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The effect of cyclic AMP analogues and glucagon on cholesteryl ester synthesis and hydrolysis in cultured hamster hepatocytes

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Two cyclic AMP analogues, 8-chloro cyclic AMP and 8-(4 chlorophenylthio) cyclic AMP, were found to increase the incorporation of [^{3}H]oleate into cholereryl ester in cultured hamster hapatocytes (30–40%), while incorporation into triacylglycerol was unaffected. An increase of a similar magnitude was observed in the presence of glucagon and the phosphodiesterase inhibitor, theophylline. The cyclic AMP analogues also stimulated the activity of neutral cholesteryl ester hydrolase in the cells, and this effect was mimicked by glucagon and theophylline. These results show that cyclic AMP can affect the cholesteryl ester cycle in hamster hepatocytes, and support the idea that the enzymes involved may be co-ordinately regulated.

Cholesterol esterification; Cholesteryl ester hydrolysis; Cyclic AMP analogue; Glucagon; Hamster hepatocyte

1. INTRODUCTION

In the liver, unesterified cholesterol, originating from the plasma lipoproteins or from endogenous synthesis, may be esterified by the action of acyl Co-enzyme A:cholesterol acyl transferase (ACAT) [1] and stored within the cell or secreted into plasma in new lipoproteins. Alternatively, it may be secreted into bile either unchanged or after catabolism to bile acids. Intracellular cholesterol stores are mobilized when required by cholesteryl ester hydrolases associated with the microsomal and cytosolic sub-cellular fractions [2,3]. The enzymes involved in the cholesteryl ester cycle ensure that the intracellular concentration of unesterified cholesterol is kept within relatively narrow limits [4]. In addition, they have a role in determining whether cholesterol is eliminated via the bile or re-circulated in the plasma, thus their regulation has important implications for the maintenance of cholesterol homeostasis in the body.

Cholesteryl ester hydrolase in some non-hepatic tissues is believed to be identical to hormone-sensitive lipase, which is known to be modulated by a cyclic AMP (cAMP)-dependent mechanism [5]. Liver microsomal and cytosolic cholesteryl ester hydrolases appear not to be associated with the lipase activity [6,7], and the mechanisms regulating their activity are not well understood. Recent in vitro cell-free studies, however, have suggested that the cytosolic enzyme may also be activated by cAMP-dependent processes [8].

The evidence currently available for the involvement of cAMP in the regulation of cholesterol esterification is scant and contradictory. One in vitro study with rat liver microsomes found that the activity of ACAT was stimulated in the presence of cAMP [9], but another group were unable to demonstrate any effect [10]. Cholesterol esterification in the mouse macrophage cell line, J774, has been reported to be both increased and inhibited in two separate studies with two different cAMP analogues [11,12]. There are no previous reports, however, on the effect of cAMP on cholesterol esterification in intact hepatocytes.

Cultured liver cells have the advantage over sub-cellular fractions in the investigation of cAMP-dependent processes, as they retain the requisite intracellular organisation. Rat hepatocytes have been used to study the effects of cAMP on cholesterol and bile acid synthesis [13–15], but it has become recognised recently that the hamster may be a better model for cholesterol metabolism in the human in a number of important respects [16]. Cell-permeable cAMP analgues have been developed to facilitate studies with intact cells, and many of these compounds have the additional advantage that they bind preferentially to a specific binding site on the cAMP-dependent protein kinase and are often more potent than the parent compound [17]. In the present work we have investigated the effects of cAMP on cholesteryl ester synthesis and hydrolysis in cultured hamster hepatocytes using two such site-selective cAMP

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Abbreviations: ACAT, acyl Co-enzyme A:cholesterol acyl transferase; cAMP, cyclic AMP; 8-Cl cAMP, 8-chloro cyclic AMP; 8-CPT cAMP, 8-(4 chlorophenylthio) cyclic AMP.

analogues, 8-chloro cAMP (8-Cl cAMP), and 8-(4 chlorophenythio) cAMP (8-CPT cAMP) and the hormone, glucagon.

2. MATERIALS AND METHODS

2.1. *Animals*

Male Golden Syrian hamsters (100–150 g) were maintained on a commercial pellet diet and allowed access to food and water ad libitum.

2.2. *Preparation and culture of hepatocytes*

Hepatocytes were prepared essentially as described by Strom et al. [18], and maintained in monolayer culture in Williams medium E on collagen-coated dishes as described previously [19]. After adhesion of the cells to the dishes the medium was replaced. cAMP analogues, glucagon or theophylline were added at this time unless indicated otherwise.

2.3. *Determination of cholesterol synthesis, cholesterol esterification and neutral cholesteryl ester hydrolase activity*

Cholesterol synthesis was determined by measuring the incorporation of 1-[^{14}C]acetate into unesterified cholesterol by the cells during 4 h of incubation [20]. Cholesterol esterification was estimated from the amount of radioactivity associated with esterified cholesterol after incubation of the hepatocytes with potassium [^{3}H]oleate for 3 h, as described previously [21]. Radioactivity associated with triacylglycerol was determined in the same experiments. The activity of neutral cholesteryl ester hydrolase was measured as before [22], except that the substrate, cholesteryl 1-[^{14}C]oleate, was delivered in acetone. Activity was determined in the supernatant resulting from the centrifugation of homogenates of hamster cells at 8,800 × *g* in a microfuge for 5 min [21].

2.4. *Other determinations*

Proteins were determined by the method of Lowry et al. [23]. Significance limits were calculated using Student's *t*-test.

2.5. *Materials*

Collagenase, insulin, antibiotics, bovine serum albumin, sera and culture media were from Sigma (Poole, Dorset, UK). Radiochemicals were supplied by Amersham International (Aylesbury, Bucks., UK).

3. RESULTS

Table I shows the effects of 8-Cl cAMP and 8-CPT cAMP on the incorporation of [^{3}H]oleate into cholesteryl ester and triacylglycerol by cultured hamster hepatocytes over 15 and/or 24 h incubation. Incorporation into cholesteryl ester was increased significantly by both analogues, while incorporation into triacylglycerol was unaffected. Cholesterol esterification is known to be stimulated when the supply of unesterified cholesterol is increased [1], thus changes in cholesterol synthesis in the cells may indirectly affect the process. For this reason, the effects of 8-Cl cAMP and 8-CPT cAMP on the incorporation of [^{14}C]acetate in unesterified cholesterol by the hamster hepatocytes were tested, and the results are shown in Table II. A marked decrease in cholesterol synthesis was found with both cAMP analogues.

The effects of 8-Cl cAMP and 8-CPT cAMP on cholesteryl ester hydrolysis were determined by measuring the activity of cholesteryl ester hydrolase in the cells after 5 h incubation with or without the analogues. In these experiments the hepatocyte cultures were incubated for 16 h before addition of the agents. Activity of the enzyme was too low to detect in the cytosolic fraction prepared from hamster hepatocytes at the end of incubations either in the presence or absence of 8-Cl cAMP. The post-mitochondrial supernatant, however, did show cholesteryl ester hydrolase activity, and this was used in subsequent experiments. Incubation with 8-Cl cAMP for 5 h led to a significant increase in activity (1 μM, 148 ± 6%; 10 μM, 147 ± 7%; 100% =

Table I

The effect of cAMP analogues on the incorporation of [^{3}H]oleate into cholesteryl ester and triacylglycerol by cultured hamster liver cells

	Incorporation (% control value)		
	Cholesteryl ester		Triacylglycerol
Incubation time	15 h	24 h	15 h
Additions			
None	100	100	100
8-Cl cAMP (1 μM)	119 ± 5	141 ± 5	100 ± 1
8-Cl cAMP (10 μM)	136 ± 3	143 ± 7	99 ± 4
8-CPT cAMP (10 μM)	121 ± 2	133 ± 2	100 ± 2

Cultured hamster hepatocytes were incubated in the presence or absence of 8-Cl cAMP or 8-CPT cAMP for 12 h or 21 h. After this time [^{3}H]oleate was added and the incubation continued for a further 3 h. The amount of radioactivity associated with cholesteryl ester and triacylglycerol was then determined. Data shown are the mean ± S.E.M. from 5 (8-Cl cAMP) or 3 (8-CPT cAMP) hamsters. In the absence of cAMP analogues, absolute (control) values (dpm/mg protein) were: 8-Cl cAMP, 15 h, 2,201 ± 322 (cholesteryl ester), 568,925 ± 24,822 (triacylglycerol), 24 h, 1,745 ± 636; 8-CPT cAMP, 15 h, 2,119 ± 114 (cholesteryl ester), 607,299 ± 27,844 (triacylglycerol), 24 h, 1,562 ± 80.

Table II

The effect of cAMP analogues on the incorporation of [^{14}C]acetate into unesterified cholesterol in cultured hamster hepatocytes

	Incorporation (% control value)	
Incubation time	15 h	24 h
Additions		
None	100	100
8-Cl cAMP (1 μM)	43 ± 3	43 ± 2
8-Cl cAMP (10 μM)	38 ± 1	33 ± 4
8-CPT cAMP (10 μM)	56 ± 3	68 ± 5

Cultured hamster hepatocytes were incubated in the presence or absence of 8-Cl cAMP or 8-CPT cAMP for 11 h and 20 h. After this time [^{14}C]acetate was added and the incubation was continued for a further 4 h. Radioactivity associated with unesterified cholesterol was then determined. Data shown are the mean ± S.E.M. from 4 (8-Cl cAMP) or 3 (8-CPT cAMP) hamsters. In the absence of cAMP analogues, absolute (control) values (dpm/mg protein) were: 8-U cAMP, 15 h, 10,410 ± 593, 24 h, 9,132 ± 279; 8-CPT cAMP, 15 h, 10,266 ± 1,291, 24 h, 9,302 ± 1,093.

12.8 ± 1.3 pmol/min/mg protein, $n = 7$ hamsters), and a similar effect was seen with 8-CPT cAMP (10 μM, 180 ± 5%; 100% = 12.7 ± 1.6 pmol/min/mg protein, $n = 6$ hamsters).

The effects of glucagon and the phosphodiesterase inhibitor, theophylline, on cholesteryl ester synthesis and hydrolysis in cultured hamster hepatocytes are shown in Table III. Glucagon alone had little effect on the incorporation of [^{3}H]oleate into cholesteryl ester, but theophylline increased this parameter significantly. In addition, a further significant increase was seen when both agents were present. Cholesteryl ester hydrolase activity was stimulated both by glucagon and by theophylline when added separately, but in this case no synergistic effect was apparent.

4. DISCUSSION

Dibutyryl cAMP and glucagon have been shown to inhibit cholesterol synthesis in isolated rat hepatocytes [13,14,24], the human liver cell line, $HepG_2$ [25], and rat liver in vivo [26]. This effect is believed to involve the phosphorylation and inactivation of 3-hydroxy 3-methylglutaryl Co-enzyme A reductase (HMG CoA reductase) [14], although the enzyme is not a substrate for cAMP-dependent protein kinase [27], and the exact mechanism by which this occurs remains unclear. Our experiments demonstrate that relatively low concentrations of 8-Cl cAMP and 8- CPT cAMP are able to bring about similar effects in cultured hamster hepatocytes (Table II). Despite this decrease in cholesterol synthesis, cholesterol esterification was markedly enhanced in the presence of similar concentrations of these analogues (Table I), and when intracellular cAMP levels were raised using glucagon and theophylline (Table III). Cholesteryl ester hydrolysis in the cells was also increased by these agents, but 8-CPT cAMP had a significantly greater effect ($P < 0.005$) than 8-Cl cAMP on this process, and a lesser effect on cholesterol esterification. Furthermore, incorporation of fatty acid into triacylglycerols was unaffected (Table I), indicating that cAMP does not alter the activity of acyl CoA synthetase, which is required for both processes.

There is some evidence from cell-free in vitro studies to suggest that, in the liver, ACAT may be activated by conditions favouring phosphorylation [9,10,28]. Recently, however, in experiments with rat liver microsomes in vitro, Corton and Hardie have shown that some of the effects reported in the earlier work could be explained by artefacts in the ACAT assay systems used, and that a number of purified phosphoprotein phosphatases did not alter ACAT activity [29]. Nevertheless, it is clear from our experiments that cAMP does influence cholesterol esterification in cultured hamster hepatocytes, and 8-CPT cAMP has been shown to have a similar effect in J774 macrophages [11]. Whether or not cAMP exerts its effect via phosphorylation of the enzyme or an indirect route will be difficult to ascertain until the enzyme has been purified and can be studied at the molecular level.

Hormone-sensitive lipase, which is also responsible for cholesteryl ester hydrolysis in some cell types, which have an important role in cholesterol metabolism such as macrophages [30], adrenal cortex and adipose tissue [5], is regulated by a well-characterized cyclic AMP-dependent reversible phosphorylation system [5]. Cholesteryl ester hydrolases in the liver, however, appear to be different enzymes [6,7], and whether or not they are regulated by phosphorylation mechanisms is open to question. Limited evidence for this has come from studies in vitro with sub-cellular fractions from rat liver. The microsomal (B. Ochoa, personal communication) and the cytosolic [8] activities have been found to be activated in the presence of cAMP-dependent protein kinase, among other conditions favouring phosphorylation. In our work both the microsomal and the cytosolic enzymes were present in the assay used to estimate cholesteryl ester hydrolase activity in the hepatocytes. The microsomal activity in hamster liver, however, is very much higher (about 20-fold) than that of the cytosolic enzyme, and appears to predominate in this species [31]. As we were unable to detect any cholesteryl ester hydrolase activity in the 105,000 × g supernatant prepared from hepatocyte cultures, it seems likely that the changes observed in our experiments were due to modulation of the activity of the microsomal cholesteryl ester hydrolase. Our findings that cAMP analogues, glucagon and theophylline, increase its activity (Table

Table III

The effect of glucagon on cholesteryl ester synthesis and hydrolysis in cultured hamster liver cells

Additions	Cholesterol esterification (% control value)	Cholesterylester hydrolysis (% control value)
None	100	100
Glucagon (0.2 μM)	103 ± 1	122 ± 6
Theophylline (1 mM)	124 ± 5	167 ± 14
Glucagon (0.2 μM) + theophylline (1 mM)	140 ± 8	110 ± 2

For experiments on cholesterol esterification, cultured hamster hepatocytes were incubated with or without glucagon (0.2 μM) or theophylline (1 mM) or both for 1 h. After this time [^{3}H]oleate was added and the incubation was continued for a further 3 h. Radioactivity associated with cholesteryl ester was then determined. For experiments on cholesteryl ester hydrolysis, the cells were incubated with or without similar concentrations of glucagon and/or theophylline for 5 h. The activity of cholesteryl ester hydrolase was then determined as described in section 2. Data shown are the mean ± S.E.M. from 4 (cholesterol esterification) or 5 (cholesteryl ester hydrolase) hamsters. In the absence of glucagon or theophylline the absolute (control) values were 2,821 ± 161 dpm/mg protein for cholesterol esterification and 11.9 ± 0.7 pmol/min/mg protein for the activity of cholesteryl ester hydrolase.

III) in cultured hamster liver cells are consistent with the results of the work with sub-cellular fractions, and provide further evidence for a role for cAMP in the regulation of the activity of this enzyme.

The cholesteryl ester cycle in liver cells plays an important part in the regulation of the supply of cholesterol for excretion from the body in the bile as unesterified cholesterol or bile acids, as well as for secretion into plasma in lipoproteins. The findings reported here show that cAMP can influence the cycle at the level of both cholesteryl ester synthesis and hydrolysis, effectively increasing the flux of cholesterol through the system, and possibly making it more responsive to regulatory mechanisms. Our results suggest that site-selective cAMP analogues are potentially useful agents for the modification of cholesterol flux in the liver, and support the idea [8] that the enzymes involved in the cholesteryl ester cycle may be co-ordinately regulated to help maintain the balance of cholesterol metabolism within the hepatocyte.

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