The role of pancreatic hormones in the regulation of lipid storage, oxidation and secretion in primary cultures of rat hepatocytes

Short- and long-term effects

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Exposure of cultured rat hepatocytes to a high concentration of insulin (78 nM) for 24 h in the presence of extracellular oleate (0.75 mM) resulted in a decrease in the secretion of apoprotein B (apoB) and triacylglycerol associated with very-low-density lipoprotein (VLDL). However, continuous exposure of the cells to insulin for longer periods (72 h) stimulated the secretion of apoB and triacylglycerol. Treatment of hepatocytes with glucagon (0.1 µM) for 24 h also suppressed the secretion of VLDL apoB, cholesterol and triacylglycerol. The cells remained responsive to the inhibitory effect of glucagon for at least 3 days. In contrast with insulin, however, exposure of the cells to glucagon for a continuous period of 72 h did not lead to a reversal of the initial inhibition. Glucagon also stimulated ketogenesis, and in this regard the cells were responsive for at least 3 days in culture. These changes were accompanied by a transient increase in intracellular cyclic AMP (cAMP) concentration, which reached a peak 10 min after addition of glucagon. Between 12 h and 24 h after glucagon addition, cAMP levels had returned almost to normal, but the secretion of VLDL remained suppressed during this period.

INTRODUCTION

The distribution of hepatic lipid between the secretory, storage and oxidative pathways is dependent on the nutritional state of the animal, and thus on the demand of extrahepatic tissues for lipid-derived energy [1]. Changes in plasma insulin and glucagon levels are important signals by which information about food intake is transmitted to the liver. The secretion of lipid as very-low-density lipoprotein (VLDL) makes an important contribution to hepatic lipid balance, and the hormonal control of this process is essential for ensuring an appropriate response of hepatic lipid metabolism to dietary change. Isolated rat hepatocytes maintained in culture medium have so far provided a useful model for studying the direct effects of insulin on VLDL secretion for periods up to 24 h [2–8]. Much less information is currently available about the direct longer-term effects of insulin on the secretion of VLDL, particularly of apoprotein B (apoB). This may be of importance under conditions in which the liver is chronically exposed to high concentrations of insulin, for example in non-insulin-dependent diabetes. Although short-term exposure of perfused liver and freshly prepared hepatocytes to glucagon suppresses the secretion of VLDL triacylglycerol [9–11], its detailed time-dependent effects on the lipid and apoprotein constituents of VLDL have not been studied in any great detail; in particular, the longer-term effects remain to be established. This information may be useful in relation to the mechanism underlying the suppressed secretion of hepatic VLDL observed in insulin-deficient diabetes [12], fat ingestion, [12] and during suckling [13,14], states which are all characterized by chronic hyperglucagonaemia. The present studies describe the effects of glucagon on the secretion of VLDL lipids and apoB, how these relate to changes in the intracellular concentration of cyclic AMP (cAMP), and how they are integrated with other glucagon-mediated changes in hepatic lipid metabolism.

MATERIALS AND METHODS

Preparation of hepatocyte cultures

Animals were fed and housed as described previously [15]. Hepatocytes were prepared under sterile conditions [6] and were suspended at a concentration of $(0.5\text{--}0.75) \times 10^6$ cells/ml in Waymouth's medium MB752/1 containing added amino acids, penicillin, streptomycin and foetal-calf serum [6]; 3.0 ml of this suspension was added to each culture dish. After 3–4 h, most of the cells had attached to the dish, and the serum-containing medium (containing unattached cells) was removed. The cell monolayer was washed, and serum-free Waymouth's medium (3.0 ml) containing added amino acids, antibiotics [6], dexamethasone (1 µM), lactate (10 mM), pyruvate (1 mM) and oleate (0.75 mM) were added. This is referred to as the supplemented medium. Cells were then cultured for periods of 24 h up to a total of 72 h. At the end of each 24 h period (i.e. day 1, day 2, or day 3) the experiment was either terminated by removal of the medium and harvesting the cells (see below) or continued after removal of the medium, followed by addition of fresh supplemented medium [16]. Hormone additions [insulin (78 nM) or glucagon (0.1 µM)] were made either for the whole of the 72 h culture period or for periods of 24 h only (see individual Table and Figure legends).

Harvesting of cells and preparation of VLDL

At the end of the culture period, the cells were cooled to 4 °C and the medium was removed. The cells were washed with phosphate-buffered saline and removed from the dish as described previously [16]. VLDL was isolated from the cell medium as described previously [16]. For storage of VLDL samples, a portion (0.2 ml) was added to a solution of BSA (1%) in phosphate-buffered saline (0.2 ml) and kept at –20 °C to –80 °C before assay of apoB.

Abbreviations used: VLDL, very-low-density lipoprotein; cAMP, cyclic AMP; apoB, apoprotein B.

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Table 1. Effect of short- and longer-term exposure to insulin on triacylglycerol and apoB secretion by hepatocyte cultures

Hepatocytes were cultured for 24 h or 72 h in the supplemented medium in the absence or presence of insulin (78 nM). At the end of the 24 h period, the medium was removed and the VLDL isolated. For the 72 h cultures, the medium was changed at 24 h intervals and replaced with identical medium with or without insulin. At the end of the final 24 h period, the medium was removed and the VLDL and the VLDL infranatant ($d > 1.006$) fractions were isolated by ultracentrifugation. Rates of secretion of VLDL apoB (ng/24 h per mg of protein) and triacylglycerol ($\mu\text{g}/24 \text{ h}$ per mg of protein) were determined. Total apoB represents the sum of the apoB measured in the VLDL and in the VLDL infranatant (ng/24 h per mg of protein). Each value represents the mean $\pm$ S.E.M. of 5 (24 h) or 6 (72 h) independent experiments. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding control values.

Incubation conditions	Exposure period (h)	VLDL		Total apoB
		Triacylglycerol	apoB	
No insulin	24 h	161.6 $\pm$ 6.9	901.2 $\pm$ 238	2220 $\pm$ 257
+ Insulin		101.2 $\pm$ 17.4*	484 $\pm$ 187	962 $\pm$ 339*
Insulin effect (% of control)		62.2 $\pm$ 9.9*	51.7 $\pm$ 8.6**	47.4 $\pm$ 9.7**
No insulin	72 h	107.7 $\pm$ 14.6	445 $\pm$ 95	1799 $\pm$ 487
+ Insulin		182 $\pm$ 30.0*	1074 $\pm$ 142**	1879 $\pm$ 341
Insulin effect (% of control)		172 $\pm$ 24.2*	328 $\pm$ 102*	114.6 $\pm$ 20.3

Measurement of cellular and VLDL triacylglycerol and cholesterol

The total lipid fraction of the cell pellet and of the VLDL were obtained as described previously [6]. Portions of the lipid extracts were used for enzymic measurements of the mass of triacylglycerol [17] and cholesterol (non-esterified plus esterified). Each of these assays utilized kits obtained from Boehringer-Mannheim ('Triglycerides GPO-PAP' and 'Cholesterol C-System' respectively). Where required, the triacylglycerol was purified from the total lipid extract by t.l.c. [18]. In all cases, manipulative losses of triacylglycerol were accounted for by addition of glycerol [^{14}C]trioleate as internal standard.

Measurement of apoB in VLDL and $d > 1.006$ infranatant

The apoB content of VLDL and of $d > 1.006$ infranatants was assayed by a competitive solid-phase monoclonal-antibody radioimmunoassay with VLDL apoB as a standard [19]. Control experiments demonstrated that the immunoreactivity of the apoB epitope recognized by this monoclonal antibody was not adversely affected by freezing or storage at -20°C or -80°C of VLDL samples after dilution in 1% BSA. Addition of BSA to infranatants was not required, because infranatants already contained sufficient BSA to stabilize the epitope upon freezing.

Statistical methods

All values are presented as means $\pm$ S.E.M. of several independent experiments. Significant differences were calculated by using a paired or unpaired Student's t test.

Other methods

Cellular protein was measured by the method of Lowry *et al.* [20]. Oleate bound to BSA (essentially fatty-acid-free) was prepared by the method of van Harken *et al.* [21]. The BSA concentration in the culture medium was 0.5%. The concentrations of β -hydroxybutyrate and acetoacetate were measured in the $d > 1.006$ infranatant by the method of Williamson *et al.* [22]. The results are expressed as the sum of these ketone bodies in the original culture medium ($\mu\text{mol}/\text{ml}$). Intracellular cAMP was determined by radioimmunoassay using a kit purchased from Amersham International (Little Chalfont, Bucks., U.K.) (product code RPA.509) according to the manufacturer's instructions.

RESULTS

Short- and longer-term effects of insulin and glucagon on the secretion of VLDL

Treatment of hepatocytes with insulin for a period of 24 h suppressed the secretion of VLDL triacylglycerol, as has been shown previously [2–4,6]. Although insulin inhibits the secretion of apoB from hepatocytes incubated in the absence of extra-cellular fatty acid [3,5,7,8], the present demonstration of a similar effect in the presence of this important source of hepatic triacylglycerol has not, so far, been reported previously in untransformed hepatocytes. In contrast, addition of insulin to cultures which had been pre-treated with the hormone for 48 h previously resulted, during the next 24 h, in a stimulation of VLDL triacylglycerol secretion. This was accompanied by a significant increase in the secretion of VLDL apoB (Table 1). These changes in VLDL apoB did not result from a redistribution of immunoreactive apoB between the VLDL fraction and the VLDL infranatant, since total apoB in the cell medium (i.e. the sum of VLDL and infranatant apoB) showed a similar trend after short-term and long-term insulin treatment (Table 1).

Addition of glucagon to the culture medium of the cells for 24 h immediately after removal of serum (i.e. day 1 of culture) also suppressed the secretion of VLDL apoB and triacylglycerol (Table 2). This suppression was maintained when glucagon was added for the final 24 h of a 48 h or 72 h culture period (i.e. when added on day 2 or day 3 of culture respectively). In other words, the cells retained their susceptibility to glucagon-mediated inhibition for at least 3 days of culture. Although this was true for cholesterol for up to 48 h, addition of glucagon for the final 24 h of a 72 h culture period failed to suppress the secretion of VLDL cholesterol (Table 2). However, in the presence of isobutylmethylxanthine, a phosphodiesterase inhibitor, glucagon suppressed the secretion of VLDL cholesterol to $2.3 \pm 0.6 \mu\text{g}/\text{mg}$ of protein during this period ($34.0 \pm 9.0\%$ of control). In contrast with the long-term stimulatory effect of insulin on the secretion of VLDL, continuous exposure of the cells to glucagon for 72 h resulted in an increase in the degree of suppression of VLDL output compared with that observed after 24 h exposure (Table 3). A substantial proportion of immunoreactive apoB was again detected in the VLDL infranatant in these experiments. However, glucagon had a similar inhibitory effect on the appearance of this non-VLDL apoB (Table 2).

Table 2. Effect of short-term exposure to glucagon on the secretion of VLDL apoB, triacylglycerol and cholesterol by hepatocyte cultures

Hepatocytes were cultured for a total of 72 h in the supplemented medium. During each 24 h period (day 1, day 2 or day 3) glucagon ($0.1 \mu\text{M}$) was either present or absent. For hepatocytes cultured longer than 24 h (i.e. day 2 and day 3), the medium was changed at intervals of 24 h. At the end of each 24 h period the medium was removed and the VLDL and VLDL infranant ($d > 1.006$) fractions were obtained. Rates of secretion of VLDL triacylglycerol and cholesterol ($\mu\text{g}/24 \text{ h}$ per mg of protein) and of apoB ($\text{ng}/24 \text{ h}$ per mg of protein) were determined. Total apoB ($\text{ng}/24 \text{ h}$ per mg of protein) is the sum of the apoB in the VLDL and the infranant. Each value represents the mean $\pm$ S.E.M. of 4 or 5 independent experiments. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from their corresponding control values.

Incubation conditions	Exposure period	VLDL			Total apoB
		Triacylglycerol	Cholesterol	apoB	
No glucagon		212 $\pm$ 29.5	10.1 $\pm$ 2.3	1234 $\pm$ 439	2407 $\pm$ 467
+ Glucagon	4–28 h	112 $\pm$ 20.9*	4.7 $\pm$ 1.0	655 $\pm$ 378	1447 $\pm$ 575
Glucagon effect (% of control)	(day 1)	52.2 $\pm$ 5.6**	47.3 $\pm$ 6.8**	43.3 $\pm$ 10.6*	55.8 $\pm$ 11.2*
No glucagon		192.4 $\pm$ 39.6	6.7 $\pm$ 0.9	700 $\pm$ 268	1882 $\pm$ 364
+ Glucagon	24–48 h	135.2 $\pm$ 27.3	4.8 $\pm$ 1.2	533 $\pm$ 209	1329 $\pm$ 232
Glucagon effect (% of control)	(day 2)	72.4 $\pm$ 4.7**	69.5 $\pm$ 13.6*	57.7 $\pm$ 7.2**	72.9 $\pm$ 7.8**
No glucagon		188.8 $\pm$ 39.6	6.2 $\pm$ 1.4	714 $\pm$ 171	1489 $\pm$ 208
+ Glucagon	48–72 h	112.8 $\pm$ 21.9	6.2 $\pm$ 1.3	281 $\pm$ 72*	883 $\pm$ 256
Glucagon effect (% of control)	(day 3)	64.7 $\pm$ 10.4*	104.0 $\pm$ 22.9	37.6 $\pm$ 5.4**	59.1 $\pm$ 12.8*

Table 3. Effect of long-term (72 h) exposure of hepatocytes to glucagon on the secretion of VLDL apoB and triacylglycerol

Hepatocytes were cultured in the presence or absence of glucagon ($0.1 \mu\text{M}$) for 72 h. During the final 24 h of this period the amounts of VLDL apoB (ng/mg of cell protein) and triacylglycerol ($\mu\text{g}/\text{mg}$ of cell protein) were determined. Values represent means $\pm$ S.E.M. of 4–8 independent experiments. Values marked *, ** and *** are significantly different ($P < 0.05$, $P < 0.005$ and $P < 0.001$ respectively) from their corresponding controls.

Incubations conditions	VLDL	
	Triacylglycerol	apoB
No glucagon	138.9 $\pm$ 23.6	1298 $\pm$ 895
+ Glucagon	50.7 $\pm$ 12.7*	579 $\pm$ 385
Glucagon effect (% of control)	39.0 $\pm$ 9.8***	42.6 $\pm$ 5.8**

Effects of glucagon on ketogenesis, triacylglycerol synthesis and fatty acid synthesis

The rate of ketogenesis declined somewhat when measured during successive 24 h periods (i.e. on days 1, 2 and 3), but this decrease was not significant (Fig. 1). Glucagon significantly increased the rate of ketone-body production when added at the beginning of each 24 h period of culture (Fig. 1). Glucagon, on the other hand, had little or no effect on the overall rate of triacylglycerol synthesis from exogenous fatty acid (Table 4). This parameter is represented by the sum of cellular and VLDL triacylglycerol labelled from exogenously added [^3H]oleate. Table 4 also shows that the capacity of the cell to synthesize triacylglycerol increased with time of culture. Thus, when labelled oleate was present during the final 24 h of the 72 h culture period, the cell esterified $1.22 \pm 0.19 \mu\text{mol}$ of fatty acid/mg of protein, whereas during the first 24 h the corresponding value was 0.66 ± 0.05 . The most important point, however, was that glucagon had no significant effect on these values.

Contribution of exogenous oleate to VLDL and cellular triacylglycerol

In some experiments, [^3H]oleate instead of non-radioactive oleate was added to the medium during the final 24 h of culture.

Fig. 1. Effects of time and glucagon ($0.1 \mu\text{M}$) on ketogenesis in cultured hepatocytes

Hepatocytes were cultured for a total of 72 h according to the procedure described in the legend to Table 2. At the start of each 24 h culture period (day 1, day 2 or day 3) glucagon was either absent or present. The results are presented as total ketone bodies (acetoacetate + β -hydroxybutyrate) present in the medium at the end of each 24 h period. Values in parentheses represent the numbers of independent determinations carried out at each time point. The percentage values refer to the effect of glucagon as a percentage of the control values at each time point. Key: Δ , glucagon absent; $\blacktriangle$, glucagon present. Values marked *, ** and *** are significantly different ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively) from the corresponding control values.

Its contribution to the cellular and VLDL triacylglycerol at the end of this period was calculated from its specific radioactivity. Table 5 shows that glucagon had no effects on the contribution

Table 4. Effect of glucagon on the synthesis of triacylglycerol from oleate

Hepatocytes were cultured for a total period of 72 h according to the legend in Table 2. [^3H]Oleate (0.75 mM; 9.78×10^5 d.p.m./ μmol) was present only during the final 24 h of each culture period. At the end of each 24 h period the cellular and VLDL triacylglycerol was isolated and its radioactivity was determined. Numbers in parentheses represent the numbers of individual experiments in each group. In each experiment the sum of the label in the cellular and VLDL triacylglycerol was taken to represent total triacylglycerol synthesis. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values at 4–28 h.

Culture period (h)	Glucagon	Triacylglycerol synthesized (μmol of oleate/24 h per mg of cell protein)	
		Cells	Total (cells + VLDL)
4–28	–	0.36 ± 0.04 (7)	0.66 ± 0.05 (7)
	+	0.44 ± 0.07 (7)	0.61 ± 0.06 (7)
24–48	–	0.63 ± 0.08 (7)*	0.98 ± 0.06 (7)**
	+	0.64 ± 0.07 (6)	0.88 ± 0.06 (6)**
48–72	–	0.71 ± 0.08 (5)**	1.22 ± 0.19 (6)*
	+	0.75 ± 0.15 (5)*	0.92 ± 0.16 (6)

Table 5. Contribution of exogenous oleate to VLDL and intracellular triacylglycerol

Cells were cultured for various periods up to 72 h in the presence of supplemented medium. During the periods shown below, non-radioactive oleate was replaced by [^3H]oleate (0.75 mM; 9.78×10^5 d.p.m./ μmol), and glucagon (0.1 μM) was either absent or present. At the end of each period the cellular and VLDL triacylglycerol was extracted. Samples of each fraction were used for determination of total mass and ^3H radioactivity. The mass of oleate which had entered each pool was calculated on the basis of its specific radioactivity. This quantity was used to calculate its contribution (%) to the total mass of each pool. Values marked * and *** are significantly different ($P < 0.05$ and $P < 0.001$ respectively) from the corresponding values observed in the cell.

Culture period during which [^3H]oleate present (h)	Glucagon present	Contribution of [^3H]oleate triacylglycerol to (%)	
		Cells	VLDL
4–28	–	48.2 ± 5.3 (8)	53.6 ± 3.8 (9)
	+	47.2 ± 6.4 (8)	55.0 ± 6.1 (9)
24–48	–	40.8 ± 3.7 (8)	55.9 ± 4.0 (9)*
	+	40.5 ± 3.8 (8)	50.9 ± 4.9 (9)
48–72	–	30.1 ± 3.8 (4)	46.7 ± 5.9 (6)**
	+	25.6 ± 3.8 (4)	45.5 ± 4.5 (6)***

of exogenous oleate to either the intracellular or secreted pools of triacylglycerol. However, it was of interest that, although the specific radioactivities of the intracellular and secreted triacylglycerol were similar during the first 24 h of culture, when added during the final 24 h of the 72 h culture period the specific radioactivity of the VLDL triacylglycerol exceeded that of the intracellular material. This was the case irrespective of the presence or absence of glucagon. It therefore appeared that during this period exogenous oleate contributed a greater proportion to the VLDL than to the intracellular triacylglycerol.

Fig. 2. Time course of the glucagon-mediated changes in cAMP concentration in cultured hepatocytes

Hepatocytes were cultured for 4 h in the presence of Waymouth's medium plus serum. The medium was then removed, the cells were washed with phosphate-buffered saline and supplemented medium (without serum) was added. After a further 24 h, the medium was removed and replaced with fresh supplemented medium. To one set of dishes was added glucagon (final concn. 0.1 μM) in saline (0.1 ml). To another set was added saline only. At the indicated times, the medium was removed, ice-cold 65% ethanol was added and the cells were kept chilled at -20°C until intracellular cAMP was determined. Each point represents the mean $\pm$ S.E.M. of 4–5 independent experiments. Key: ●, + glucagon; ○, no glucagon. Values marked * and ** are significantly different from the corresponding control values ($P < 0.05$ and $P < 0.01$ respectively).

Changes in intracellular cAMP and correlation with variations in VLDL output

Addition of glucagon (0.1 μM) to primary cultures of rat hepatocytes produced a rapid rise in concentration of intracellular cAMP (Fig. 2). This rise reached a peak about 10 min later, and subsequently declined rapidly to $\sim 30\%$ of peak value 1 h after glucagon addition (Fig. 2). At 4 h, 12 h and 24 h after addition of glucagon, the concentration of intracellular cAMP was only 20%, 15% and 7% of peak value respectively. Despite the relatively small elevation of cAMP levels between 12 and 24 h, the inhibition of VLDL triacylglycerol secretion in the presence of glucagon during this period was maintained [147.6 ± 24.2 (control) versus 80.3 ± 20.2 (glucagon) μg of triacylglycerol/mg of protein, a decrease of $45.6 \pm 7.7\%$ ($n = 4$; $P < 0.005$)]. This decrease was greater than that observed during the first 6 h of culture in the presence of glucagon [$29.0 \pm 11.5\%$ ($n = 4$; $P < 0.05$)], a period when intracellular cAMP levels were considerably higher (Fig. 2).

DISCUSSION

Primary cultures of rat hepatocytes provide a useful model for studying the direct effects of hormones on hepatic lipid metabolism, but so far these studies have been confined to periods generally lasting less than 24 h. During this relatively short period, several groups have shown that insulin suppresses the secretion of VLDL triacylglycerol [2–4,6] and apoB [3,5,7] from primary hepatocytes. Both processes are dose-dependent with respect to insulin, and at lower insulin concentrations lipid synthesis and secretion are stimulated. At higher concentrations of insulin, consistent with post-prandial portal levels, lipid synthesis continues to be stimulated. However, secretion of VLDL lipid as well as apoB is decreased. Although lipid accumulates in the liver with insulin treatment [7,16], this is not

the case for apoB, which is intracellularly degraded [7]. In the long term, insulin has been shown to be necessary to maintain normal VLDL and apoB secretion, as both are significantly decreased in the diabetic state [23–25] as well as with prolonged fasting. When insulin is given to diabetic animals, apoB production is restored to control levels [24]. Effects of insulin in the longer term remain controversial, and initial short-term inhibitory effects decrease with time in rat hepatocytes and human HEP G2 cells [26,27]. In the present studies, we have explored longer-term effects of insulin and find these effects consistent with our previous findings that rat hepatocytes, when exposed to a high concentration of insulin for long periods, not only lose initial inhibitory effects of insulin on VLDL and apoB but stimulate VLDL triacylglycerol secretion [6,25]. The present results extend these previous studies, and show that this increased triacylglycerol output is accompanied by an increase in the output of VLDL apoB. We did not, however, discriminate between the two forms of apoB (i.e. apoB_H and apoB_L) secreted by rat liver. As each VLDL particle contains only one molecule of apoB [28], this suggests that the long-term effect of insulin is to increase the number of particles of VLDL secreted, rather than merely to increase the triacylglycerol content of each particle. Thus the response of hepatic VLDL apoB secretion to chronically high concentrations of insulin differs from that resulting from short-term exposure and may explain the relatively high rates of hepatic VLDL output under conditions of chronic hyperinsulinaemia [29–31].

The appearance of a substantial amount of non-VLDL apoB in the $d > 1.006$ fraction of the medium (Table 1) was puzzling, especially as this was not accompanied by a significant quantity of triacylglycerol (results not shown). Previous studies [3] have also demonstrated a relatively large proportion of medium apoB associated with lipoprotein fractions of $d > 1.006$. Also, Fukuda *et al.* [32] showed that a substantial proportion of cholesterol was associated with the LDL fraction of rat liver perfusates. These studies [3,32] concluded that this non-VLDL apoB arose from direct secretion rather than from extrahepatic catabolism of VLDL secreted into the medium. This latter process results from the lipolysis of VLDL triacylglycerol by hepatic lipase, and is inhibited by Triton WR-1339 [33]. However, in the present work Triton did not decrease the amounts of apoB in the $d > 1.006$ infranant when cells were cultured for 24 h (results not shown).

It has been known for some time that glucagon and cAMP each suppress the secretion of VLDL and enhance ketogenesis in short-term perfusion of rat livers [10,34,35]. Detailed longer-term studies using cultured cells have not been carried out, despite the implications for an increased understanding of the effects of chronic hyperglucagonaemia [12,36]. The HEP-G2 cell model does not appear to be very useful in this respect, since it is insensitive to glucagon- and cAMP-mediated metabolic effects [37]. Rat hepatocytes, on the other hand, show some promise as a model for studying the mechanism underlying the glucagon-mediated effects on hepatic VLDL metabolism. Fig. 2 illustrates the sensitivity of intracellular cAMP concentration to a glucagon challenge. The transient nature of the increased cAMP level, which reached a peak 10 min after glucagon addition, may be attributed to glucagon breakdown and to the glucagon-mediated desensitization of adenylate cyclase to glucagon [38,39]. The cells are also sensitive to the effects of glucagon on lipid metabolism after culturing for up to 3 days, as shown by the reciprocal changes in ketogenesis and VLDL output. The rate of ketogenesis [$2.73 \pm 0.63 \mu\text{mol}/24 \text{ h}$ per mg of cell protein; calculated from Fig. 1 (day 1)] was similar to those calculated from previous reports using perfused livers (range 0.63 – $6.0 \mu\text{mol}/24 \text{ h}$ per mg of protein; assuming a linear rate during this period) [32,40,41] and in freshly isolated hepatocytes ($4.3 \mu\text{mol}/24 \text{ h}$ per mg) [42].

Net accumulation of newly synthesized triacylglycerol by the cultured cells ($0.36 \pm 0.04 \mu\text{mol}$ of oleate/ 24 h per mg of protein; Table 3) was similar to that reported in short-term-perfused livers ($0.28 \mu\text{mol}/24 \text{ h}$ per mg of protein), assuming a linear rate [32].

The suppression, by glucagon, of the secretion of VLDL apoB has not been demonstrated previously, and suggests that the hormone diminishes the actual number of VLDL particles secreted rather than merely decreasing the amount of triacylglycerol per particle. The mechanism by which this occurs remains obscure. ApoB is secreted as a phosphoprotein [8,43–45], and both serine and tyrosine residues appear to be phosphorylated [45]. Further, the extent of hepatic apoB phosphorylation is increased in the diabetic state [45]. In addition to phosphorylation, apoB is known to be fatty-acylated with palmitic and stearic acids as thioester bonds with cysteine residues [46,47]. The specific roles of phosphorylation and fatty acylation in apoB metabolism have not been determined, but preliminary evidence suggests that insulin may have an effect on apoB phosphorylation state. In these cases, insulin suppressed phosphorylation of apoB in short-term hepatocyte suspensions [8], whereas in primary hepatocyte culture this process was increased [48]. There is currently no direct evidence of glucagon control over apoB phosphorylation or fatty acylation. However, it is reasonable to speculate that glucagon could mediate post-translational changes in apoB affecting degradation pathways, intracellular movement or association of apoB with preformed lipids. It is of interest that there is continued suppression of VLDL secretion when intracellular cAMP levels have returned almost to normal (Fig. 2). However, it is possible that even a small increase in cAMP above basal could produce this metabolic effect. Alternatively, glucagon, in terms of its activation of AMP-dependent protein kinase, could effect apoB phosphorylation similarly to that described for acetyl-CoA carboxylase [49] and HMG-CoA reductase [50]. Glucagon may also act via changes in intracellular Ca^{2+} concentration [51] and, in this respect, it has previously been shown that α -adrenergic stimulation of hepatocytes, which results in a redistribution of intracellular Ca^{2+} , also inhibits VLDL triacylglycerol secretion [52].

Although the shorter-term effects (i.e. 24 h) of glucagon and insulin on the secretion of VLDL are similar, the longer-term effects differ. Whereas chronic exposure of hepatocytes to insulin leads to a stimulation of VLDL triacylglycerol and apoB secretion (Table 1), continuous exposure to glucagon maintains or even exacerbates the initial inhibition (Table 3). Either or both of these effects may explain the decreased rate of secretion of VLDL [15,41,53,54] and apoB [23,24] observed *in vitro* in livers from diabetic animals, since diabetes is characterized by chronic hypoinsulinaemia and is associated with chronically elevated levels of plasma glucagon [12].

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