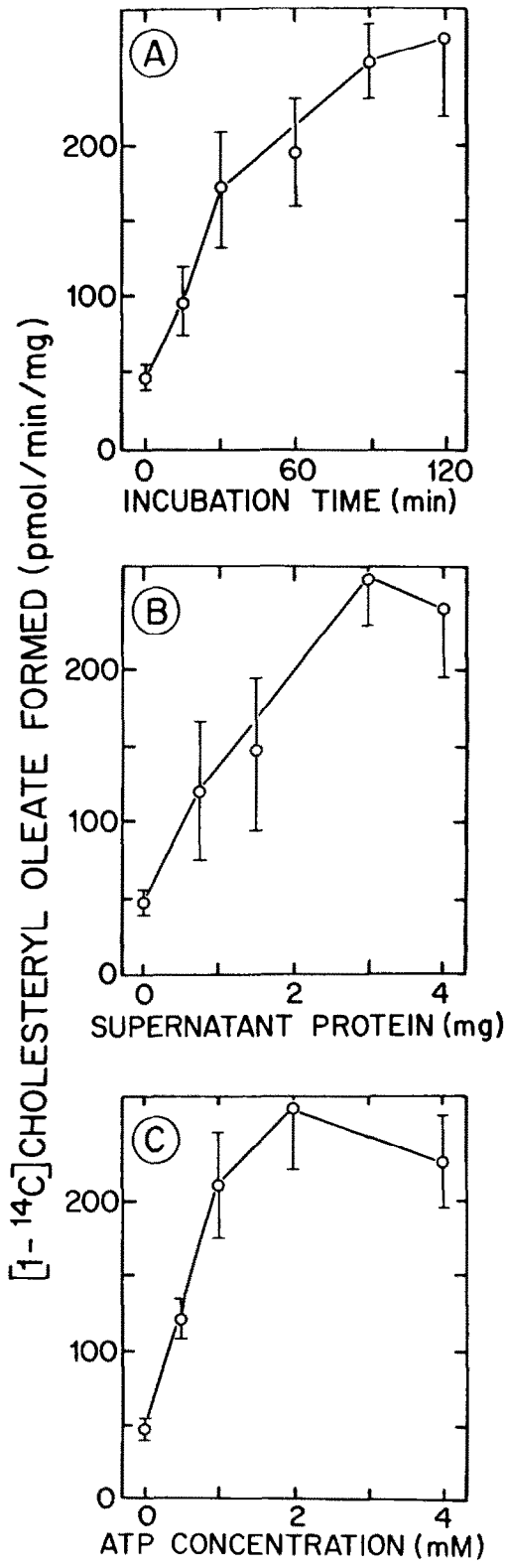
The kinetics of this process are shown in fig.1. Panel A shows that the time course for activation was linear for about 30 min and reached a plateau at 90 min. The activation was dependent on the

Table 1

In vitro activation of rat liver acyl-CoA:cholesterol acyltransferase

Additions	Cholesteryl oleate formed (pmol. $min^{-1}.mg^{-1}$)
1. None	37
2. ATP (4 mM)	57
3. 2 + 100000 × *g* supernatant (3 mg)	65
4. 3 + NaF (40 mM)	140
5. 4 + $MgCl_2$ (4 mM)	168

Rat liver microsomal protein (0.5 mg) was preincubated for 90 min at 37°C with the additions shown. [1-^{14}C]oleoyl-CoA was added and incubations continued for a further 2 min to measure ACAT activity



amount of supernatant protein present up to 3 mg supernatant protein added to 0.5 mg microsomal protein (panel B). Maximal activation by ATP was achieved at 2 mM (panel C).

These results suggest that an ATP- and protein kinase-dependent reaction is involved in the activation process which takes place during the preincubation period. The effect of the fluoride suggests that maximal activation is only revealed when protein phosphatase activity is inhibited. These effects are opposite to those which occur with HMG-CoA reductase [1]. The addition of cyclic AMP (100 μM) in the presence of theophylline (4 mM) failed to increase the maximal extent of the ATP-dependent activation (256 ± 25 pmol.min^{-1}.mg^{-1} compared with a control of 275 ± 28 at 90 min) or the rate at which it was reached (170 ± 8 pmol.min^{-1}.mg^{-1} compared with 189 ± 21 at 30 min).

Recombination of rat intestinal microsomes with the 100000 × *g* supernatant in the same way as described for the liver did not result in an ATP-dependent activation of the rate of cholesteryl ester formation. However, if the preincubations were carried out using the 8500 × *g* supernatant fraction with subsequent isolation of the microsomal fraction for assay, a similar activation was observed (table 2). Activation was complete after a 60 min preincubation in the presence of 2 mM ATP/$MgCl_2$ and 150 mM NaF.

Fig.2 shows that it was possible to inhibit the rate of cholesteryl ester formation in hepatic microsomes by preincubation in the presence of supernatant protein in a time-dependent (panel A) and protein-dependent way (panel B). The extent of inhibition (fig.2) was much less than the activation (fig.1) and clearly the ACAT activity measured in freshly isolated microsomes lies near

Fig.1. Activation in vitro of rat liver acyl-CoA:cholesterol acyltransferase. A: 0.5 mg rat liver microsomal protein was preincubated at 37°C for the times shown with ATP/$MgCl_2$ (4 mM), NaF (40 mM) and 3 mg 100000 × *g* supernatant protein; B and C: Incubations carried out for 90 min in the presence of additions as for panel A with (B) varying amounts of 100000 × *g* supernatant protein and (C) ATP/$MgCl_2$. Error bars indicate either the SEM of 3–5 independent measurements or the range of 2 independent measurements.

Table 2

Activation of rat intestinal acyl-CoA:cholesterol acyltransferase by two mechanisms

	Cholesteryl oleate formed (pmol. $min^{-1}.mg^{-1}$)
1. Microsomes 37°C, 30 min	297 ± 11
2. 1 + liposomes	1054 ± 66
3. Activated microsomes	677 ± 25
4. 3, 37°C, 30 min	488 ± 170
5. 4 + liposomes	1429 ± 300

Activated microsomes were prepared by incubating the 8500 × *g* supernatant fraction at 37°C with 2 mM ATP and 25 mM NaF for 30 min to minimize the extent of non-specific inactivation followed by centrifugation at 100000 × *g* for 1 h. The activated and control microsomes were incubated for 30 min at 37°C with 100 μl of a sonicated liposome preparation containing egg yolk phosphatidylcholine, beef brain phosphatidylserine and cholesterol (molar ratio 3:1:4; 8 mg cholesterol/ml)

Results are the mean ± SEM of 3 experiments (1,2) or mean ± range of 2 experiments (3–5)

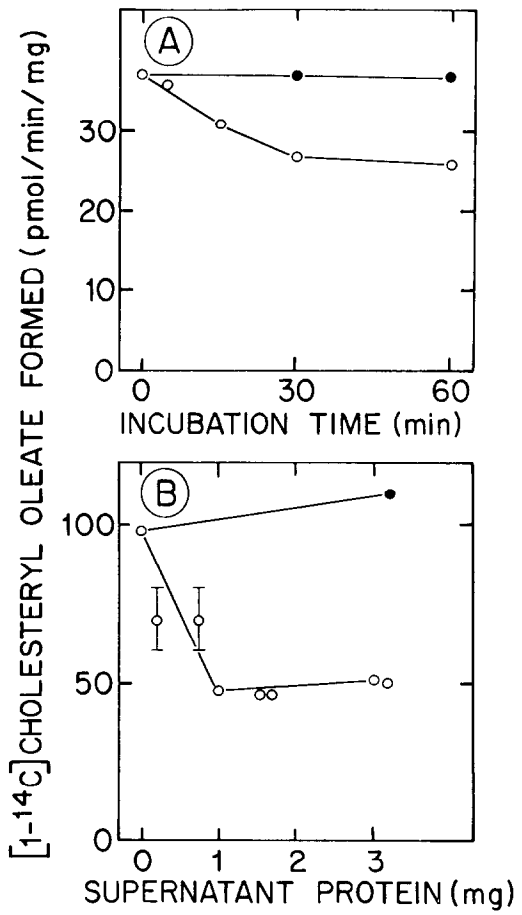


Fig.2. Inhibition in vitro of rat liver acyl-CoA:cholesterol acyltransferase. (A) 0.5 mg rat liver microsomal protein was preincubated at 37°C for the times shown with 1.5 mg 100000 × *g* supernatant protein in the presence of 2 mM $MgCl_2$ (○), supernatant protein omitted (●). (B) Incubations were carried out at 37°C for 30 min with varying amounts of 100000 × *g* supernatant in the presence of 2 mM MgCl (○), 40 mM NaF added (●).

the lower end of the range of activity, similar to HMG-CoA reductase in liver and intestine [1,6]. The inhibition could itself be blocked by 40 mM NaF (panel B) and in some cases preincubation of liver microsomes with fluoride alone led to a small activation of ACAT. Preparation of rat liver microsomes in the presence of NaF (50 mM) led to identical activation of the rate of cholesteryl ester formation. However, this preparation was not inhibitable by incubation with the 100000 × *g* supernatant.

Several experiments were performed to show that the ATP-dependent activation of the liver microsomal ACAT was reversible (table 3). Microsomes were activated by incubation with 100000 × *g* supernatant in the presence of ATP/$MgCl_2$ and NaF. The microsomes were resedimented, washed once with buffer, and the washed activated preparation preincubated with 100000 × *g* supernatant for up to 1 h. The ACAT activity was then measured. Table 3 shows that the activity of the activated preparation could be inhibited to the same extent as the control preparation and that the inhibition observed in both cases could be prevented by NaF. The 100000 × *g* supernatant was then partially fractionated and the fraction used in [12] to assay activation of purified HMG-CoA reductase by dephosphorylation was used in similar experiments. The ACAT activity of the activated microsomes was again inhibited and this effect was also prevented by the presence of NaF. In the intestinal preparations a small decrease

Table 3

Inhibition of rat liver acyl-CoA:cholesterol acyltransferase

	Cholesteryl oleate formed relative to control	
	Untreated microsomes	Activated microsomes
1. No additions	1.00	2.00 ± 0.14 (14)
2. 100000 × *g* supernatant 2 mM $MgCl_2$	0.75 ± 0.07 (14)	0.92 ± 0.05 (8)
3. 2 + 40 mM NaF	1.41 ± 0.16 (10)	1.89 ± 0.26 (9)
4. Fractionated 100000 × *g* supernatant, 2 mM $MgCl_2$		1.56 ± 0.26 (4)
5. 4 + 40 mM NaF		2.37 ± 0.47 (3)

Activated microsomes were prepared by incubating freshly prepared microsomes with 100000 × *g* supernatant (3 mg supernatant protein/mg microsomal protein) in the presence of ATP/$MgCl_2$ (4 mM) and NaF (40 mM) for 60–90 min at 37°C. The activated microsomes were obtained by centrifugation at 100000 × *g* for a further 50 min followed by resuspension in buffer and recentrifugation to give a washed microsomal pellet. In the preincubations 3 mg 100000 × *g* supernatant protein was used per mg microsomal protein (2 and 3, 60 min preincubation) or 300 μg of the fractionated 100000 × *g* supernatant/mg microsomal protein (4 and 5, 30 min preincubation). Oleoyl-CoA was then added to initiate the assay of ACAT activity. Results are expressed relative to a control incubation of untreated microsomes and are given as the mean ± SEM (*n*)

(35%) in the ACAT activity was observed compared with a control preincubation in the presence of NaF (150 mM) suggesting a minor phosphatase-dependent inhibition.

Finally, we examined a group of rats to determine whether the ATP-dependent activation catalysed by the supernatant fraction occurred independently of the activation which is known to take place when additional substrate cholesterol is presented to the microsomes from cholesterol-rich liposomes [5]. The results are given in table 4.

The addition of liposomes alone to the microsomes caused a 10-fold increase in the amount of ester formed, and a 2.5-fold increase was found when liposomes were included in the preincubation with ATP/$MgCl_2$ and supernatant protein. The two effects were additive over the whole 90 min incubation period, suggesting that they can operate independently. An increase in the rate of cholesteryl ester formation was also observed when the intestinal microsomes were incubated with liposomes (table 2). Activation by both ATP/$MgCl_2$ and liposomes was less in the intestine than in the liver.

The present studies raise the possibility that two mechanisms, covalent modification and substrate supply, may operate independently to regulate ACAT activity in vitro. ACAT activity is known to be highly sensitive to membrane composition and

Table 4

Activation of rat liver acyl-CoA:cholesterol acyltransferase by two mechanisms

Additions	Cholesteryl oleate formed ($pmol.min^{-1}.mg^{-1}$)
1. None	45 ± 3 (15)
2. 1 + liposomes (100 μl)	443 ± 55 (5)
3. 1 + ATP/$MgCl_2$/NaF/100000 × *g* supernatant	245 ± 19 (11)
4. 3 + liposomes (100 μl)	635 ± 86 (5)

Preincubations were carried out for 90 min at 37°C with 0.5 mg microsomal protein. Liposomes were prepared as in table 2. Incubations 3 and 4 contained ATP/$MgCl_2$ 4 mM, NaF 40 mM and 3 mg 100000 × *g* supernatant protein. Results are the mean ± SEM (*n*)

physical state [5]. Whilst the results suggest that phosphorylation of the enzyme is the regulatory event, phosphorylation of other membrane components, causing an indirect modification of ACAT activity, can not be ruled out. The present studies show that ACAT, like cholesterol 7α-hydroxylase, can be regulated in the opposite sense to HMG-CoA reductase. Thus a single regulatory mechanism (i.e., phosphorylation/de-

phosphorylation) may operate in many cells to coordinate cholesterol metabolism. Under appropriate conditions, e.g., in response to uptake of cholesterol, ACAT, and in the liver, cholesterol 7α-hydroxylase, may be activated, promoting storage and utilization of cholesterol, respectively, and HMG-CoA reductase may be inhibited, preventing endogenous synthesis.

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