

Chronic exogenous hyperinsulinaemia does not modify the acute inhibitory effect of insulin on the secretion of very-low-density lipoprotein triacylglycerol and apolipoprotein B in primary cultures of rat hepatocytes

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Male Wistar rats were fitted with subcutaneous osmotic minipumps that delivered insulin at a constant rate of 0.20 i.u./h for 7 days. This treatment raised the plasma insulin concentration from 31 ± 4 to $201 \pm 64 \mu\text{-i.u./ml}$. Hepatocytes prepared from the hyperinsulinaemic animals secreted very-low-density lipoprotein (VLDL) triacylglycerol (TAG) at a higher rate ($172 \pm 21 \mu\text{g}$ per 24 h per mg cell protein) than did those from sham-operated controls ($109 \pm 12 \mu\text{g}$ per 24 h per mg) ($P < 0.05$). However, chronic exogenous hyperinsulinaemia had no stimulatory effect on the secretion of VLDL apolipoprotein B (apoB) in derived hepatocytes compared with those from the sham-operated controls (2.32 ± 0.38 compared with $3.09 \pm 0.40 \mu\text{g}$ per 24 h per mg). Hepatocytes from the hyperinsulinaemic rats thus secreted larger

VLDL particles as evidenced by the increased TAG:apoB ratio (78.4 ± 13.1 compared with 38.4 ± 7.6 ; $P < 0.05$). In hepatocytes from the hyperinsulinaemic rats a larger proportion of the newly synthesized TAG was secreted as VLDL. Hepatocytes from the hyperinsulinaemic and the sham-operated control animals were equally sensitive to the inhibitory effect of insulin added *in vitro* on the secretion of VLDL TAG. Insulin added *in vitro* to the culture medium of hepatocytes from hyperinsulinaemic animals significantly decreased the TAG:apoB ratio of the secreted VLDL. This change did not occur in hepatocytes from sham-operated rats. These results suggest that, *in vivo*, chronic hyperinsulinaemia is not in itself sufficient to desensitize the liver to the acute inhibitory effect of insulin on the secretion of VLDL.

INTRODUCTION

Pathophysiological states associated with chronic hyperinsulinaemia are usually accompanied by a decreased sensitivity of body tissues to the normal metabolic effects of insulin [1,2]. Although this resistance to insulin is normally assessed in terms of defective carbohydrate metabolism, the metabolism of lipids is also grossly disturbed [3,4]. At the hepatic level, the secretion of very-low-density lipoprotein (VLDL) is normally suppressed when liver is exposed for short periods to an elevated concentration of insulin. This hepatic effect of insulin occurs at all levels of biological organization and is apparent *in vivo* [5–7], in the intact perfused liver [8,9] and in cultured hepatocytes (reviewed in [10,11]). The inhibitory effect of insulin *in vivo* is not simply a result of a decreased flux of fatty acids to the liver secondary to suppression of adipose tissue lipolysis [5,7]. Although there is currently no direct evidence, it has been proposed that this inhibitory effect on the secretion of VLDL would occur maximally *in vivo* in the post-prandial state when plasma insulin levels are highest. This effect would then serve to limit the entry of hepatic VLDL into the plasma post-prandially (and thus to attenuate post-prandial lipaemia) when fat absorption from the gut increases [9,12]. Alternatively, or in addition, suppression of hepatic lipid secretion post-prandially could contribute to insulin's overall role in promoting lipid storage when food intake is high [10,13,14].

When the liver becomes insensitive to insulin, in states such as obesity and non-insulin-dependent diabetes mellitus, for instance, insulin is unable to act normally to suppress the output of glucose [15,16]. These conditions are associated with chronic hyperinsulinaemia and are accompanied by unusually high outputs of hepatic VLDL both in human subjects [4,17–20] and

in experimental animals [1,21]. The question therefore arises as to whether, under these circumstances, the high VLDL output is due at least in part to a failure of insulin to suppress the secretion of VLDL, as with glucose. Support for this idea comes from studies with animal models of insulin resistance in which insulin added *in vitro* was less effective in the suppression of VLDL secretion in hepatocyte cultures [22–24]. Insulin also failed to acutely suppress the secretion of hepatic apolipoprotein B (apoB) in obese subjects who were chronically hyperinsulinaemic [6]. Resistance to the normal acute inhibitory effect of insulin on the secretion of VLDL may also be induced *in vitro* by chronic exposure of hepatocytes from normal animals to elevated concentrations of insulin [25–28]. Because insulin resistance is almost invariably associated with chronic hyperinsulinaemia [1,2] the question arose as to whether the insensitivity of VLDL secretion to the inhibitory effect of insulin in hepatocytes from insulin-resistant animals was a direct secondary effect of chronic exposure of the liver to high insulin concentrations *in vivo*. The present study was designed to address this question by culturing hepatocytes from donor animals that had been fitted subcutaneously with osmotic minipumps. In this way insulin was delivered at a pre-determined constant rate for up to 14 days. Hepatocytes were challenged with increasing concentrations of insulin after various periods in culture and the response of VLDL secretion was determined. This was compared with that observed in hepatocytes from age-matched and sham-operated control animals. The use of osmotic minipumps to deliver insulin is superior to that in other models of exogenous hyperinsulinaemia in which elevated insulin concentrations are achieved by insulin injection. It is impossible to achieve a constant insulin infusion by these latter methods. To our knowledge the present report is

the first study of VLDL metabolism in isolated liver preparations derived from animals maintained under conditions of controlled exogenous hyperinsulinaemia.

MATERIALS AND METHODS

Maintenance of animals and preparation of hepatocytes

Male Wistar rats were fed and housed as described previously [29]. Seven days before preparation of hepatocytes a group of rats were each fitted with osmotic minipumps filled with a solution of insulin (see below). The pump was inserted subcutaneously into the scapular region of the back under halothane anaesthesia. Control animals were sham-operated at the same time. After the operation the animals were returned to their cages and fed *ad lib.* as before. Animals fitted with the minipumps were given glucose (10% w/v) in their drinking water. This was done to prevent an excessive hypoglycaemia. The consumption of food, water and glucose solution was monitored daily for the next 7 days and the daily weight gain was recorded. Immediately before the hepatocytes were prepared, the animals were weighed (final weight) and a blood sample was taken for determination of plasma insulin, glucose and triacylglycerol (TAG) concentrations. Hepatocytes were prepared simultaneously (under sterile conditions [27]) from one of each group of animals. After addition of serum-free supplemented Waymouth's medium [27] the cells were cultured for various periods of time up to a total of 48 h. To the batches of cells cultured for 24 h only was added [³H]oleate (0.75 mM; 0.98×10^6 d.p.m./ μ mol) with or without various concentrations of insulin ranging from 0.5 nM to 780 nM. At the end of this period the medium was collected and the cells were harvested. To the cells cultured for a total of 48 h unlabelled oleate (0.75 mM) was added for the first 24 h period. Insulin was not present during this time. At the end of this period the medium was removed and fresh, supplemented Waymouth's medium was added containing [³H]oleate (0.75 mM; 0.98×10^6 d.p.m./ μ mol) in the presence or absence of the above concentrations of insulin. The cells were cultured for periods of 6, 12, 18 or 24 h, after which the medium was collected and the cells were harvested.

Harvesting of cells, preparation of VLDL, and measurement of TAG and apoB

At the end of each culture period, the medium was removed. The cells were harvested and the secreted VLDL was obtained as

previously described [27]. The total lipid fractions of the VLDL and of the cell pellets were obtained as previously described [26]. TAG in the extracts was assayed [30] with a kit from Boehringer-Mannheim (Triglycerides GPO-PAP). For the measurement of the ³H content of the labelled TAG, the total lipid fraction was separated by TLC [31] and the band corresponding to TAG was isolated. The amount of newly synthesized TAG was calculated from the specific radioactivity of the [³H]oleate in the medium (0.98×10^6 d.p.m./ μ mol). In some cases, labelled phospholipids were also isolated from the TLC plate. ApoB was measured by using an ELISA assay [32]. Rat plasma VLDL was used to prepare the standard curve for this assay and anti-human apoB antiserum was used as the primary antibody.

Other methods

Cellular protein was measured by the method of Lowry et al. [33]. Oleate bound to BSA (essentially fatty-acid free) was prepared as described previously [27]. The albumin concentration in the culture medium was 0.5%. Osmotic minipumps (see below) were filled with bovine insulin (200 i.u./ml) dissolved in a solution (pH 3.0) containing glycerol (16 mg/ml), phenol (2.5 mg/ml) and glutamic acid (7 mg/ml). After the insulin had dissolved the pH of the solution was adjusted to 3.5.

Statistical methods

All values are expressed as the means \pm S.E.M. for six independent experiments, each containing one hyperinsulinaemic and one sham-operated animal whose livers were perfused simultaneously. Differences between means were analysed by a paired or unpaired Student's *t*-test. Responses of each group to the different insulin concentrations were analysed by repeated-measures analysis of variance (ANOVA) with the SPSS programme for Microsoft Windows.

Materials

All tissue culture media were obtained from Gibco Ltd. (Paisley, Scotland, U.K.). Radiochemicals were obtained from Amersham International (Aylesbury, Bucks., U.K.). Anti-human apoB antiserum was obtained from Boehringer-Mannheim (Lewes, Sussex, U.K.) and anti-sheep IgG antibody was obtained from Sigma (Poole, Dorset, U.K.). Osmotic minipumps ('Alzet', model 2001; 0.22 ml capacity) were obtained from Alza Corp., (Palo Alto, CA, U.S.A.) and delivered 1.03 ± 0.04 μ l/h.

RESULTS

The constant subcutaneous delivery of insulin resulted in a plasma insulin concentration of 201 ± 64 μ -i.u./ml 7 days after implantation of the minipump. This compared with a concentration of 31 ± 4 μ -i.u./ml in the sham-operated control animals (Table 1). The consumption of solid food by the hyperinsulinaemic rats was significantly less than that of the sham-operated controls. However, the former animals also consumed an average of 58.3 ± 6.7 g of glucose per week in the drinking water so that total energy intake was greater than in the control animals. This resulted in a significantly greater rate of weight increase and a higher final weight in the hyperinsulinaemic animals. This was probably due, at least in part, to an increase in adipose tissue mass if the epididymal fat pad weight was representative of changes in other depots (Table 1). Despite the

Table 1 Characterization of rats used in the study

The values below were obtained from six animals in each group except for the values for plasma insulin (three animals), triacylglycerol (four) and fat pad weight (four). Significant differences were determined by a paired *t*-test. Values marked *, ** and *** were significantly different ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively).

	Sham-operated	Hyperinsulinaemic
Weight gain (g/week)	41.5 ± 3.9	$57.8 \pm 6.0^*$
Final weight (g)	236 ± 7.9	$252 \pm 10.0^{**}$
Chow consumption (g/week)	164 ± 5.8	$142 \pm 6.3^*$
Glucose consumption (g/week)	—	58.5 ± 6.7
Plasma glucose (mM)	9.6 ± 0.29	$3.41 \pm 0.59^{***}$
Plasma insulin (μ -i.u./ml)	31 ± 4	$201 \pm 64^*$
Plasma TAG (mg/dl)	45.9 ± 3.7	$28.9 \pm 5.5^*$
Epididymal fat pad weight (g)	1.39 ± 0.23	$2.16 \pm 0.22^*$

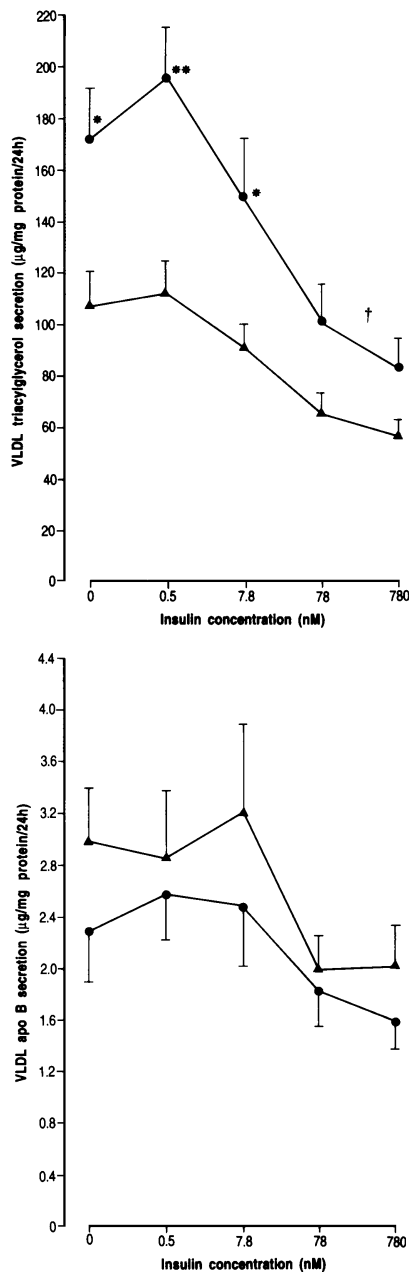


Figure 1 VLDL TAG and apoB output: the effects of insulin during the first 24 h of culture

Hepatocytes from hyperinsulinaemic and sham-operated animals were cultured for 24 h in the presence of oleate (0.75 mM) and various concentrations of insulin (0–780 nM). Each group contained six animals. Key: ●, hyperinsulinaemic animals; ▲, sham-operated control animals. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding values obtained in the control group. Lines marked † are significantly different ($P < 0.01$) from corresponding lines of the sham-operated rat (ANOVA).

high glucose intake, the hyperinsulinaemic animals had significantly lower levels of plasma glucose than the control animals and were also relatively hypotriglyceridaemic (Table 1).

When hepatocytes from the chronically hyperinsulinaemic rats were cultured for 24 h in the absence of insulin, the secretion of VLDL TAG was increased by 60% compared with hepatocytes derived from control animals (Figure 1; $P < 0.05$). Increasing the concentration of insulin in the culture medium of these cells

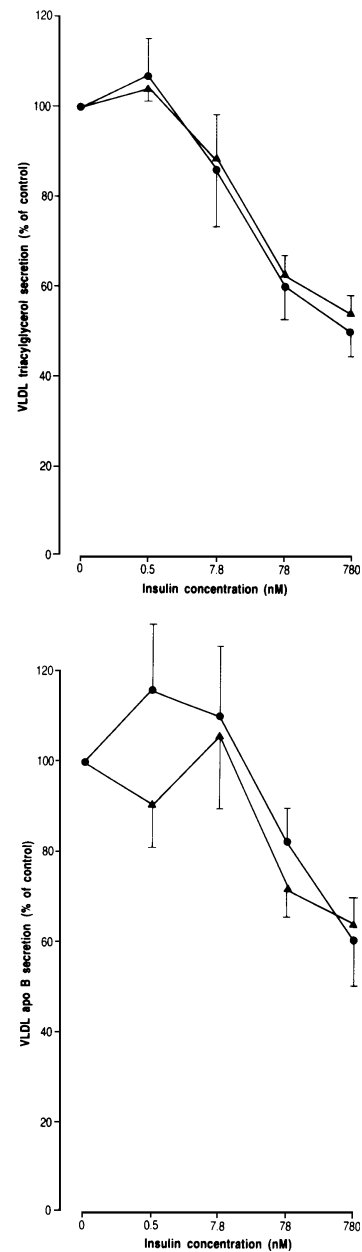


Figure 2 Fractional suppression of VLDL TAG and apoB output by insulin during the first 24 h of culture

Data are taken from Figure 1. Each value is calculated as a percentage of the rate observed in each group when insulin was absent from the culture medium. There were no significant differences in the fractional suppression of either TAG or apoB between each group at any of the concentrations of insulin tested.

suppressed the secretion of VLDL TAG by up to $49.8 \pm 6.1\%$. The pattern of inhibition of VLDL TAG secretion by insulin in the cells from the sham-operated animals was virtually identical with that in the hepatocytes from the hyperinsulinaemic rats, reaching a maximum of $46.0 \pm 3.7\%$ at the highest concentration of insulin (Figure 2). Thus statistical analysis by ANOVA revealed that there was no significant differences between the lines either for VLDL TAG or apoB (Figure 2). In contrast with the elevated secretion of TAG, chronic hyperinsulinaemia *in vivo* did not lead to an increased secretion of VLDL apoB in the

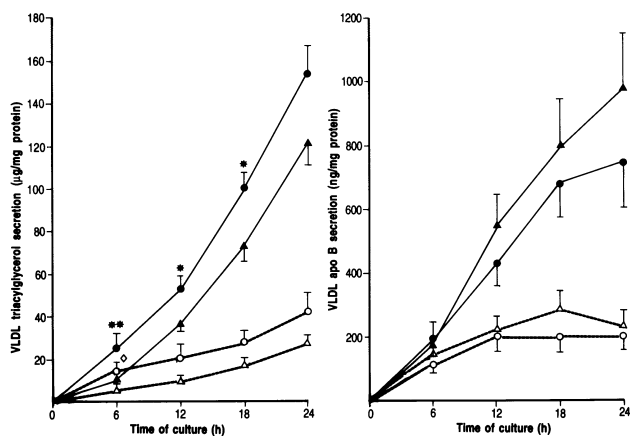


Figure 3 Time course of VLDL TAG and apoB output: the effects of insulin during the second 24 h period of culture

After 24 h in culture medium containing 0.75 mM oleate, the medium was removed and replaced by medium of an identical composition in the presence (78 nM) or absence of insulin. After 6, 12, 18 and 24 h the medium was removed and the cells were harvested. Each group consisted of hepatocytes obtained from six individual rats. Key: ●, hyperinsulinaemic rats, insulin absent; ▲, control rats, insulin absent; ○, hyperinsulinaemic rats, insulin present; △, control rats, insulin present. Insulin significantly ($P < 0.05$) inhibited the secretion of apoB and TAG in each group at all time points studied. Values marked * and ** are significantly different ($P < 0.05$ or $P < 0.01$ respectively) from the corresponding control group when insulin was absent from the culture medium. Values marked ◇ are significantly different from the corresponding control group when insulin was present in the culture medium.

derived hepatocytes (Figure 1). If anything, there was a tendency towards a slight, but not significant, decrease in apoB secreted in association with VLDL. Addition of insulin to the culture

medium suppressed the secretion of VLDL apoB from cells derived from each type of animal (Figure 1). Quantitatively, however, there was no significant difference in the extent of this response; cells from the hyperinsulinaemic animals remained as sensitive to insulin in this respect as cells from the sham-operated control animals (Figure 2).

The increased output of VLDL TAG by cells from the hyperinsulinaemic animals persisted during the second day of culture (Figure 3). However, the difference became less pronounced towards the end of the time-course studied and was not significant at the end of the second 24 h period (i.e. 48 h after isolation of the cells). As observed during the previous 24 h, the secretion of apoB was somewhat lower in the cells from the hyperinsulinaemic animals than in those from the sham-operated (Figure 3). However, analysis by ANOVA showed that this difference was not statistically significant. Insulin (78 nM) suppressed the secretion of VLDL TAG and apoB at all time points during the second day of culture in hepatocytes from both types of animals. Again, as observed 24 h previously (Figure 2) chronic hyperinsulinaemia *in vivo* did not influence the sensitivity of the hepatocytes to the subsequent inhibitory effect of insulin on the secretion of VLDL TAG and apoB. Thus, during the second day of culture, insulin maximally suppressed the secretion of VLDL TAG to $17.8 \pm 3.1\%$ and $18.6 \pm 3.0\%$ of the rates observed in the absence of insulin in cell cultures derived from the hyperinsulinaemic and sham-operated animals respectively. The corresponding values for the suppression of apoB secretion were $30.3 \pm 8.3\%$ and $22.9 \pm 4.5\%$ respectively.

The overall rate of TAG synthesis from exogenous [3 H]oleate in the hepatocytes from the hyperinsulinaemic rats was slightly (but not significantly) higher than in the sham-operated controls. Insulin added to the culture medium had little or no effect on TAG synthesis in either case. As expected, however, insulin inhibited the secretion of newly synthesized TAG irrespective of

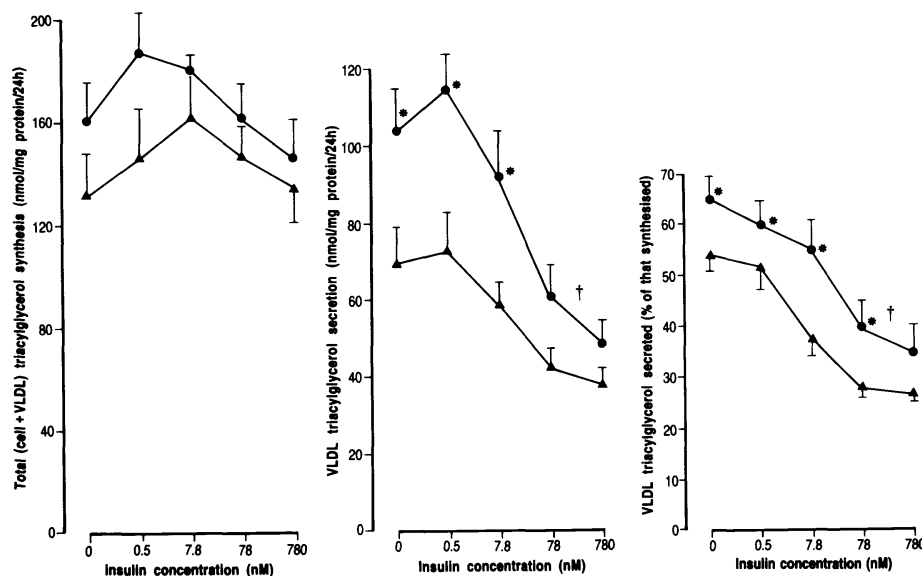


Figure 4 Recruitment of newly synthesized TAG for VLDL assembly

Hepatocytes were cultured with [3 H]oleate (0.75 mM; 0.98×10^6 d.p.m./ μ mol) in either the absence or the presence of various concentrations of insulin for 24 h immediately after the removal of serum from the medium. For each dish, labelled cellular and VLDL TAG was isolated. Total TAG synthesis (left panel) represents the sum of these two values. The centre panel shows the amounts of newly synthesized TAG secreted as VLDL. The right panel shows the proportion of newly synthesized TAG secreted as VLDL. There were six animals in each group. Key: ●, hyperinsulinaemic animals; ▲, control animals. Values marked * are significantly greater ($P < 0.05$) than the corresponding values for the sham-operated control group. Lines marked † are significantly different ($P < 0.01$) from corresponding lines in the sham-operated rat (ANOVA).

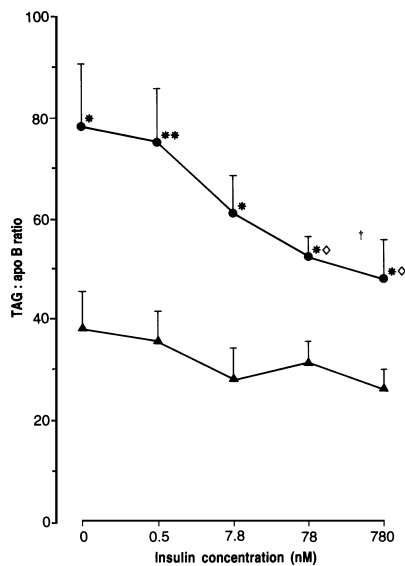


Figure 5 TAG:apoB ratios of secreted VLDL

Data are taken from Figure 1. Key: ●, hyperinsulinaemic group; ▲, control group. Values marked * and ** are significantly greater ($P < 0.05$ and $P < 0.01$ respectively) than the corresponding values in the sham-operated group. Values marked ◇ are significantly lower ($P < 0.05$) than that obtained in the absence of insulin. The line marked † is significantly different ($P < 0.05$) from the corresponding line in the sham-operated rat (ANOVA).

the state of the donor animals, and the pattern of this change was similar in both types of cells (Figure 4). Nevertheless, of the total TAG synthesized, a greater proportion was secreted as VLDL by hepatocytes from the hyperinsulinaemic animals compared with those from the controls. This pattern persisted at all concentrations of insulin (Figure 4).

The differential effect of chronic hyperinsulinaemia on the secretion of TAG on the one hand, and apoB on the other, gave rise to a higher ratio of TAG:apoB in the secreted VLDL than was observed in the sham-operated controls. This difference remained at all concentrations of insulin (Figure 5). In the hepatocytes from the hyperinsulinaemic rats, but not in those from the controls, insulin significantly decreased the VLDL:apoB ratio of the secreted VLDL (Figure 5).

DISCUSSION

Pathophysiological states associated with chronic hyperinsulinaemia are often accompanied by an elevated rate of hepatic VLDL output [1,10,11,18]. Under these conditions the liver is resistant to the normal acute inhibitory effect of insulin on the secretion of VLDL. This decreased sensitivity to insulin has been shown for VLDL apoB in human subjects [6] and for VLDL TAG and apoB in animal models *in vitro* [22–24]. A similar desensitization of the liver can be induced directly *in vitro* by chronic exposure of hepatocytes of normal rats to high concentrations of insulin [14,25–28]. This observation suggested that the effects of chronic hyperinsulinaemia *in vivo* are a direct consequence of long-term exposure of the liver to a high-insulin environment.

The present results, however, do not support this hypothesis. Chronic exogenous hyperinsulinaemia induced over a 7-day period was insufficient, in itself, to modify the normal acute inhibitory effect on VLDL apoB and TAG secretion when

hepatocytes were challenged with insulin *in vitro* (Figures 1 and 2). Other workers have investigated the causes of the disturbances of carbohydrate metabolism that accompany chronic endogenous hyperinsulinaemia and insulin resistance. In these cases the high rate of hepatic glucose output is insensitive to suppression acutely by insulin [15,16]. Again, however, the effects cannot be reproduced simply by chronic infusion of exogenous insulin alone into normal animals. Thus the liver retained normal sensitivity to insulin delivered acutely via a hyperinsulinaemic, euglycaemic clamp [34,35]. Nor could the resistance to insulin-stimulated glucose uptake in adipose tissue in animal models of chronic endogenous hyperinsulinaemia be reproduced in normal animals chronically infused with exogenous insulin [36].

In the present work, the major effect of chronic insulin infusion was to increase the rate of hepatic VLDL TAG output without affecting the sensitivity of this process to acute inhibition by insulin. There was no suggestion, in the current work, that chronic hyperinsulinaemia significantly stimulated hepatic TAG synthesis from exogenous fatty acids (Figure 4). Thus it may be calculated from Figure 4 that, in the hepatocytes from the hyperinsulinaemic animals, in the absence of insulin, 483 ± 46 nmol of oleate per mg of cell protein was removed from the medium for the synthesis of TAG. In the presence of the highest concentration of insulin, the corresponding value was 441 ± 41 nmol of oleate. The corresponding values for hepatocytes from the sham-operated animals were 396 ± 50 and 405 ± 45 nmol of oleate respectively. The increased output of TAG probably arose, at least in part, from an increased recruitment of newly synthesized TAG from the cellular pool [37] for participation in the assembly of VLDL (Figure 4). Although exogenous fatty acids make only a small direct contribution to VLDL TAG in the shorter term [41], over the longer period involved in the present studies, sufficient TAG newly synthesized from exogenous oleate has accumulated within the cell to maintain the high rates of secretion observed [37]. The efficiency of this indirect pathway probably accounts for the high proportion of fatty acids incorporated into cellular TAG that are eventually secreted as VLDL TAG (Figure 4). It should also be noted that fatty acid synthesis *de novo* increased in rats with chronic hyperinsulinaemia [34] and this condition is usually associated, in some as yet unknown way, with an increased rate of VLDL output [38–41]. The increased secretion of VLDL TAG in hepatocytes from the chronically hyperinsulinaemic rats persisted for up to 42 h in culture, whereafter any differences became non-significant (Figure 3). It therefore appears that the metabolic and hormonal environment of the liver created *in vivo* by chronic exogenous hyperinsulinaemia is essential for the maintenance of a high TAG output and that these conditions eventually lose their impact after removal of the cells into a different setting *in vitro*. The hyperinsulinaemic environment as it persists *in vivo*, however, is not conducive to the development of insulin resistance, but the possibility that a much longer period of hyperinsulinaemia may be required for insulin resistance to develop cannot be ruled out. In this respect it should be noted that, despite the hyperinsulinaemia of young obese Zucker rats, adipose tissue remains sensitive to glucose uptake and is followed only later by the well-documented insulin-resistance that develops after longer periods of hyperinsulinaemia in the older rats [36]. We have, however, implanted minipumps for a total of 14 days with results very similar to those obtained after 7 days of hyperinsulinaemia (C. Bourgeois, unpublished work). Thus the present findings in liver and those of others in adipose tissue cannot explain the relatively rapid direct desensitization by insulin, of insulin responsiveness in hepatocytes [25–28] and adipocytes [42–45] *in vitro*.

In the present work the increased output of VLDL TAG was not accompanied by an increase in the secretion of VLDL apoB in the hepatocytes from the chronic hyperinsulinaemic rats. Because each particle of VLDL contains only one molecule of apoB, chronic hyperinsulinaemia did not lead to an increase in the number of particles secreted but each particle transported an increased amount of TAG. This lack of coupling between apoB and TAG output resembles the effect of chronic consumption of a high-carbohydrate diet in animals [23] and in man [46]. Whether chronic hyperinsulinaemia and insulin resistance in man gives rise to an increase in VLDL particle size, particle number, or both, remains controversial and probably depends on the precise nature of the metabolic disturbance involved [47,48].

Finally, the low plasma TAG that occurs in association with a high hepatic VLDL TAG output in the hyperinsulinaemic animals is suggestive of an increased rate of clearance of VLDL TAG by peripheral organs such as adipose tissue. This is supported by the observed increase in the weight of the epididymal fat pads (Table 1) and by the increased sensitivity of adipose tissue to net lipid storage under conditions of chronic exogenous hyperinsulinaemia [34,49].

In conclusion, one of the most important physiological functions of insulin is the enhancement of whole-body energy storage when food consumption is high. This function is achieved by increased net storage of glycogen in the liver, and of TAG in the liver and adipose tissue. One of the consequences of insulin resistance is an impairment of insulin's ability to continuously promote TAG and glycogen storage beyond a certain level, a defect that contributes to an increased net release of carbohydrate (as glucose) and lipid (as VLDL) from the liver and an increased lipid release (as fatty acids) from adipose tissue. Our present results show that the impairment of lipid release from the liver is not the direct consequence of chronic hyperinsulinaemia, at least not over the relatively short term. At a physiological level, this observation is consistent with previous findings that chronic hyperinsulinaemia is not in itself responsible for defective insulin regulation of hepatic carbohydrate and adipose tissue lipid metabolism [34,35,38,49].

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