Stimulation of apolipoprotein secretion in very-low-density and high-density lipoproteins from cultured rat hepatocytes by dexamethasone

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The effects of dexamethasone (a synthetic glucocorticoid) and insulin on the secretion of very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) were investigated. Rat hepatocytes in monolayer culture were preincubated for 15 h in the presence or absence of combinations of 100 nM-dexamethasone and 2 nM-, 10 nM- or 50 nM-insulin. Dexamethasone increased [³H]oleate incorporation into secreted triacylglycerol by 2.7-fold and the mass of triacylglycerol secreted by 1.5-fold. Insulin alone decreased these parameters and antagonized the effect of dexamethasone. Dexamethasone increased the secretion of [³H]leucine in apolipoprotein (apo) E, and in the large (B_H) and small (B_L) forms of apo B in VLDL by about 7.1-, 3.6- and 4.0-fold respectively. Insulin alone decreased the secretion of these ³H-labelled apolipoproteins in VLDL. However, 2 nM-insulin with dexamethasone increased the secretion of ³H-labelled apo B_H and apo B_L by a further 0.8- and 3.2-fold respectively; 50 nM-insulin decreased the secretions of apo E, apo B_H and apo B_L in VLDL. Similar effects for dexamethasone or insulin alone were also obtained for the masses of apo E and apo B_{L+H} secreted in VLDL. Albumin secretion was not significantly altered by either dexamethasone or insulin alone, but in combination they stimulated by 2.1–2.6-fold. Insulin or dexamethasone alone had little effect on the secretion of apolipoproteins in the HDL fraction. However, dexamethasone plus 2 nM-insulin increased the incorporation of [³H]leucine into apo A_I, apo A_{II} plus apo C, apo A_{IV} and apo E of HDL by about 1.8-, 1.6-, 1.7- and 2.0-fold respectively. The apo E in the bottom fraction represented about 69% of the total ³H-labelled apo E secreted. The responses in the total secretion of apo E from the hepatocytes resembled those seen in HDL. The interactions of insulin and dexamethasone are discussed in relation to the general regulation of lipoprotein metabolism, the development of hyperlipidaemias and the predisposition to premature atherosclerosis.

INTRODUCTION

The liver plays a central role in the metabolism of lipoproteins. It secretes VLDL and HDL components and removes from the circulation major proportions of chylomicron remnants, intermediate-density lipoproteins, LDL and HDL. The present work is concerned with the effects of insulin and glucocorticoids in regulating lipoprotein secretion by the liver.

Glucocorticoids stimulate the secretion of VLDL *in vivo* [1–3], in perfused liver [4,5] and in isolated hepatocytes [6–8]. By contrast, insulin on its own can inhibit the secretion of triacylglycerol, phospholipid, cholesterol ester, apo B and apo E in VLDL [6,7,9–15]. However, some investigators have observed no significant short-term effects of insulin on VLDL secretion [6,16,17], whereas others have claimed that insulin can stimulate this process [18,19]. It is important to reconcile these discrepancies, since this is vital in understanding fully the hormonal control of VLDL secretion. Glucagon, via cyclic AMP [20,21], and adrenaline, via α -adrenoreceptors [22], decrease VLDL secretion from cultured hepatocytes. Hormones also modulate the secretion of components associated with HDL. Insulin and dexamethasone increase the secretion of apo A_I by cultured rat hepatocytes [23,24]. By contrast, glucagon decreases apo A_I appearance in the medium [23].

There is accumulating evidence that the mechanisms for the

secretion for VLDL and HDL by the liver are different. For example, orotic acid inhibits the secretion of VLDL, but not of HDL [25]. Also, the secretion of both lipid and apolipoprotein components of VLDL, but not of HDL, are dramatically decreased in choline-deficient, compared with choline-supplemented, hepatocytes [26].

The present studies were therefore undertaken primarily to investigate the interaction of insulin and dexamethasone on the secretion of apolipoproteins in VLDL. We have confirmed that the secretion of triacylglycerol was stimulated by dexamethasone and that insulin antagonized this effect [6]. The secretion of apo B_H, apo B_L and apo E in VLDL was then determined by measuring the incorporation of [³H]leucine and by immunotitration. Since apo E was also secreted in HDL and in the fraction of density > 1.18 g/ml, information is also provided about apolipoprotein secretion in these fractions. This provides a more complete picture of the factors controlling apo E secretion and the relation between VLDL and HDL production. The work was performed with primary monolayer cultures of rat hepatocytes, since this system has been well characterized in terms of the metabolic viability of the cells and their hormonal responsiveness in terms of lipid and lipoprotein metabolism [6,27–30]. The omission of insulin and glucocorticoid from the incubations does not decrease viability, as indicated by the retention of protein and lactate dehydrogenase activity. The cells

Abbreviations used: apo, apolipoprotein; B_H, B_L, large (heavy) and small (light) forms of apo B; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

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cultured in the absence of hormones also remain metabolically responsive and competent [6,27–30].

EXPERIMENTAL

Materials

The sources of most of the materials have been described previously [6,27–30]. Acrylamide, *NN'*-methylenebisacrylamide, goat anti-(rabbit IgG)–alkaline phosphatase conjugate, goat anti-(mouse IgG)–alkaline phosphatase conjugate and Bio-Gel A-150 M (100–200 mesh) were purchased from Bio-Rad. L-[4,5-³H]-Leucine (46–69 Ci/mmol) and [9,10(n)-³H]oleic acid (5 Ci/mmol) were from Amersham International. Cab-O-Sil (fumed silica) was from Sigma. Male Wistar rats (about 200 g) were supplied by Charles River, Québec, Canada. Mengo virus was a gift from Dr. D. G. Scraba, Department of Biochemistry, University of Alberta.

Preparation and incubation of hepatocytes

Hepatocytes were prepared from male rats, and about 2.2×10^6 cells were applied to Falcon culture dishes that had been coated with collagen [31]. The medium was modified Leibovitz L-15 containing 10% (v/v) newborn-calf serum. Cells were incubated for 1–2 h at 37 °C in air at 96% humidity, and the unattached and non-viable cells were removed by a medium change. The monolayer of hepatocytes was incubated for a further 6 h in the same medium (3 ml/dish). The medium was then changed and the calf serum was replaced by 0.2 mM fatty-acid-poor BSA. Dexamethasone and insulin were added at the concentrations indicated, and the cells were incubated for 14 h. In order to measure protein synthesis, the medium was replaced by an identical medium except that the leucine was decreased from 950 to 50 μ M. The latter concentration supported almost maximum rates of protein synthesis. After a further incubation for 1 h, the medium was replaced with 2 ml of the equivalent leucine-depleted medium/dish containing about 43 μ Ci of [³H]leucine/ml and 1 mM-oleate, and the cells were incubated for 8 h. This time was chosen because the rates of secretion for triacylglycerol [6] and for apolipoproteins [32] are constant during this period. The medium from 7 or 8 dishes was collected and centrifuged at 10000 *g* for 10 min to remove cell debris. The hepatocytes were washed twice with ice-cold 0.16 M-NaCl and scraped from the plates in 1 ml of 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes adjusted to pH 7.4 with KOH.

Lipid secretion from the hepatocytes was measured in cells treated as described above but with normal, rather than leucine-depleted, Leibovitz L-15 medium, and the final incubation contained 1 mM-[³H]oleate (1.66 Ci/mol) and 1 mM-glycerol. The specific radioactivity of the [³H]oleate was increased by 10-fold when the size of the VLDL particles was determined. The loss of viable cells from the monolayer was normally less than 5% during this period, as indicated by the leakage of lactate dehydrogenase activity.

Isolation and analysis of cell and secreted proteins

The combined medium from 7 or 8 dishes was fractionated by a single-step ultracentrifugation procedure [32]. Lipoproteins in VLDL ($d < 1.02$ g/ml), LDL ($1.02 < d < 1.06$ g/ml), HDL ($1.06 < d < 1.18$ g/ml) and the bottom fraction ($d > 1.18$ g/ml) were concentrated by absorption on to fumed silica (Cab-O-Sil). Apolipoproteins were solubilized from the Cab-O-Sil with 0.5 ml of 2% SDS containing 6 M-urea for the VLDL and LDL fractions, and with 2.5 ml of reagent for the HDL and bottom fractions [32]. Samples (200 μ l for VLDL and LDL, and 100 μ l for HDL and the bottom fraction) were separated on SDS/PAGE by using 3–15% gradient gels for VLDL and LDL and 12% gels

for HDL and the bottom fraction. The positions of the apolipoproteins on the gels were confirmed by using standards of VLDL and HDL prepared from rat serum [33] and by staining with Coomassie Blue R. The possible contamination of the HDL fraction by membrane proteins was eliminated since the medium was centrifuged at 10000 *g* for 10 min. and fluorography of the ³H-labelled proteins after SDS/PAGE revealed no major membrane protein bands. Areas containing apolipoproteins were cut from the gels, dried at 50 °C and incubated overnight with 0.5 ml of 30% (v/v) H₂O₂. The [³H]leucine incorporated was determined by scintillation counting. In the basal conditions average radioactivity isolated from the bands of apo E, apo B_L and apo B_H from VLDL was 3430, 1580 and 1213 d.p.m. of ³H respectively. The recovery of radioactivity in these apolipoproteins was about 70% of the total radioactivity applied to the Cab-O-Sil, as expected [32]. The percentage recovery did not vary with the various hormone treatments.

The incorporation of [³H]leucine into total cell protein was determined after adding 1.25 mg of BSA as carrier to homogenates of the hepatocytes. The protein was precipitated with a final concentration of 5% (w/v) of ice-cold trichloroacetic acid and collected by filtration through microfibre filters (Whatman International). The filters were washed with 6 $\times$ 1 ml of ice-cold trichloroacetic acid, and the protein was solubilized by incubating overnight in 0.8 ml of 0.1 M-NaOH. The solution was neutralized with 0.2 ml of 0.5 M-HCl containing 0.12 M-Tris, and radioactivity was determined.

Determination of lipid synthesis and secretion

Lipids were extracted from duplicate plates [34], except that 0.2 M-H₃PO₄ containing 2 M-KCl was used to separate the phases. The bottom phase was washed twice with synthetic top phase obtained by mixing equivalent volumes of solvents. The incorporation of [³H]oleate into triacylglycerol and the mass of triacylglycerol were determined [35], except that the final thin-layer chromatograph for the former assay was developed with light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (50:50:1, by vol.).

Measurement of the quantity of apo B and apo E secreted by rat hepatocytes

The concentrations of apo B and apo E in the VLDL fraction were determined by e.l.i.s.a. assays. Anti-apo E was prepared by using apo E purified from the VLDL fraction of rat serum by SDS/PAGE as described above. The apo E band was homogenized in 0.15 M-NaCl containing 20 mM-potassium phosphate buffer, pH 7.4, emulsified in Freund's complete adjuvant and injected into rabbits. Two booster doses in Freund's incomplete adjuvant were given at 4-weekly intervals. The antibody was precipitated from the serum by 40% saturation with (NH₄)₂SO₄, and the IgG was eluted from Mono Q in 10 mM-Tris/HCl (pH 8.5) with a linear gradient of NaCl up to 1 M by using an f.p.l.c. apparatus. The antibody was specific for apo E, as demonstrated by Western-blot analysis of VLDL. Anti-apo B (kindly provided by Dr. R. A. Davis, Hepatobiliary Research Center, Denver, CO, U.S.A.) was a monoclonal antibody prepared against rat apo B_L [36].

For the e.l.i.s.a. assays, polystyrene micro-titre plates (Dynatech Laboratories) were coated overnight at 4 °C with apo E contained in the VLDL fraction purified either from rat serum or from incubation medium. The proportion of protein in apo E, apo B_L and apo B_H in the standard VLDL preparation from rat serum was determined from the relative intensities of the bands at 630 nm after SDS/PAGE followed by staining with Coomassie Brilliant Blue G-250 [37]. The VLDL was diluted in 0.5 M-Na₂CO₃ adjusted to pH 9.6 with HCl and containing

Fig. 1. Standard immunotitration curves for apo B_{H+L} and apo E

The curves for apo B_{H+L} (■) and for apo E (□) were established as described in the Experimental section and by incubating the alkaline phosphatase reaction for 15 h and 1 h respectively.

0.1 % NaN₃. Unoccupied protein-binding sites were saturated by incubating the wells for 4 h at room temperature with 1 % (w/v) BSA in 10 mM-phosphate buffer, pH 7.4, containing 0.15 M-NaCl and 0.05 % Tween 20. The BSA solution was removed, antibody against apo E or apo B was added, and the plates were incubated for 2 h at room temperature. The wells were then washed three times with 10 mM-potassium phosphate buffer, pH 7.4, containing 0.15 M-NaCl and 0.05 % Tween 20. The second antibody, which was either goat anti-(rabbit IgG) or goat anti-(mouse IgG), both conjugated to alkaline phosphatase, was then applied to the wells in 200 µl of the same buffer, and the plates were incubated for 2 h at room temperature. The wells were washed three times with the same buffer, and 0.67 mM-*p*-nitrophenyl phosphate in 10 % (w/v) diethanolamine (adjusted to pH 9.5 with HCl) containing 0.5 mM-MgCl₂ was added. The A₄₀₅ of the wells were determined after 1–15 h of incubation at 22 °C for apo E, or at 37 °C for apo B, during which time the alkaline phosphatase reaction rate was constant. The concentrations of apo E and apo B_{H+L} in VLDL from the incubation media were calculated from the slopes of the regression lines

obtained with five concentrations of secreted VLDL, compared with those for standard VLDL (Fig. 1).

Measurement of the relative size of the VLDL particles

Hepatocytes were incubated in the absence or presence of insulin and dexamethasone as described above and for the last 8 h with 1 mM-[³H]oleate (16.6 Ci/mol). The combined medium (5 ml) was concentrated by about 5-fold in a dialysis bag by exposure to dry Sephadex G-100. Samples (0.5 ml) were then applied to a column (1.6 cm × 32 cm) of Bio-Gel A-150M agarose and eluted in 1 ml fractions by using f.p.l.c. at 0.16 ml/min with 0.16 mM-NaCl containing 0.01 % NaN₃ and 0.1 % Na⁺ EDTA, pH 7.4. Lipids were extracted [34], but without adding H₃PO₄ and KCl and by using olive oil as a carrier [35]. Samples of the bottom phase of these extractions were diluted with 2 ml of chloroform, and then shaken with 1 g of basic alumina to remove residual oleate [28]. Portions (2 ml) of the chloroform extract were dried and ³H was determined by scintillation counting.

Determination of protein concentration and lactate dehydrogenase activity

These were measured as described previously [6].

Expression of results and calculation of statistical significance

All results have been calculated relative to the units of lactate dehydrogenase present in the hepatocytes. This compensates for small variations among dishes of cells better than does the determination of protein or DNA [27,28], since some non-viable cells may remain attached to the plate and contribute to the last two values. Loss of viability is accompanied by leakage of lactate dehydrogenase into the incubation medium. The content of lactate dehydrogenase per dish of cells was 1.0 ± 0.1 ($n = 9$) µmol of lactate oxidized/min, equivalent to 1 unit of activity at 22 °C. One dish of cells contains about 1 mg of protein, so that results can be readily expressed relative to protein if this is required for comparison with other work. The present incubations also contained about 13 mg of BSA/ml, and therefore the measurement of the relatively small concentration of hepatocyte protein might have been inaccurate for the routine determination of cell number. All results throughout the paper are given as means ± S.E.M. for the numbers of independent experiments with hepatocytes from different rats that are shown in parentheses. *P* values were calculated by using a paired *t* test. In some instances the error bars in the Figures were too small to be indicated.

Fig. 2. Effects of insulin and dexamethasone on the incorporation of triacylglycerol into VLDL secreted by rat hepatocytes

Hepatocytes were preincubated for 15 h in the absence (□) or presence (■) of 100 nM-dexamethasone and the indicated concentration of insulin. The cells were then incubated with 1 mM-[³H]oleate (1.66 µCi/ml) for a further 8 h under the same hormonal conditions. The incorporations of [³H]oleate into secreted triacylglycerol (a) and the relative mass of triacylglycerol (b) are expressed relative to the control incubation where no hormone was added. There were 3–6 independent experiments for the different conditions. The absolute value for the incorporation of [³H]oleate into secreted triacylglycerols over 8 h in the absence of hormones was 7.4 ± 0.35 ($n = 3$) nmol/unit of lactate dehydrogenase, whereas the secretion of triacylglycerol was 47.9 ± 6.1 ($n = 6$) nmol/unit of lactate dehydrogenase.

RESULTS

Effects of dexamethasone and insulin on the secretion of triacylglycerol by rat hepatocytes

Preincubation of the hepatocytes with dexamethasone alone increased ($P < 0.025$) the incorporation of [^3H]oleate into the secreted triacylglycerol by about 2.7-fold, whereas the mass of triacylglycerol secreted was increased ($P < 0.005$) by about 1.5-fold (Fig. 2). This apparent discrepancy probably reflects the preferential secretion of newly formed versus existing triacylglycerol, as observed previously [38]. Insulin at 10 nM and 50 nM decreased ($P < 0.025$) the [^3H]triacylglycerol secretion by 35% in each case, and decreased the mass of triacylglycerol in the medium by 38 and 60% respectively. Insulin (2–50 nM) also reversed ($P < 0.01$) the stimulating effects of dexamethasone (Fig. 2). These results are similar to those reported previously [6,7].

Effects of insulin and dexamethasone on protein synthesis and albumin secretion

Dexamethasone decreased ($P < 0.005$) the incorporation of [^3H]leucine into the proteins of the hepatocytes by about 24% (Fig. 3a). By contrast, insulin (2 nM) increased ($P < 0.025$) this incorporation by 40–70%, and this increase was maintained ($P < 0.05$) in the presence of 100 nM-dexamethasone with 10 nM- and 50 nM-insulin (Fig. 3a). The appearance of [^3H]albumin in the bottom fraction obtained by ultracentrifugation of the incubation medium was not significantly affected by dexamethasone or insulin alone, but was increased by 2.1–2.6-fold when insulin was present with dexamethasone (Fig. 3b).

Effects of insulin and dexamethasone on the secretion of apo E, apo B_L and apo B_H in VLDL

Incubation of the hepatocytes with dexamethasone increased ($P < 0.05$) the secretion of apo E (7.1-fold), apo B_H (3.6-fold) and apo B_L (4.0-fold) in the VLDL fraction (Figs. 4a–4b) as measured by the incorporation of [^3H]leucine. Conversely, the secretion of the three apolipoproteins was inhibited ($P < 0.025$) by insulin in a dose-dependent manner. At 50 nM, insulin inhibited the secretion of apo E, apo B_H and apo B_L by 68, 52 and 48% respectively (Figs. 4a–4c).

The interaction of insulin with dexamethasone depended on the concentration of insulin that was used. At 2 nM-insulin there was a further stimulation of apo B secretion above that obtained with dexamethasone alone (Figs. 4a–4b). The increases in the

presence of the two hormones were 4.4-fold ($P < 0.05$) and 7.2-fold ($P < 0.005$) for apo B_H and apo B_L respectively (Figs. 4a–4b). However, there was no synergistic effect of insulin for the secretion of apo E. With 10 nM- and 50 nM-insulin these synergistic effects for apo B_L and apo B_H were lost, and the stimulations were not significantly different from those obtained with dexamethasone alone, probably because of the relatively large S.E.M. for the incorporations with dexamethasone alone. This degree of variation is to be expected when hepatocytes prepared from different animals are being used. However, the inhibitory effects of the higher insulin concentrations can be seen when the results with 2 nM-insulin are compared with those with 50 nM-insulin, both in the presence of dexamethasone. In the latter instance, the secretions of apo E, apo B_H and apo B_L were decreased significantly ($P < 0.025$, $P < 0.05$ and $P < 0.05$ respectively).

The masses of apo E and apo B_{H+L} were determined by immunoassay. The results are essentially compatible with the effects observed with [^3H]leucine incorporation. Insulin alone decreased ($P < 0.05$) the secretion of apo E (Fig. 4d), whereas dexamethasone showed a striking increase ($P < 0.025$). The addition of 2 nM-insulin with dexamethasone produced no significant change in apo E secretion, as was found with [^3H]leucine incorporation. Increasing insulin concentrations to 10 nM and 50 nM significantly antagonized the stimulation produced by dexamethasone. This latter effect appeared to be even more dramatic than that seen with the incorporation of [^3H]leucine. However, it should be stressed that entirely different preparations of hepatocytes were used for the mass measurements, and so a direct comparison is not possible. We have also observed differences in the sensitivity to insulin with different hepatocyte preparations with respect to the secretion of [^3H]apolipoproteins. It seems likely that this sensitivity was particularly high in the three preparations used for the immunotitration experiments, in light of the dramatic reversal of the dexamethasone effect on apo E secretion by insulin. However, the results do confirm the [^3H]leucine measurements, in that dexamethasone markedly stimulates apo E secretion whereas insulin decreases it and antagonizes the effects of dexamethasone.

The rate of apo B_{H+L} secretion in the absence of hormones was 35 ± 12 ($n = 3$) ng/unit of lactate dehydrogenase in 8 h. Dexamethasone increased ($P < 0.05$) this secretion by 5.3 ± 1.4 - and 5.5 ± 1.5 -fold in the absence and presence of 2 nM-insulin respectively. In this case the apparently higher secretion of apo B mass in the presence of dexamethasone and 2 nM-insulin was not

Fig. 3. Effects of insulin and dexamethasone on the incorporation of [^3H]leucine into total proteins of rat hepatocytes and into albumin in the medium

Rat hepatocytes were incubated for 15 h in the absence (□) or presence (■) of 100 nM-dexamethasone with the concentrations of insulin indicated. The cells were then incubated for a further 8 h under the same hormonal conditions with 50 μM -[^3H]leucine (about 43 $\mu\text{Ci}/\text{ml}$). The recovery of ^3H -labelled protein in the hepatocytes (a) and of albumin in the bottom fraction of density > 1.18 g/ml obtained from the medium after centrifugation (b) is expressed relative to that in the absence of dexamethasone and insulin. There were 3–8 independent experiments for each condition. The absolute rates of incorporation of [^3H]leucine into the protein of the hepatocytes and into albumin of the bottom fraction of the medium in the absence of hormones were 10800 ± 1700 ($n = 8$) and 315 ± 65 ($n = 7$) pmol/unit of lactate dehydrogenase in 8 h respectively.

Fig. 4. Effects of insulin and dexamethasone on the secretion by rat hepatocytes of apo E, apo B_H and apo B_L in the VLDL fraction

Rat hepatocytes were incubated in the absence (□) or presence (■) of 100 nM-dexamethasone as described in Fig. 2. The recoveries of [³H]leucine in apo B_H (a), apo B_L (b) and apo E (c) in the VLDL fraction obtained after ultracentrifugation are expressed relative to the values obtained in the absence of insulin and dexamethasone. There were 3–8 independent experiments for each condition. The absolute incorporations of [³H]leucine into apo E, apo B_H and apo B_L in the control incubations in the absence of hormones were 1.97 ± 0.43 , 0.65 ± 0.12 and 0.85 ± 0.17 ($n = 9$) pmol of leucine incorporated/unit of lactate dehydrogenase in 8 h respectively. Apo E mass was determined by immunotitration (d); 20.9 ± 9.3 ($n = 6$) ng of apo E/unit of lactate dehydrogenase was secreted after 8 h in the incubations performed in the absence of hormones.

statistically significant. However, there were only three independent experiments, compared with five for leucine incorporation. Also, apo B_H and apo B_L were measured together by immunotitration rather than separately. With the [³H]leucine incorporation the major effect was observed with the incorporation of apo B_L. Furthermore, the results for apo E secretion (Fig. 4d) indicate a high insulin-sensitivity of the three preparations of hepatocytes used for the mass measurements and a possible displacement of the insulin-response curve to the left. Despite the lack of significant synergism between 2 nM-insulin and dexamethasone from the mass measurements, the latter results do confirm the substantial effect of dexamethasone in stimulating the secretion of apo B_{H+L} in VLDL. With 2 nM-insulin alone the secretion of apo B_{H+L} was 64 ± 21 % of the control value, but with only three experiments this was not statistically different. However, the inhibition of apo B secretion from rat hepatocytes by insulin has been demonstrated in other work [11,13,15,39]. The major reason for measuring apo B mass in the present work was to confirm the stimulation produced by dexamethasone.

Apo B_H, apo B_L and apo E were also detected in the fraction that was isolated from the incubation medium in the density range of LDL. The incorporation of [³H]leucine into apo B_H, apo B_L and apo E of the LDL fraction for the control incubation was 0.51 ± 0.12 , 0.62 ± 0.11 and 0.89 ± 0.21 ($n = 9$) pmol/unit of lactate dehydrogenase respectively. These values were 78 %, 73 % and 45 % respectively of the incorporations into the equivalent apolipoproteins isolated in the VLDL fraction (see legend to Fig. 4). The effects of dexamethasone and insulin on these secretions in the density range of LDL followed similar patterns to those observed for VLDL. However, the changes were smaller in magnitude. For example, the stimulations in the

Fig. 5. Effects of insulin and dexamethasone on the size of the triacylglycerol-rich particles secreted by rat hepatocytes

Rat hepatocytes were incubated as described in Fig. 2, except that the specific radioactivity of the [³H]oleate was 16.6 Ci/mol. The incubation medium from four dishes was concentrated and chromatographed on a column (1.6 cm × 32 cm) of Bio-Gel A-150 M agarose. The [³H]triacylglycerol in the fractions was determined and expressed as d.p.m./ml of original incubation medium after taking into account the extent of the concentration of the incubation medium. The additions of hormones are indicated by: □, none; ■, 2 nM-insulin; ◆, 100 nM-dexamethasone; ◇, 2 nM-insulin plus 100 nM-dexamethasone. Large quantities of unesterified [³H]oleate and albumin were eluted from the column after fraction 36 (results not shown). The values are from a representative experiment that was repeated two more times. The separation range given by the manufacturer for Bio-Gel A-150 M is (1–150) × 10⁶. The column was partially calibrated with samples of Mengo virus, for which the centre of the elution peak was at fraction 33.

Fig. 6. Effects of insulin and dexamethasone on the secretion by rat hepatocytes of apo A_I, apo A_{II} plus apo C, apo A_{IV} and apo E in HDL

Rat hepatocytes were incubated in the absence (□) or presence (■) of 100 nM-dexamethasone as described in Fig. 2. The recoveries of [³H]leucine in apo A_I (a), apo A_{II} plus apo C (b), apo A_{IV} (c) and apo E (d) are expressed relative to the values obtained in the absence of insulin and dexamethasone. There were 3–7 independent experiments for each condition. The absolute incorporations of [³H]leucine into these apolipoproteins in the absence of hormones were 13.7 ± 2.0 ($n = 7$), 15.8 ± 3.6 ($n = 6$), 24.3 ± 5.2 ($n = 7$) and 44.5 ± 10.5 ($n = 7$) pmol/unit of lactate dehydrogenase respectively in 8 h.

appearance of ³H-labelled apo B_H, apo B_L and apo E in LDL in the presence of 100 nM-dexamethasone were 1.5-, 1.6- and 1.9-fold, compared with 3.6-, 2.4- and 7.1-fold respectively in VLDL. There was no evidence for the hormones causing a significant shift of the particles between the VLDL and LDL density ranges.

Effects of insulin and dexamethasone on the sizes of VLDL particles

The results in Figs. 2 and 4 show that the ratio of triacylglycerol to apo B_{H+L} secreted in VLDL was changed by treatment of the hepatocytes with insulin and dexamethasone, which suggested that there were changes in the sizes of the VLDL particles in response to hormones. This was therefore investigated by labelling the triacylglycerol in the VLDL with [³H]oleate. Pre-incubation with dexamethasone, in the absence or presence of insulin, caused an increased secretion of both large and small particles (Fig. 5). An impression of the size of the secreted lipoproteins is obtained by reference to Mengo virus, which has a diameter of 30 nm and an apparent $M_r = 8.4 \times 10^6$ [40]. This was eluted with a peak in fraction 33.

Effects of insulin and dexamethasone on the secretion of apolipoproteins in HDL and in the fraction of density > 1.18 g/ml

There was no significant change in the secretion of the various apolipoproteins associated with HDL when the hepatocytes were incubated with insulin alone (Fig. 6). Dexamethasone alone also

had little significant effect, although there was a significant 37% increase ($P < 0.05$) in the secretion of [³H]apo A_{IV}. However, with dexamethasone plus 2 nM-insulin there were larger increases of 77%, 55%, 74% and 104% for the fractions containing apo A_I, apo A_{II} plus apo C, apo A_{IV} and apo E ($P < 0.01$, $P < 0.05$, $P < 0.005$ and $P < 0.025$ respectively). Similarly, significant increases were seen when 10 nM- or 50 nM-insulin was added with dexamethasone (Fig. 6).

The amount of apo E in the bottom fraction was even higher

Table 1. Distribution of apo E in fractions obtained from the incubation medium of rat hepatocytes

Rat hepatocytes were incubated as described in Fig. 2. The incubation medium was fractionated by ultracentrifugation and the secretion of apo E was determined by measuring the incorporation of [³H]leucine into apo E isolated by PAGE. The results are for seven independent incubations performed in the absence of insulin and dexamethasone.

Fraction	Leucine incorporated (pmol/unit of lactate dehydrogenase)	Distribution of apo E (%)
VLDL	2.16 ± 0.54	1.4
LDL	0.93 ± 0.27	0.6
HDL	44.5 ± 10.5	29.1
Bottom fraction	105 ± 26	68.6
Total	153 ± 30	100

than that in the HDL fraction. For example, in the incubations that did not contain insulin or dexamethasone an average of 69 % of the total [^3H]apo E was in the bottom fraction, compared with about 29 % in the HDL fraction (Table 1). The addition of insulin and dexamethasone separately did not significantly alter the total secretion of apo E. However, when added together these hormones stimulated ($P < 0.05$) total apo E secretion to a similar extent (results not shown) to that in Fig. 6 for the HDL fraction.

DISCUSSION

Effects of hormones on VLDL secretion

The most striking novel findings from this work are the relatively large increases of 7.1-, 4.0- and 3.6-fold in the secretions in VLDL of apo E, apo B_L and apo B_H respectively when the hepatocytes were exposed to 100 nM-dexamethasone (Fig. 4). The secretions of apo B_H , and particularly apo B_L , were further enhanced by the presence of 2 nM-insulin in the incubations. However, insulin alone inhibited the secretion of the three apolipoproteins in VLDL, and at 50 nM partly reversed the stimulatory effects of dexamethasone. These results were obtained by labelling of apolipoproteins with [^3H]leucine. Similarly, the results from the measurements of the masses of apo B_{H+L} and apo E were compatible with the effects demonstrated by the leucine incorporations. Insulin and dexamethasone, either together or alone, have little significant effect on the synthesis of triacylglycerols in the hepatocyte system [6], and therefore their effects appear relatively selective for the secretion process.

Changes in apolipoprotein secretion in VLDL were not simply non-specific effects on protein synthesis, because dexamethasone decreased the accumulation of total protein labelled with [^3H]leucine in the hepatocytes, and insulin had the opposite effect (Fig. 3), as expected. Furthermore, the effects on the secretion of apolipoproteins in VLDL did not follow changes in overall protein secretion, since that of [^3H]albumin was not significantly changed by incubating the hepatocytes separately with insulin or dexamethasone. However, incubation with both hormones in combination increased albumin secretion by 2.1–2.6-fold (Fig. 3). Higher concentrations of insulin did not reverse the effects of dexamethasone.

The inhibition of apo B secretion by insulin that we observed resembles that described by other investigators [11,13,15,39]. However, Sparks *et al.* [15] failed to demonstrate a significant stimulation of apo B secretion when they incubated hepatocytes for 12–14 h with 100 nM-cortisol. This concentration of natural glucocorticoid is at the low end of the physiological range, since the basal concentration of corticosterone in unstressed rats varies between about 50 and 400 nM, depending upon the diurnal cycle [41]. The addition of 100 nM-corticosterone to rat hepatocytes fails to increase the activity of phosphatidate phosphohydrolase, but an increase can be readily seen with 1 μM - and 10 μM -corticosterone [42]. It is probable that higher concentrations of corticosterone were required because hepatocytes degrade glucocorticoids. The present work employed a synthetic glucocorticoid, dexamethasone, which is relatively resistant to degradation. At the chosen concentration of 100 nM it stimulates the activities of tyrosine aminotransferase and phosphatidate phosphohydrolase [27,28,42], increases triacylglycerol secretion [6], and decreases the binding and degradation of LDL by hepatocytes [29,30,43].

The mechanism by which dexamethasone stimulates apo B secretion (Fig. 4) is not known. The present work clearly demonstrates hormonal effects on VLDL secretion, although information on total apolipoprotein synthesis is not provided. Pullinger *et al.* [39] claimed that the apo B gene in Hep G2 cells

is constitutively expressed and that regulation must involve co- or post-translational processes. However, these authors failed to observe an effect of 1 μM -dexamethasone on apo B secretion. Furthermore, neither glucagon nor 8-bromo cyclic AMP inhibited apo B secretion [39], although these agents will inhibit VLDL secretion by rat hepatocytes [20,21]. Pullinger *et al.* [39] raised the possibility that Hep G2 cells may not be responsive to glucagon in the absence of phosphodiesterase inhibitors. It is also possible that Hep G2 cells do not respond to glucocorticoids or control VLDL secretion in the same way as do primary cultures of rat hepatocytes.

Effects of insulin and dexamethasone on the secretion of HDL and albumin

The secretion of apolipoproteins in the HDL fraction showed a response to insulin and dexamethasone (Fig. 6) that differed from that for VLDL (Fig. 4). These results demonstrate distinct mechanisms of hormonal control for these two lipoprotein classes. The secretion in HDL of apo A_I , apo A_{II} plus apo C, apo A_{IV} and apo E all responded in a similar way to the effects of dexamethasone and insulin. Individually these hormones had little effect on the secretion of the apolipoproteins in HDL. However, when added together the two hormones stimulated apolipoprotein secretion in HDL by 1.5–2-fold (Fig. 6). This pattern of response was similar to that for the secretion of albumin (Fig. 3b). In other work with rat hepatocytes, insulin above 100 pM stimulated albumin secretion, and at concentrations above 10 nM inhibited apo A_I secretion [23]. Part of the discrepancy between that and our work may have been caused by the preincubation of all hepatocytes with 1 μM -dexamethasone when they were plated by Masumoto *et al.* [23]. It is feasible that this high dexamethasone concentration could have produced persistent effects, possibly by retention of dexamethasone in the cells. In that case the subsequent insulin effect may have been caused by an interaction with dexamethasone, as seen in Fig. 5. However, if this were the case, a stimulation of apo A_I secretion would have been expected, but this was not found by Masumoto *et al.* [23]. It should also be noted that Crane & Miller [44] observed a stimulation of albumin secretion when insulin was added alone to cultures of rat hepatocytes. We therefore cannot fully explain the differences between our work and that of others on the effects of insulin on the secretion of albumin and apo A_I , except to say that the experimental conditions, including the presence of oleate in our culture media, were different from the work of others [23].

The effects of dexamethasone in Fig. 3 are compatible with those found by Crane & Miller [44], who reported no change in albumin secretion when hepatocytes were incubated with 78 μM -cortisol. Similarly, Masumoto *et al.* [23] showed no significant effect on the secretion of albumin and apo A_I at 100 nM-dexamethasone. However, apo A_I secretion was increased when the concentration of dexamethasone was above 1 μM .

Lin [24] showed that incubating hepatocytes with either 1 μM -insulin or 1 μM -dexamethasone separately for 20 h had little effect on the secretion of apo A_I . Insulin alone had little effect on apo E secretion, and dexamethasone alone decreased this process. However, when the hormones were added together there was a synergistic stimulation of the secretion of apo E and apo A_I . Our results obtained from a 16 h preincubation with hormones resembled those of Lin [24], except that we did not see inhibition of apo E secretion with dexamethasone. Lin [24] also extended the incubation period with hormones to 44 h. Insulin and dexamethasone each separately stimulated the secretion of apo A_I and apo E. Synergism between the hormones was seen in the secretion of apo A_I and apo E, but the effect on apo A_{IV} secretion was only marginal. Those discrepancies that do exist between our

work and that of Lin [24] probably relate to the period of exposure to hormones and the fact that Lin [24] used concentrations of dexamethasone and insulin that were, respectively, 10- and 20–500-fold higher.

The mechanism by which insulin and glucocorticoids control the secretion of apolipoproteins associated with HDL is incompletely understood. However, it has been shown that insulin and dexamethasone, either separately or together, can increase the concentration of the mRNA for apo A_I and apo A_{IV}, but not that of apo E [45]. Mangeney *et al.* [46] concluded that the expression of the apo E gene is controlled by the glucagon/insulin ratio.

Control of apo E secretion

The majority of apo E secreted from the hepatocytes was probably not associated with lipoproteins, as has been shown from previous work [47,48]. In our studies (Table 1) 69% and 29% of the apo E was recovered in the bottom and HDL fractions respectively, with only 1.4% in the VLDL fraction.

Fasting [49,50] and refeeding [50] rats with carbohydrate (which may involve hormonal regulation) increases the production of apo E by the liver [43]. The increased production of apo E after fasting [49], or sucrose feeding [50], was accompanied by increases in apo E mRNA. However, addition of insulin and dexamethasone, separately or together, to hepatocyte cultures had no significant effects on the concentration of apo E mRNA [50]. Our own work shows no significant effects of these hormones when added separately on the total secretion of apo E, but increases ($P < 0.05$) of about 2-fold were obtained where these hormones were added together. The larger increases in apo E secretion in VLDL were probably a consequence of the increased secretion of VLDL particles and a corresponding increase in the association of apo E with these particles.

General effects of insulin and glucocorticoids on lipoprotein metabolism

The results in Figs. 2 and 4 confirm previous results [1–8] showing that glucocorticoids stimulate the secretion of VLDL. However, the major contribution of the present work is that the secretion of apolipoproteins in VLDL (Fig. 4) was increased by dexamethasone to an even greater extent than that of the triacylglycerol (Fig. 2). Furthermore, although dexamethasone stimulated the secretion of both large and small VLDL particles (Fig. 5), a low concentration of insulin (2 nM) further enhanced the secretion of apo B_L (Fig. 3b).

Synergistic stimulations between insulin and glucocorticoids also occur for the synthesis of glycogen [51] and fatty acids [52–56] and the activity of lipoprotein lipase in adipose tissue [57]. These reactions are all involved in energy storage, and the combined increase in both insulin and glucocorticoids which occurs in the circulation after meals [58–60] probably provides a signal for energy deposition [56,61]. This same signal could be used for stimulating VLDL secretion, except that we observed a statistically significant synergism (in the sense of a mutually co-operative effect) only with respect to the secretion of apo B_H, and particularly of apo B_L (Fig. 3b), but not for apo E (Figs. 4c and 4d) and triacylglycerols (Fig. 2). However, we do not know the mechanism by which 2 nM-insulin was apparently increasing the effectiveness of the dexamethasone in terms of apo B_L secretion, and further work on the interactions of glucocorticoids and insulin on VLDL secretion is warranted.

The results in Fig. 4 demonstrate that insulin can inhibit the secretion of apolipoproteins associated with VLDL. By contrast, 2 nM-insulin also stimulated the secretion of ³H-labelled apo B_H and apo B_L provided that dexamethasone was also present. In

other work where insulin stimulated VLDL secretion [18,19], fresh liver was perfused with whole blood. It is possible that the effects of glucocorticoids initiated *in vivo* could have also 'permitted' insulin to produce this stimulation. Furthermore, there may have been some residual glucocorticoids in the blood used for these perfusions, although the blood was extensively dialysed. Insulin has been proposed to produce two opposing effects on VLDL secretion: first to inhibit this process, but secondly to increase triacylglycerol availability [62]. This observation and the possible interaction with glucocorticoids could provide a partial reconciliation for the two diametrically opposing views of insulin action. The inhibitory effects of insulin are compatible with the decrease in the hepatic release of VLDL observed in rats injected with insulin [63,64] and in humans with high portal insulin concentrations [65]. Furthermore, subcutaneous insulin infusion in humans suppresses hepatic triacylglycerol output [66]. Thus, insulin might decrease VLDL secretion in the post-prandial period, when triacylglycerol is being supplied to peripheral tissues via chylomicrons [10,15,44,67]. Conversely, as insulin concentrations decrease and glucocorticoid concentrations rise, VLDL secretion would increase to replace chylomicrons as a source of fatty acids to muscle and adipose tissue.

The effects of insulin and glucocorticoids in controlling VLDL secretion are also important in understanding the development of hypertriglyceridaemia. This abnormality has been thought to be a result of the effects of insulin, since hyperinsulinaemia and hypertriglyceridaemia are often related [68–70]. However, an alternative explanation is that the hypertriglyceridaemia is caused by a lack of insulin action on the liver as a result of insulin-resistance and an enhanced glucocorticoid action [42,45]. The present study emphasizes that glucocorticoids stimulate the secretion of VLDL particles. Insulin can suppress this process, but at low concentrations may act synergistically, as seen with apo B_H and apo B_L (Figs. 4a and 4b).

The observed glucocorticoid-induced increase in the secretion of apo B in VLDL would be expected to result in an increased number of VLDL particles secreted, since there is assumed to be 1 molecule of apo B per VLDL particle [71]. Consequently, efficient insulin control in the liver could decrease the availability of VLDL particles in the circulation that ultimately will be converted to intermediate-density lipoprotein and LDL. Glucocorticoids would produce the opposite effect by increasing LDL production. The accumulation of LDL particles would also be increased by these latter hormones, since they decrease the availability of LDL receptors at the hepatocyte surface and the subsequent uptake of LDL [29,30,42]. In addition, glucocorticoids suppress the stimulatory action of insulin in this respect [29,30,42]. The combined results further strengthen the hypothesis that an increased control of metabolism by glucocorticoids can predispose humans to premature atherosclerosis [61]. However, the situation may be further complicated by the possible synergism between insulin and glucocorticoids in stimulating both apo B secretion, which is potentially atherogenic, and HDL secretion, which should be protective. It now seems important to investigate the interactions of insulin and glucocorticoids in insulin-resistant states.

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REFERENCES

1. Reaven, E. P., Kolterman, C. L. & Reaven, G. M. (1974) *J. Lipid Res.* **15**, 74–83
2. Krausz, Y., Bar-On, H. & Shafir, E. (1981) *Biochim. Biophys. Acta* **663**, 69–82
3. Taskinen, M. R., Nikkilä, E. A., Pelkonen, R. & Sane, T. (1983) *J. Clin. Endocrinol. Metab.* **57**, 619–626
4. Klausner, H. & Heimberg, M. (1967) *Am. J. Physiol.* **212**, 1236–1246
5. Cole, T. G., Wilcox, H. G. & Heimberg, M. (1982) *J. Lipid Res.* **23**, 81–91
6. Mangiapane, E. H. & Brindley, D. N. (1986) *Biochem. J.* **233**, 151–160
7. Bartlett, S. M. & Gibbons, G. F. (1988) *Biochem. J.* **249**, 37–43
8. Duerden, J. M., Bartlett, S. M. & Gibbons, G. F. (1989) *Biochem. J.* **262**, 313–319
9. Nikkilä, E. A. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tystrup, N., eds.), pp. 306–378, Munksgaard, Copenhagen
10. Durrington, P. N., Newton, R. S., Weinstein, A. D. & Steinberg, D. (1982) *J. Clin. Invest.* **70**, 63–73
11. Patsch, W., Franz, S. & Schonfeld, G. (1983) *J. Clin. Invest.* **71**, 1161–1174
12. Pullinger, G. L. & Gibbons, G. F. (1985) *Biochim. Biophys. Acta* **833**, 44–51
13. Sparks, C. F., Sparks, J. D., Bolognino, M., Salhanick, A., Strumph, P. S. & Amatruda, J. M. (1986) *Metab. Clin. Exp.* **35**, 1128–1136
14. Woodside, W. F. & Heimberg, M. (1976) *J. Biol. Chem.* **251**, 13–23
15. Sparks, J. D., Sparks, C. E. & Miller, L. L. (1989) *Biochem. J.* **261**, 83–88
16. Beynen, A. C. & Geelen, M. J. H. (1982) *Vet. Res. Commun.* **5**, 223–236
17. Haagsman, H. P. & Van Golde, L. M. G. (1984) *Vet. Res. Commun.* **8**, 157–171
18. Topping, D. L. & Mayes, P. A. (1972) *Biochem. J.* **126**, 295–311
19. Laker, M. E. & Mayes, P. A. (1984) *Biochim. Biophys. Acta* **795**, 427–430
20. Kempen, H. J. M. (1980) *J. Lipid Res.* **21**, 671–680
21. Gibbons, G. F. & Pullinger, C. R. (1987) *Biochem. J.* **243**, 487–492
22. Brindle, N. P. J. & Ontko, J. A. (1988) *Biochem. J.* **250**, 363–368
23. Masumoto, A., Koga, S., Uchida, E. & Ibayashi, H. (1988) *Atherosclerosis* **70**, 217–223
24. Lin, R. C. (1988) *Metab. Clin. Exp.* **37**, 745–751
25. Hamilton, R. L., Guo, S. S., Felker, R. E., Chao, Y.-S. & Havel, R. M. (1986) *J. Lipid Res.* **27**, 967–978
26. Yao, Z. & Vance, D. E. (1988) *J. Biol. Chem.* **263**, 2998–3004
27. Pittner, R. A., Fears, R. & Brindley, D. N. (1985) *Biochem. J.* **230**, 525–534
28. Pittner, R. A., Fears, R. & Brindley, D. N. (1986) *Biochem. J.* **240**, 253–257
29. Salter, A., Fisher, S. C. & Brindley, D. N. (1987) *FEBS Lett.* **220**, 159–162
30. Salter, A., Fisher, S. C. & Brindley, D. N. (1988) *Atherosclerosis* **71**, 77–80
31. Cascales, C., Mangiapane, E. H. & Brindley, D. N. (1984) *Biochem. J.* **219**, 911–916
32. Vance, D. E., Weinstein, D. B. & Steinberg, D. (1984) *Biochim. Biophys. Acta* **792**, 39–47
33. Oschry, Y. & Eisenberg, S. (1982) *J. Lipid Res.* **23**, 1099–1106
34. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
35. Graham, A., Bennett, A. J., McLean, A. A. M., Zammit, V. A. & Brindley, D. N. (1988) *Biochem. J.* **253**, 687–692
36. Davis, R. A., Chan, D. C. F., Thompson, J. J., Borchardt, R. A. & Gallaher, W. R. (1989) *J. Lipid Res.* **30**, 1185–1196
37. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Appendix I, Cold Spring Harbor Laboratory, New York
38. Ide, T. & Ontko, J. A. (1981) *J. Biol. Chem.* **256**, 10247–10255
39. Pullinger, C. E., North, J. D., Teng, B.-B., Rifci, V. A., Ronhild de Brito, A. E. & Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1076
40. Luo, M., Vriend, G., Kamer, G., Minor, I., Arnold, E., Rossmann, M. G., Bolge, U., Scraba, D. G., Duke, G. M. & Palmenberg, A. C. (1987) *Science* **235**, 182–191
41. Knox, A. M., Sturton, R. G., Cooling, J. & Brindley, D. N. (1979) *Biochem. J.* **180**, 441–443
42. Jennings, R. J., Lawson, N., Fears, R. & Brindley, D. N. (1981) *FEBS Lett.* **133**, 119–122
43. Brindley, D. N., Brown, N. F., Salter, A. M., Fisher, S. C. & Fears, R. (1989) *Biochem. Soc. Trans.* **17**, 43–46
44. Crane, L. J. & Miller, D. L. (1977) *J. Cell Biol.* **72**, 11–25
45. Elshourbagy, N. A., Boguski, M. S., Liao, W. S., Jefferson, L. S., Gordon, J. I. & Taylor, J. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8242–8246
46. Mangeney, M., Cardot, P., Lyonnet, S., Coupe, C., Benarous, R., Munnich, A., Girard, J., Chambaz, J. & Bereziat, G. (1989) *Eur. J. Biochem.* **181**, 225–230
47. Krul, E. S., Dolphin, P. J. & Rubinstein, D. (1981) *Can. J. Biochem.* **59**, 676–686
48. Davis, R. A., Druz, S. M., Leighton, J. K. & Brengaze, V. A. (1989) *J. Biol. Chem.* **264**, 8970–8977
49. Kim, M. H., Nakayama, R., Manos, P., Tomlinson, J. E., Choi, E., Ng, J. D. & Holton, D. (1989) *J. Lipid Res.* **30**, 663–671
50. Strobl, W., Gorden, N. L., Fienup, G. A., Lin-Lee, Y.-C., Gotto, A. M. & Patsch, W. (1989) *J. Biol. Chem.* **264**, 1190–1194
51. Whitton, P. D. & Hems, D. A. (1976) *Biochem. J.* **156**, 585–592
52. Diamant, S. & Shafir, E. (1975) *Eur. J. Biochem.* **53**, 541–546
53. Kirk, C. J., Verrinder, T. R. & Hems, D. A. (1976) *Biochem. J.* **156**, 593–602
54. Amatruda, J. M., Donahy, S. A. & Cheng, C. L. (1983) *Biochem. J.* **121**, 135–141
55. Minshall, M. & Strong, C. R. (1985) *Int. J. Biochem.* **17**, 529–532
56. Al-Sieni, A. I. I., Plested, C. P., Rolland, Y. & Brindley, D. N. (1989) *Biochem. Pharmacol.* **38**, 3661–3667
57. Ashby, P. & Robinson, D. S. (1980) *Biochem. J.* **185**, 185–192
58. Quigley, M. E. & Yen, S. S. C. (1979) *J. Clin. Endocrinol. Metab.* **49**, 945–947
59. Slag, M. F., Ahmed, M., Gannon, M. C. & Nutall, F. O. (1981) *Metab. Clin. Exp.* **30**, 1104–1108
60. Follenius, M., Brandenberger, G., Hielter, B., Siméoni, M. & Reinhard, B. (1982) *J. Clin. Endocrinol. Metab.* **55**, 757–761
61. Brindley, D. N. & Rolland, Y. (1989) *Clin. Sci.* **77**, 453–461
62. Duerden, J. M., Bartlett, S. M. & Gibbons, G. F. (1989) *Biochem. J.* **263**, 937–943
63. Penhos, J. C., Wu, C. H., Lemberg, A., Daunas, J., Brodoff, B., Sodero, A. & Levine, R. (1968) *Metab. Clin. Exp.* **17**, 246–259
64. Alcindor, L. G., Infante, R., Soler-Argilaga, C. & Polonovski, J. (1973) *Biochim. Biophys. Acta* **306**, 347–352
65. Vogelberg, K. H., Gries, F. A. & Moschinski, D. (1980) *Horm. Metab. Res.* **12**, 688–694
66. Pietri, A. O., Dunn, F. L., Grundy, S. M. & Raskin, P. (1983) *Diabetes* **32**, 75–81
67. Gibbons, G. F. (1989) *Biochem. Soc. Trans.* **17**, 49–51
68. Yulkin, J. & Szanto, S. (1971) *B. Med. J.* **1**, 349
69. Sleder, J., Chen, Y.-D. I., Culley, M. D. & Reaven, G. M. (1980) *Metab. Clin. Exp.* **29**, 303–305
70. Tobey, T. A., Greenfield, M., Kraemer, F. & Reaven, G. M. (1981) *Metab. Clin. Exp.* **30**, 165–171
71. Elovson, J., Chatterton, J. E., Bell, G. T., Schumaker, V. N., Reuben, M. A., Puppione, D. L., Reeve, J. R. & Young, N. L. (1988) *J. Lipid Res.* **29**, 1461–1473

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