Biphasic effects of glucagon and cyclic AMP on the synthesis and secretion of lipids by rat hepatocytes

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Cultured rat hepatocytes were preincubated with glucagon or a cyclic AMP analogue for up to 24 h and lipid synthesis and secretion were determined during the next 2 h. Glucagon or cyclic AMP did not change the incorporation of choline or glycerol into phosphatidylcholine, or choline into sphingomyelin, in the cells after 0–12 h of preincubation. After 12 h these incorporations were increased. Incorporations into hepatic lysophosphatidylcholine were decreased after preincubation with glucagon or cyclic AMP for 0–12 h, but by 24 h they increased. There was no change in the lysophosphatidylcholine in the medium after preincubation with glucagon or cyclic AMP for up to 6 h, but increases occurred after preincubation from 12 to 24 h. The secretion of triacylglycerol was decreased after preincubation for 0–1 h, but it returned to control values after 4 h. After preincubation for 18–24 h the incorporation of glycerol into secreted triacylglycerol was increased. The results are discussed in relation to the control of lipid metabolism in starvation and diabetes.

INTRODUCTION

The role of glucagon in lipid metabolism in the liver is still unclear. On the one hand, it is well established that glucagon, acting through cyclic AMP, decreases fatty acid synthesis in the liver, *in vivo* [1,2] as well as *in vitro* [3,4]. The process is paralleled by a decrease in very-low-density-lipoprotein (VLDL) secretion that would be a direct effect of glucagon, independent of the rate of triacylglycerol synthesis [5–8]. This latter effect is mediated by cyclic AMP [9].

On the other hand, the high levels of glucagon that may occur *in vivo* in fasting and diabetes increase lipolysis in adipose tissue, resulting in an increased fatty acid supply to the liver that may counteract the lack of fatty acid synthesis. The liver is able to maintain a high rate of triacylglycerol synthesis in order to protect itself against the potentially toxic effects of accumulated fatty acids and acyl-CoA esters that occur after accelerated lipolysis [10,11]. Glucagon and cyclic AMP are involved in this process by enhancing the activity of phosphatidate phosphohydrolase in the long term, especially in the presence of glucocorticoids [10–12]. This, together with smaller increases in glycerol-phosphate acyltransferase, results in an enhanced potential for triacylglycerol synthesis [13–15]. In contrast with this, glucagon via cyclic AMP displaces phosphatidate phosphohydrolase from the endoplasmic reticulum [16], and this is paralleled by a decrease in triacylglycerol synthesis [17]. This effect is overcome provided that fatty acid availability is high [17], thus maintaining a high potential for triacylglycerol synthesis in starvation and diabetes. If the supply of fatty acids from adipose tissue exceeds the capacity for VLDL secretion, then this can produce the fatty liver that often accompanies diabetes, stress or long-term starvation.

The present experiments were conducted to investigate the effects of short- and long-term exposure of hepatocytes to glucagon and thereby provide further insight into the regulation of VLDL production. Perfused rat liver [18] and rat hepatocytes [19–22] also release lysophosphatidylcholine into the incubation medium, and this lipid associates with albumin rather than with

VLDL [21,22]. This may provide a mechanism for the transport of choline and unsaturated fatty acids to other organs [20] in non-ruminants [23]. We therefore also decided to study the effects of glucagon and cyclic AMP on this process.

EXPERIMENTAL

Animals and materials

The sources of most of the materials have been described previously [10,24,25]. Radiochemicals were purchased from Amersham International. Male Wistar rats (about 200 g body wt. each) were supplied by Charles River, Québec, Canada.

Preparation and incubation of hepatocytes

Hepatocytes were prepared as described previously [10,24,25], and approx. 2×10^6 cells were applied to tissue-culture dishes that had been previously coated with collagen. The medium was modified Leibovitz L-15 containing 10% (v/v) newborn-calf serum (3 ml/dish). Cells were incubated for 1 h at 37 °C in air at 96% humidity, and the unattached and non-viable cells were removed by changing the medium and incubating for a further 4 h. The medium was changed again and the monolayer of cells was incubated overnight in 3 ml of medium in which newborn-calf serum was replaced by 0.03 mM fatty-acid-poor BSA. The cells were then incubated for 24 h in medium containing 0.1 mM fatty-acid-poor BSA. During this period, glucagon (100 nM) or 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) (100 μ M) were added without changing the medium at times indicated to produce the desired length of preincubation. The medium was then changed, and the cells were incubated for a further 2 h in 2 ml of the same medium containing 100 nM-glucagon, or 100 μ M-CPT-cAMP as indicated, plus 1 mM-oleic acid, 1 mM-[1,3-³H]glycerol (9 Ci/mol) and 100 μ M-[¹⁴C]choline (12 Ci/mol). The Leibovitz medium contained relatively high concentrations of amino acids, 5 mM-pyruvate, 5 mM-galactose and 5 mM-glucose [10,24,25]. These substrates, plus the 1 mM-glycerol, should prevent an action of glucagon in decreasing

Abbreviations used: CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate; (V)LDL, (very-)low-density lipoprotein.

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Table 1. Synthesis and secretion of lipids by rat hepatocytes

Control hepatocytes were preincubated for 24 h in 0.1 mM-BSA, and the incorporation of [3 H]glycerol and [14 C]choline was measured after 2 h (see the Experimental section). These results are from cells where no additions of glucagon or CTP-cAMP were made, and the time corresponds to 0 h of preincubation. The values are means $\pm$ S.E.M. for eight independent experiments and were not statistically different from the values found in control cells corresponding to the other times of preincubation.

	Precursor	Incorporation (nmol/unit of lactate dehydrogenase)				Concentration of triacylglycerol (nmol/unit of lactate dehydrogenase in cells)
		Triacylglycerol	Phosphatidylcholine	Lysophosphatidylcholine	Sphingomyelin	
Cells	Glycerol	25.8 $\pm$ 1.4	7.19 $\pm$ 0.87	0.019 $\pm$ 0.003	Not appropriate	120 $\pm$ 18
	Choline	Not appropriate	3.82 $\pm$ 0.53	0.013 $\pm$ 0.006	0.014 $\pm$ 0.001	
Media	Glycerol	0.160 $\pm$ 0.039	0.010 $\pm$ 0.001	0.093 $\pm$ 0.011	Not appropriate	18.9 $\pm$ 7.9
	Choline	Not appropriate	0.003 $\pm$ 0.004	0.032 $\pm$ 0.004	Not detected	

glycerol phosphate concentrations [26]. Glucagon under these conditions should not inhibit triacylglycerol synthesis, and this is confirmed in Table 1 and Fig. 1 (below). Choline was also added, at a saturating concentration of 100 μ M, to avoid hormonal effects on the choline pool.

The medium was collected from triplicate dishes after 2 h of incubation, centrifuged at 7800 g (r_{av} 7 cm) for 10 min at 4 $^{\circ}$ C to remove detached cells and cell debris, and then pooled. The monolayer of hepatocytes was washed with 2 $\times$ 2.5 ml of ice-cold 0.15 M-NaCl and then scraped from the dishes in 1 ml of ice-cold 0.25 mM-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes, pH 7.4. The cell suspensions from triplicate dishes were pooled, sonicated, and cell viability was assessed by determination of lactate dehydrogenase, as described previously [27].

Extraction and analysis of lipids

For determination of incorporated radioactivity, 0.5 ml of cell suspension and 1.2 ml of medium were extracted using a modification of the procedure of Bligh & Dyer [28]. The phases were separated using 0.2 M-KH₂PO₄ and 2 M-KCl instead of water. The bottom phase of the extracts was washed three times with the appropriate volume of synthetic top phase prepared from chloroform/methanol/(0.2 M-KH₂PO₄/2 M-KCl) (10:10:9, by vol.) and containing 1% (v/v) glycerol. Butylated hydroxytoluene (2,6-di-*t*-butyl-*p*-cresol) was added as an antioxidant to samples at a final concentration of 0.05% of the final bottom phase (0.5 ml for cells and 1 ml for media). The incorporation of radioactive precursors into lipids was determined by t.l.c. on plastic-backed plates of silica-gel 60 G (Merck, Darmstadt, Germany) as previously described [16], except that the second development was performed in light petroleum (b.p. 40–60 $^{\circ}$ C)/diethyl ether/acetic acid (50:50:1, by vol.). Lipids were cut from the appropriate areas of the plates, and 0.5 ml of water and 5 ml of Amersham ACS scintillant were added. Radioactivity was determined after waiting for 24 h.

Triacylglycerol concentrations in cells were determined essentially as described previously [20] after extraction [28]. The lipid residue was dissolved in 20 μ l of propan-2-ol before adding 0.35 ml of the Triglyceride GPO-PAP reagent purchased from Boehringer, Mannheim, Germany. Triacylglycerol in medium could not be assayed directly after extraction in the bottom phase owing to the turbidity caused by the fatty acids [20]. The whole bottom phase (about 2.5 ml) was thus shaken with 1 g of basic alumina to remove residual oleate [21]. After a 30 min centrifugation at 10000 g and room temperature, 1.6 ml of the supernatant was taken and the triacylglycerol concentration was determined as described above.

Expression of results

All results were expressed relative to the units of lactate dehydrogenase in the hepatocytes. This was considered to give a better indication of cell number and viability than the determination of DNA or protein, since non-viable cells remain attached to the dishes and the incubations contain high BSA concentrations [11,25]. Each dish of cells contains about 1.5 mg of hepatocyte protein. The results in Figs. 1 and 2 below are expressed relative to the control value for each time of preincubation. This compensates for differences in the absolute values in different experiments. However, the absolute values for 0 h preincubation are given in Table 1. Since preincubation in the absence of cyclic AMP and glucagon did not significantly change these absolute values, the results in the Figures accurately reflect the effects of cyclic AMP or glucagon.

RESULTS AND DISCUSSION

The absolute incorporations for lipids in control hepatocytes are shown in Table 1. As expected, triacylglycerol and phosphatidylcholine were the major lipids found in cells (Table 1). It is noteworthy that, after only 2 h incubation with [3 H]glycerol as a substrate, labelled triacylglycerol contributed about 22% to the total mass of triacylglycerol that accumulated in the cells. The latter value was equivalent to about 180 nmol/mg of cell protein and remained constant during the preincubation period. The incorporation of [3 H]glycerol in phosphatidylcholine was about two times higher than for [14 C]choline. The amounts of [3 H]glycerol and [14 C]choline incorporated into the lysophosphatidylcholine fraction of the cells were very small in relation to that in other lipids, and were only just detectable. This result agrees with previous findings [20,21].

The total amount of cell triacylglycerol, or the amount of triacylglycerol synthesized *de novo* during 2 h from [3 H]glycerol, were not significantly modified by glucagon or CPT-cAMP, whatever the duration of preincubation (Figs. 1a and 1b). On the other hand, long-term preincubation (18 and 24 h) with both glucagon and CPT-cAMP resulted in a significant increase in the incorporation of both [3 H]glycerol and [14 C]choline in phosphatidylcholine in the hepatocytes. By contrast, short-term incubation (up to 12 h) had no significant effect (Figs. 1c and 1d). The effects of glucagon and CPT-cAMP on the incorporation of [14 C]choline into sphingomyelin were similar (Figs. 1c and 1d) to those observed with phosphatidylcholine (Fig. 1e). Surprisingly, preincubation for up to 12 h with glucagon and CPT-cAMP resulted in a significant decrease of [14 C]choline incorporation in

Fig. 1. Effects of different times of preincubation with glucagon and CPT-cAMP on lipid accumulation in hepatocytes

Cells were incubated for 2 h with [^3H]glycerol and [^{14}C]choline after preincubation with 100 nM-glucagon (○) or 100 μM -CPT-cAMP (■) for times varying from 0 h to 24 h, as described in the Experimental section. Results are means $\pm$ S.E.M. for four to eight independent experiments and are expressed as percentages of values found for control cells (□) at each time. Absolute values for control cells at 0 h preincubation are given in Table 1. The significance of the difference between the treated hepatocytes and the controls was calculated by a paired *t* test and is indicated by: **P* > 0.05; ***P* < 0.01; ****P* < 0.001.

lysophosphatidylcholine (Fig. 1e). Similar results were obtained for incorporation of [^3H]glycerol into lysophosphatidylcholine (results not shown).

Our results on the synthesis of phosphatidylcholine (Figs. 1c and 1d) are compatible with previous work that showed stimulations of phosphatidylcholine synthesis after incubating hepatocytes for 24–72 h with glucagon [13] or for 12 h with cyclic AMP analogues and phosphodiesterase inhibitors [29]. The stimulation is related to an increase in the activity of CTP:phosphocholine cytidyltransferase, which is believed to be the rate-controlling enzyme for phosphatidylcholine synthesis [30]. In our work we also observed a stimulation in the incorporation of [^{14}C]choline into sphingomyelin (Fig. 1e). This is expected, because phosphatidylcholine is a precursor for sphingomyelin synthesis, although

the increased labelling of sphingomyelin appeared to be even higher than that for phosphatidylcholine.

Short-term incubation of hepatocytes with glucagon or cyclic AMP analogues decreases the rate of phosphatidylcholine synthesis in hepatocytes [17,31]. This effect depends upon the phosphorylation of the cytidyltransferase, which tends to displace it from the endoplasmic reticulum, thus causing its inactivation [17,32]. A similar effect is believed to regulate the activity of phosphatidate phosphohydrolase, and this can explain the short-term effects of cyclic AMP analogues in inhibiting triacylglycerol synthesis [11,16]. However, these acute effects on the synthesis of triacylglycerols and phosphatidylcholine can be overcome by the addition of fatty acids to the incubation medium [17]. This increases the amount of active phosphatidate phospho-

Fig. 2. Effects of different times of preincubation with glucagon and CPT-cAMP on lipid secretion in medium

Experimental conditions and the expression of statistical significance were the same as in Fig. 1. Absolute values for control medium at 0 h of preincubation are given in Table 1.

hydrolase on the endoplasmic reticulum [11,16], which maintains diacylglycerol concentrations. The diacylglycerol activates the cytidyltransferase ([33]; H. Jamil & D. E. Vance, unpublished work) and itself serves as the precursor for the synthesis of both triacylglycerol and phosphatidylcholine. The presence of 1 mM-oleate in the present incubations has this effect [11,16], and it explains why there was no significant decrease in the synthesis of triacylglycerol and phosphatidylcholine in cells incubated for up to 6 h with glucagon or CPT-cAMP (Figs. 1a–1d).

Lipids secreted into medium by control cells after only 2 h incubation with labelled substrates were in very low amounts compared with the accumulation in the cells (Table 1). The values obtained for sphingomyelin and phosphatidylcholine were too low to be measured accurately, and were thus omitted. The amount of triacylglycerol labelled with [^3H]glycerol that was secreted represented less than 1% of the total triacylglycerol (Table 1). This indicated that most of the substrate for VLDL secretion at this time and at subsequent times of preincubation was derived from pre-existing triacylglycerol rather than that synthesized *de novo*. The incorporation of [^3H]glycerol into lysophosphatidylcholine of the medium was 3-fold higher than for [^{14}C]choline (Table 1).

Figs. 2(a) and 2(b) show the expected decrease in triacylglycerol secretion after a short-term preincubation with glucagon and CPT-cAMP. A long-term preincubation with the same compounds showed a reversal of this effect. There were increases in the incorporation of [^3H]glycerol in secreted triacylglycerol, which was maximal after 18 h preincubation (Fig. 2b). However, a long-term preincubation with glucagon or CPT-cAMP had no significant effect on the total mass of secreted triacylglycerol

(Fig. 2a). These agents (together with glucocorticoids) would also be expected to increase the capacity of the liver to synthesize triacylglycerols by increasing phosphatidate phosphohydrolase activity [10–12,14]. This effect may be important *in vivo*, especially if accompanied by an increased mobilization of fatty acids from adipose tissue [14] resulting from starvation, stress or diabetes, since fatty acids activate the phosphohydrolase [14,24]. An increased capacity of the liver to secrete VLDL is generally paralleled by an increased activity of the Mg^{2+} -stimulated (*N*-ethylmaleimide-sensitive) phosphatidate phosphohydrolase [14,34]. However, whether these events are causally related is not known. Only a very small proportion of newly synthesized triacylglycerol was secreted after only 2 h in our experiments (Table 1), and there was no significant change in the triacylglycerol content of the hepatocytes after long-term incubation with glucagon or CPT-cAMP (Table 1; Figs. 1a and 1b).

The accumulation of lysophosphatidylcholine in the medium was also increased by the long-term exposure of the hepatocytes to glucagon or CPT-cAMP (Figs. 2c and 2d). This occurred when the labelling of phosphatidylcholine in the hepatocytes was increased (Figs. 1c and 1d). The mechanism whereby lysophosphatidylcholine appears in the medium is not fully understood, but it probably involves the breakdown of phosphatidylcholine in the hepatocytes, mainly by a phospholipase A_1 , but with probably some intervention by phospholipase A_2 [23]. It is therefore possible that glucagon, acting through cyclic AMP, may increase the activity of these phospholipases in the long term, whereas in the short term there may be decreases (Fig. 1f).

In the short term, glucagon and CPT-cAMP inhibited triacylglycerol secretion (Figs. 2a and 2b) as expected [5–8],

although it has also been reported that glucagon does not inhibit the secretory process *per se* [35]. It is surprising that the inhibitions of VLDL secretion by glucagon are superficially similar to the effects of insulin [6,19,25,36–40], because antagonism between these two hormones is normally expected. However, both insulin [40,41] and glucagon [42] also stimulate the binding and degradation of LDL by hepatocytes. It may also be significant that insulin has a biphasic effect on VLDL secretion in that it produces an initial inhibition, whereas after long periods a stimulation is seen [43,44]. In the present work, the inhibitory effects of glucagon and the cyclic AMP analogue on triacylglycerol secretion only lasted about 2 h. After 6 h, there was an increase in the secretion of newly synthesized triacylglycerol, although the total mass of triacylglycerol secreted after incubation with glucagon or cyclic AMP was not significantly different from control values. Consequently, glucagon might not counteract the effects of increased fatty acids [45] and glucocorticoids [19,25,44,46] in stimulating VLDL production in the longer term. This could be important in diabetes and after a prolonged stress reaction. However, in contrast with this, Emmison & Agius [8] reported that preincubation of hepatocytes for 14 h with 100 nM-glucagon in the presence of 10 nM-dexamethasone decreased the mass of triacylglycerol secreted.

Our results demonstrate, for the first time, a long-term stimulation by glucagon of the secretion of lysophosphatidylcholine and newly synthesized triacylglycerol by cultured rat hepatocytes. This effect cannot be accounted for by a degradation of glucagon or a desensitization of the cells to its effect, and is probably mediated by cyclic AMP, since the stable cyclic AMP analogue had consistently produced a larger effect than glucagon itself. It is hoped that the present studies will improve our understanding of the control of VLDL secretion and lysophosphatidylcholine production by the liver in conditions such as starvation, stress and diabetes.

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