Regulation of very-low-density-lipoprotein lipid secretion in hepatocyte cultures derived from diabetic animals

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Hepatocytes were derived from 2–3-day streptozotocin-diabetic rats and maintained in culture for up to 3 days. Compared with similar cultures from normal animals, these hepatocytes secreted less very-low-density-lipoprotein (VLDL) triacylglycerol, but the decrease in the secretion of VLDL non-esterified and esterified cholesterol was not so pronounced. This resulted in the secretion of relatively cholesterol-rich VLDL particles by the diabetic hepatocytes. Addition of insulin for a relatively short period (24 h) further decreased the low rates of VLDL triacylglycerol secretion from the diabetic hepatocytes. The secretion of VLDL esterified and non-esterified cholesterol also declined. These changes occurred irrespective of whether or not exogenous fatty acids were present in the culture medium. Little or no inhibitory effect of insulin was observed after longer-term (24–48 h) exposure to the hormone. Both dexamethasone and a mixture of lipogenic precursors (lactate plus pyruvate) stimulated VLDL triacylglycerol and cholesterol secretion, but not to the levels observed in hepatocytes from normal animals. The low rate of hepatic VLDL secretion in diabetes contrasts with the increase in whole-body VLDL production rate. This suggests that the intestine is a major source of plasma VLDL in insulin-deficient diabetes.

INTRODUCTION

Insulin-dependent (Type I) diabetes is often associated with an increase in the concentration of the plasma triacylglycerol, most of which is associated with the very-low-density lipoprotein (VLDL) fraction [1–3]. The cause(s) of the hypertriglyceridaemia is not clear, although there is substantial evidence that defective lipoprotein lipase-mediated clearance is an important contributory factor [4–8]. Increased production of plasma VLDL may also occur [9–12]. Since the liver is generally believed to be the major source of the plasma VLDL [13–15], it might be expected that the increased VLDL production observed under conditions of insulin deficiency is of hepatic origin. This concept is supported by the numerous observations that insulin suppresses hepatic VLDL secretion both *in vivo* [16,17] and *in vitro* [18–24]. However, in short-term perfusion of livers from diabetic animals *in vitro*, VLDL secretion decreased [25–29]. A major objective of the present work was to determine the characteristics of VLDL lipid secretion by hepatocyte cultures derived from diabetic animals, to establish a model for studying the longer-term regulation of this process in insulin-deficient diabetes. This condition is associated with a range of hormonal and metabolic disturbances (for a review, see [3]) secondary to insulin deficiency. The direct, longer-term, effects of each of these changes on hepatic lipid metabolism are unknown. One of the obvious advantages of the hepatocyte culture model is that it permits investigation of the direct effects of a single hormone or metabolite without the complications brought about by other simultaneous, often complex, changes which occur in diabetes *in vivo*.

Apart from quantitative changes in the plasma VLDL,

diabetes is often associated with qualitative changes in the lipid composition of lipoprotein fractions [30–34]. This is thought to contribute to the decreased rate of clearance of these particles, thereby enhancing their atherogenic potential. Another aim of the present work was to investigate whether any such changes occurred in the newly formed VLDL particles secreted by hepatocyte cultures derived from diabetic animals.

MATERIALS AND METHODS

Materials

All culture-media components were obtained from Gibco (Paisley, Scotland, U.K.). Bovine insulin, dexamethasone, sodium pyruvate, L(+)-lactic acid and streptozotocin were obtained from Sigma (Poole, Dorset, U.K.). Radiochemicals were purchased from Amersham International, Little Chalfont, Bucks., U.K.

Maintenance of animals

Male Wistar rats were housed in a windowless room artificially lit between 08:00 h and 20:00 h. Animals were fed on a commercially available pelleted diet (Diet 41B; Dixon and Sons, Ware, Herts., U.K.) with the following composition (by wt.): 46.7% carbohydrate (mainly starch), 15.4% protein, 3% fat and 4.4% crude fibre. Food and water were given *ad libitum* [24]. Animals were used for hepatocyte preparation at 11:00 h, and weighed between 230 and 300 g. Where appropriate, insulin deficiency was induced by injection of a solution of streptozotocin (50 or 75 mg/kg body wt.) in 0.01 M-citrate buffer (pH 4.5) via the tail vein under light diethyl ether anaesthesia. Since hypoglycaemia occurs 7 h after

Abbreviation used: VLDL, very-low-density lipoprotein.

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injection of the drug [35], glucose was added to the drinking water between 7 and 20 h after streptozotocin treatment. The plasma glucose concentration of the diabetic animals was 17.4 ± 4.2 mM. Similarly treated animals had plasma insulin, acetoacetate and β -hydroxybutyrate concentrations of 9.40 ± 3.58 μ units/ml, 0.62 ± 0.20 mM and 1.73 ± 0.42 mM respectively. The animals were not ketotic, as judged by both plasma and urine ketone-body concentrations. Animals were used for hepatocyte preparation 2–3 days after streptozotocin treatment. This timing was selected in order to compare results with those of others who have previously investigated the effects of diabetes on VLDL metabolism [25,27,29,31].

Preparation of hepatocyte cultures and incubation procedures

Hepatocytes were prepared from normal and diabetic animals as described previously [24]. The hepatocytes were suspended at a concentration of $(0.5\text{--}1.0) \times 10^6$ cells/ml in Waymouth's medium MB 752/1 containing glutamine (3.4 mM), serine (0.5 mM), alanine (0.4 mM), penicillin (200 units/ml), streptomycin (200 μ g/ml) and either fetal- or newborn-calf serum (10%, v/v). The cell suspension was plated out (3.0 ml per dish), and 4 h later the medium was removed, the cell monolayer washed and fresh medium (without serum) was added [24]. After addition of the serum-free Waymouth's medium (i.e. 4 h after plating), batches of cells were treated in different ways, as follows. Protocol A: where appropriate, insulin, dexamethasone, lactate and pyruvate were added to give initial concentrations of 78 nM, 1 μ M, 10 mM and 1 mM respectively. Cells were incubated for 24 h (i.e. between 4 and 28 h after isolation of the cells). Protocol B: after transfer to the serum-free medium (4 h after plating), the cells were cultured in the absence of hormones and substrates for a further 20 h. The medium was then changed and, where appropriate, hormones and/or substrates were added at the above concentrations. The cells were then incubated for 24 h (i.e. between 24 and 48 h after isolation of the cells). Protocol C: as for Protocol B, except that at the end of the 24 h treatment period (i.e. 48 h after cell isolation) the medium was removed and replaced with medium containing the appropriate hormone or substrate. The cells were then incubated for a further 24 h (i.e. between 48 and 72 h after isolation of the cells). These cells had thus been exposed to the hormones or substrates for a total of 48 h, whereas in Protocols A and B cells had been exposed to hormones or substrates for 24 h only. In each experimental protocol, the medium contained VLDL which had been secreted over a different 24 h period.

Isolation of VLDL and lipid analysis

At the end of each 24 h period, the medium from two identically treated dishes was combined, the density was adjusted to 1.006 g/ml, and EDTA and NaN_3 were added to give concentrations of 0.1 g/litre in each case. The supernatant was centrifuged at 20000 g for 10 min to remove fragments of broken cells. The supernatant from this centrifugation was then centrifuged at 40000 rev./min in a Beckman SW-50.1 rotor for 18 h. The floating fraction of $d < 1.006$ was obtained by tube slicing. A portion of this fraction was used for extraction of total lipid [36] and measurement of the triacylglycerol content [24]. Total, esterified and non-esterified chol-

esterol contents of the VLDL were determined by a combined enzymic/g.l.c. method as described previously [23,24].

Other analytical procedures

Rates of protein synthesis were determined by measurement of incorporation of [^3H]leucine into total cellular protein [24]. Protein concentration was determined by the method of Lowry *et al.* [37]. Plasma glucose was measured as described by Slein [38].

RESULTS

Comparison of VLDL lipid secretion rates in hepatocyte cultures from normal and diabetic rats

During the first 24 h period after removal of serum from the culture medium (i.e. 4–28 h after plating), the secretion of VLDL triacylglycerol from the diabetic hepatocytes was only 40% of that observed in cultures derived from normal animals ($P < 0.05$). This relative decrease persisted during the two subsequent 24 h periods, the diabetic cells secreting 31% ($P < 0.001$) and 39% ($P < 0.01$) respectively of the amounts of triacylglycerol secreted by the normal cell cultures (Fig. 1).

Irrespective of the state of the donor animals, the amounts of VLDL triacylglycerol declined with increasing age of the culture (i.e. time after plating). There was no decrease in the rate at which [^3H]leucine was incorporated into cellular protein (53.2, 38.7, 46.6 nmol/mg of cell protein during each successive 24 h period). These rates were very similar to those observed in cultures from normal animals [24]. Despite the decreased output of VLDL triacylglycerol, during the first 24 h in serum-free culture diabetes had little or no inhibitory effect on the secretion of the total, non-esterified and esterified cholesterol (Fig. 1). In older cultures from more severely diabetic animals, less cholesterol was secreted than in similar cultures from normal animals. Nevertheless, the decrease in the rate of secretion of triacylglycerol was always greater than that of cholesterol secretion. This differential effect of diabetes on VLDL lipid secretion gave rise to changes in the composition of the secreted VLDL (Table 1). Thus, compared with normal cultures, there was an increase in the quantity of total cholesterol (i.e. esterified plus non-esterified cholesterol) relative to triacylglycerol during each of the subsequent 24 h periods after removal of serum from the medium. There were also increases in the amounts of non-esterified cholesterol and of esterified cholesterol relative to triacylglycerol.

Effects of insulin on VLDL lipid secretion

To determine whether insulin was capable of restoring rates of VLDL lipid secretion in diabetic hepatocytes to those observed in normal cells, cultures of different ages were incubated in the presence or absence of the hormone either for 24 h or for 48 h (Table 2). Exposure to insulin for a 24 h period immediately after removal of the serum (4–24 h after preparation; Protocol A) resulted in a 64% decline in the output of VLDL triacylglycerol. There were also decreases in the rates of output of total, esterified and non-esterified cholesterol (Table 2). Similar effects were observed when insulin was added for 24 h to older cell cultures (i.e. between 24 h and 48 h after plating; Protocol B). However, continued exposure of these cell cultures to insulin for a further 24 h abolished the inhibitory effect and produced a slight stimulation of

Fig. 1. Effects of diabetes on the secretion of VLDL lipids by hepatocyte cultures

Hepatocytes from normal and diabetic animals were cultured for different periods of time as defined by Protocols A, B and C (see the Materials and methods section). The amounts of VLDL lipids accumulating in the medium during successive 24 h periods were determined. ●, Normal hepatocytes; ▲, diabetic hepatocytes. The vertical bars represent the S.E.M. The numbers in parentheses represent the numbers of individual hepatocyte cultures used (each from a different animal).

Table 1. Effects of diabetes on the lipid composition of the newly secreted VLDL

The quantities of triacylglycerol (TG), total cholesterol (TC), free (non-esterified) cholesterol (FC) and cholesteryl ester (CE) associated with the VLDL fraction isolated from each hepatocyte preparation were determined. Each value represents the mean $\pm$ S.E.M. of at least four independent determinations, except where noted. Values marked * in the diabetic hepatocytes are significantly different from those obtained with the normal hepatocytes ($P < 0.05$).

	TC: TG (%)	FC: TG (%)	CE: TG (%)
Normal hepatocytes			
4-28 h (Protocol A)	6.95 $\pm$ 1.24	5.42 $\pm$ 0.64	2.50 $\pm$ 1.08
24-48 h (Protocol B)	9.70 $\pm$ 2.42	5.84 $\pm$ 0.39	4.23 (2)
Diabetic hepatocytes			
4-28 h (Protocol A)	15.49 $\pm$ 2.53*	13.07 $\pm$ 3.38*	3.66 $\pm$ 1.10
24-48 h (Protocol B)	17.81 $\pm$ 2.30*	8.96 $\pm$ 2.62	8.84 $\pm$ 0.34

Table 2. Effects of insulin on the secretion of VLDL lipids in hepatocyte cultures from diabetic rats

Hepatocyte cultures were prepared from diabetic rats and insulin was added according to experimental Protocols A, B or C (see the Materials and methods section). In Protocols A and B, insulin had been present for 24 h. In Protocol C, insulin had been present for 48 h, and VLDL lipid output was measured during the final 24 h of this period. The effect of insulin is expressed as a percentage of the rate observed in cultures from which insulin was absent. The values represent the means $\pm$ S.E.M. of at least five different preparations (4–28 h) or three preparations (24–48 h and 48–72 h). The control values for triacylglycerol secretion during the periods 4–28 h, 24–48 h and 48–72 h were (in $\mu\text{g}/\text{mg}$ of protein) 32.5, 13.9 and 9.4. The corresponding values for total cholesterol were 4.80, 3.21, 1.20, for non-esterified cholesterol 3.90, 1.74, 0.78, and for cholesteryl ester 1.85, 1.49 and 0.42 respectively. Values marked *, **, *** are significantly different from the controls at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

Age of culture (h after preparation)	Triacylglycerol (% of control)	Cholesterol (% of control)		
		Total	Non-esterified	Esterified
4–28 (Protocol A)	36.2 $\pm$ 1.2***	41.7 $\pm$ 8.4**	39.2 $\pm$ 6.6**	69.1 $\pm$ 28.7
24–48 (Protocol B)	33.2 $\pm$ 2.4***	36.1 $\pm$ 4.6***	53.5 $\pm$ 20.3	36.1 $\pm$ 11.1*
48–72 (Protocol C)	139.7 $\pm$ 29.2	149.9 $\pm$ 19.7*	111.4 $\pm$ 17.3	256.0 $\pm$ 70.8

Table 3. Effect of insulin on VLDL triacylglycerol secretion in the presence of oleate

Hepatocytes were plated out in Waymouth's medium containing serum for 4 h. The medium was removed and the monolayer was washed three times with fresh medium (without serum). Fresh Waymouth's medium (no serum) was added, and the cells were incubated in the presence or absence of insulin and oleate as indicated for 24 h. The numbers of individually prepared cell cultures are shown in parentheses. Values marked ** are significantly different from the respective controls (no insulin) at $P < 0.01$ (paired *t* test). Values marked † and †† are significantly different from the incubations containing oleate only ($P < 0.05$ and $P < 0.01$ respectively). VLDL triacylglycerol output is expressed as $\mu\text{g}/24$ h per mg of cell protein.

Hepatocytes	Additions to culture medium			
	None	Insulin	Oleate	Oleate + insulin
Normal	68.9 $\pm$ 9.0 (6)	41.4 $\pm$ 8.6 (6)**	117.7 $\pm$ 16.3 (5)	67.6 $\pm$ 9.4 (4)†
Diabetic	9.7 $\pm$ 2.4 (3)	3.6 $\pm$ 0.8 (3)**	30.1 $\pm$ 9.0 (3)	11.8 $\pm$ 2.6 (3)††

VLDL lipid output. At no time did insulin have any effect on the rates of protein synthesis from [^3H]leucine (49.7, 47.2 and 58.3 nmol of leucine incorporated/mg of cellular protein for Protocols A, B and C respectively; corresponding values for the non-insulin-treated cells were 53.2, 38.7 and 46.6 nmol/mg of protein).

Insulin-dependent diabetes is almost invariably associated with an increased concentration of plasma non-esterified fatty acids [4]. Exogenous fatty acids are well-established precursors of hepatic VLDL [39–44], and it may be argued that the decreased VLDL triacylglycerol output in the diabetic hepatocytes compared with normal was a result of extracellular fatty acid deficiency rather than of diabetes itself. To investigate this possibility, hepatocyte cultures from normal and from diabetic animals were incubated with or without 0.75 mM-oleate for 24 h (Protocol A; 4–24 h after plating). In both cases, there was a substantial increase in the rate of output of VLDL triacylglycerol (Table 3), but under these conditions the output from the diabetic cells remained considerably lower than that from the normal hepatocytes. Furthermore, the oleate-stimulated rate of output in the diabetic cells failed even to match the basal rates observed in the normal cells. In the group of diabetic

animals used for this experiment the basal rates of VLDL secretion were lower than those observed previously. However, the increased plasma glucose concentrations of these animals suggested that the diabetes was of greater severity.

In view of the important precursor role of exogenous fatty acids in VLDL production and secretion, it was important to compare the effects of insulin under these conditions of elevated VLDL triacylglycerol output with that on the basal rates of secretion. Table 3 shows that insulin retained its inhibitory effect when added for 24 h to cultures in which VLDL secretion was stimulated by exogenous oleate. This occurred in cultures from both normal and diabetic animals. To our knowledge this is the first report of an inhibitory effect of insulin on fatty acid-stimulated rates of VLDL output.

Effects of dexamethasone and of lipogenic precursors on VLDL lipid output

Glucocorticoid levels are increased in diabetes [3,45], so it was important to determine the effects of this class of hormone on VLDL secretion in diabetic hepatocytes. Addition of the synthetic glucocorticoid dexamethasone to cultures either 4 h (Protocol A) or 24 h (Protocol B)

Table 4. Effects of dexamethasone and of a mixture of lactate and pyruvate on VLDL lipid secretion from diabetic hepatocytes

Hepatocyte cultures were obtained from diabetic animals and treated with dexamethasone (1 μ M) or with a mixture of lactate (10 mM) and pyruvate (1 mM) at various times after preparation (Protocols A, B and C; see also legend to Table 2). The values represent means $\pm$ S.E.M. of at least three hepatocyte preparations unless otherwise stated. Values marked * or ** are significantly different from the controls (no addition to culture medium) at $P < 0.05$ and $P < 0.01$ respectively. For absolute control values, see the legend on Table 2.

Additions to culture	Triacylglycerol (% of control)	Cholesterol (% of control)		
		Total	Non-esterified	Esterified
Dexamethasone				
4–28 h	158.9±10.0**	167.6±30.9*	157.8±9.1**	361±175
24–48 h	141.2±11.8*	115.6±25.4	138.9±43.3	198.1 (2)
48–72 h	193.0±57.0	274.2±113.3	213±33.8*	124.9 (2)
Lactate plus pyruvate				
4–28 h	127.0±10.7*	131.5±35.9	133.3±57.3	336.8 (2)
24–48 h	121.8±16.7	78.5±21.4	104.9±17.4	99.0 (2)
48–72 h	153.6±44.6	195.5±64.8	130.9 (2)	132.3 (2)

after plating gave rise to a significant increase in triacylglycerol output during a subsequent 24 h period (Table 4). The output of total, esterified and non-esterified cholesterol also increased. During the period 48–72 h after isolation of the cells, dexamethasone remained stimulatory, but the extent varied widely from one culture to another. At no time was the stimulatory effect of dexamethasone sufficiently great to restore VLDL output to that observed in normal cells.

In livers from normal animals, precursors such as lactate and pyruvate increase rates of fatty acid synthesis *de novo* [46–48], and this is accompanied by an increase in the rates of secretion of VLDL triacylglycerol and cholesterol [23,49]. In the present work, addition of a mixture of the lipogenic precursors to cultures from diabetic animals also stimulated somewhat the secretion of VLDL lipids (Table 4), but the effects were small and rather variable.

DISCUSSION

Previous investigations into the effects of diabetes on hepatic VLDL lipid secretion *in vitro* have been restricted to relatively short-term experiments using the isolated perfused liver model [25–29]. The present results suggest that cultured hepatocytes from diabetic animals may provide a useful model for studying the longer-term regulation of this process by hormones and metabolic substrates. During relatively brief perfusions of livers from diabetic rats there was a decline in the rate of VLDL lipid output compared with that observed with normal livers [28,29]. The present results show that this relatively low output persists for at least several days after isolation of the liver cells. Treatment of the diabetic hepatocytes at various stages after isolation with a variety of hormones and precursors known to increase triacylglycerol synthesis failed to restore VLDL secretion rates to those observed in cells from normal animals. However, it is noteworthy that proportionately oleate was a more effective stimulator of basal VLDL triacylglycerol secretion in diabetic than in normal hepatocytes. This may reflect the greater importance of exogenous fatty acids as

precursors of VLDL triacylglycerol in diabetes. As regards the role of insulin, the low circulating concentration of this hormone *in vivo* in the diabetic animals raised the question as to whether the low rate of VLDL secretion by the liver was a direct or indirect consequence of insulin lack. The present observations show that insulin added for a period of 24 h was not only incapable of restoring normal rates of VLDL secretion but actually further depressed the already low basal rates observed in the diabetic cells. This effect persisted even in the presence of oleate, an important precursor of hepatic triacylglycerol and promoter of VLDL lipid secretion (Table 3). The suppressive effect of insulin on the diabetic hepatocytes was similar to that observed previously in hepatocyte cultures from normal animals [18–24]. In contrast with the short-term effects (up to 24 h), chronic treatment of the diabetic hepatocytes with insulin (48 h exposure) abolished the initial suppression of VLDL secretion. In fact, during this period there was a slight stimulation of this process. This again resembled the stimulatory effect of chronic insulin exposure in normal hepatocytes [24]. Other workers have also observed quantitative differences in the effects of insulin on VLDL secretion, depending on the duration of exposure of normal hepatocyte cultures to the hormone. These changes were attributed to changes in the activity of the insulin receptor [20]. These results emphasize the importance of distinguishing between the acute and chronic effects of insulin on hepatic VLDL metabolism.

It should be noted that diabetes and/or insulin may affect the re-uptake of VLDL by the hepatocytes, and this may contribute to the effects observed. However, we consider it unlikely that such a large decrease in the net output of VLDL in the diabetic hepatocytes was due mainly to an increase in the rate of VLDL re-uptake, particularly as the concentration of secreted VLDL in the medium was very low. Furthermore, Durrington *et al.* [18] have shown previously that re-uptake of VLDL by hepatocytes from normal animals was negligible, irrespective of the presence or absence of insulin.

The disproportionately large decrease in the secretion of VLDL triacylglycerol compared with non-esterified

cholesterol (Table 1) resulted in the secretion of a VLDL particle in which the surface coat was enriched in cholesterol. This compositional change would impair the interaction between VLDL and apoC-II, resulting in a decreased affinity for lipoprotein lipase [50]. This would reinforce the inactivating effect of insulin deficiency on lipoprotein lipase and may contribute to the delayed clearance of plasma VLDL in the diabetic state [31].

The question remains as to why insulin lack *in vivo* results in a suppression of hepatic VLDL triacylglycerol secretion in diabetic animals, whereas acute insulin exposure *in vitro* has a similar effect. There is no easy answer to this paradox. However, in view of the increased secretion of VLDL after chronic insulin treatment of normal cells *in vitro* [24], it might be argued that continuous, rather than acute, exposure to the hormone is required to maintain hepatic VLDL secretion *in vivo*. Continuous insulin deficiency, as in diabetes, might then be expected to suppress this process. On the other hand, insulin deficiency is almost invariably associated with hyperglucagonaemia [51–53], and glucagon is known to suppress hepatic VLDL secretion *in vivo* [54] and *in vitro* [23,49,55].

Another question which remains to be answered is the molecular mechanism by which chronic insulin lack *in vivo* ultimately suppresses hepatic VLDL triacylglycerol secretion at the cellular level. In this regard Sparks and colleagues [56], using hepatocyte cultures, have provided evidence that streptozotocin-diabetes is associated with a decline in the rate of synthesis of apoprotein B, the hepatic content of which may limit the rate of VLDL assembly and secretion.

Finally, the problem remains as to why diabetes gives rise to an increased whole-body production of VLDL in human subjects [9–11] and in the rat [12], whereas the hepatic contribution is apparently decreased. It is possible that the '*in vitro*' models so far employed do not adequately reflect the performance of the diabetic liver *in vivo*. A more likely explanation is that some source other than the liver is responsible for the excess amounts of VLDL produced by the body in the diabetic state. In this regard there is substantial evidence that the intestinal production of VLDL increases in diabetes [55]. This evidence arises from dietary considerations [57], the composition of the plasma lipoproteins in diabetes [58,59] and from direct measurements of the output of mesenteric-lymph lipoproteins in starved diabetic animals [60,61]. Similar experiments in fed animals remain to be done.

We thank Mrs M. Barber for typing the manuscript. This work was supported by a grant from the Medical Research Council (U.K.). G.F.G. is a member of the External Staff of the Medical Research Council at the University of Oxford.

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Received 7 March 1989/3 May 1989; accepted 9 May 1989