Decreased sensitivity of very-low-density lipoprotein secretion to the inhibitory effect of insulin in cultured hepatocytes from lactating rats

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Hepatocytes were prepared from 10–11-day lactating rat dams and from lactating dams which had been weaned for periods of either 1–2 days or 7 days. Hepatocytes from each group were cultured for periods of up to 48 h in a chemically defined medium. Compared with those from the 7-day weaned animals, hepatocytes from the lactating rats were resistant to the inhibitory effects of insulin on the secretion of very-low-density lipoprotein (VLDL) triacylglycerol (TAG). These differences persisted for up to 48 h in culture. Hepatocytes from the 1–2-day weaned animals remained relatively insulin-resistant in this respect. Similar differences in the response to insulin were not observed for the secretion of VLDL apolipoprotein B. TAG production increased and ketogenesis decreased in the hepatocytes from the lactating compared with those from the 7-day weaned rats. Insensitivity of the liver to the normal effects of insulin on the secretion of VLDL TAG may arise from a need to maintain an adequate flux of hepatic lipids to the lactating mammary gland in order to meet the large demand for milk-fat production.

INTRODUCTION

Lactation is associated with widespread changes in whole-body lipid metabolism, the purpose of which is to direct lipids and lipid precursors to the mammary gland for milk-fat production [1]. A major contributory factor to this overall process is the redistribution of dietary lipid, transported as chylomicrons, away from adipose tissue towards the lactating mammary gland. This is achieved, at least in part, by a decreased activity of adipose tissue lipoprotein lipase (LPL) [2,3]. Resistance to the normal stimulatory effects of insulin on the activity of LPL plays a role here [4], and insulin resistance to carbohydrate metabolism is also observed [5,6]. The hormonal regulation of adipose tissue lipid metabolism during lactation has received considerable attention over the years (for a review, see [1]) but comparatively little is known of the hepatic contribution to the overall changes in whole-body lipid metabolism which occur as lactation becomes established. Several observations, however, suggest that the liver may play an important role. First, the increase in the rate of lipogenesis *de novo* in the liver [7–10] probably results from the hyperphagia of lactation and might be expected to stimulate the secretion of lipid as very-low-density lipoprotein (VLDL) [11–13]. Increased availability of hepatic triacylglycerol (TAG) would also arise as a result of the re-direction of plasma fatty acids away from oxidation and into the esterification pathway during lactation [7,9]. Increased secretion of hepatic VLDL during lactation has not, however, yet been conclusively demonstrated experimentally. Secondly, in the post-absorptive state, hepatic VLDL is the primary source of plasma TAG available for uptake by the lactating mammary glands, and plasma fatty acids, major precursors of VLDL, increase during lactation [14]. Finally, since hepatic VLDL release is an insulin-sensitive process (for reviews, see [15,16]), lipid output may well be affected by the low plasma insulin associated with lactation. Current knowledge of the precise effects of lactation on the secretion of VLDL is inconclusive: although experiments *in vivo* initially suggested that lactation suppressed hepatic VLDL output [17], later findings suggested that this conclusion may have been confounded because concomitant changes in VLDL uptake by mammary tissue were not adequately accounted for [18].

The rapid development of insulin resistance by adipose tissue as lactation becomes established forms part of the strategy by which plasma lipids are directed away from storage pools and thus become available for uptake by the mammary gland [1]. Evidence also exists for the development of insulin resistance in brown adipose tissue of the lactating rat [19,20] and in the hind-limb tissues of lactating sheep [21]. Whether, during lactation, the liver also becomes resistant to the normal effects of insulin is not known. Since insulin acutely suppresses the secretion of hepatic VLDL [15,16], a decreased sensitivity of the liver to the effects of insulin might be expected to result in an increased release of VLDL. In the present work, primary cultures of rat hepatocytes derived from lactating, 1–2-day weaned and 7-day weaned animals were used to provide answers to the following questions. First, is the secretion of hepatic lipid as VLDL altered during lactation? Secondly, does lactation affect the sensitivity of the liver to the acute suppression of VLDL release by insulin? Thirdly, do changes in hepatic lipid metabolism associated with lactation persist in cultured cells for long periods after removal of the *in vivo* milieu? Finally, how rapidly does hepatic lipid metabolism respond to weaning?

MATERIALS AND METHODS

Materials

All tissue culture media were obtained from Gibco Ltd. (Paisley, Scotland, U.K.). Radiochemicals were obtained from Amersham International (Little Chalfont, 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.).

Maintenance of rats and preparation of hepatocytes

Female rats of the Wistar strain were used and were housed and fed as previously described [22]. Rats were used after 10–11 days

Abbreviations used: VLDL, very-low-density lipoprotein; LPL, lipoprotein lipase; apoB, apolipoprotein B; TAG, triacylglycerol.

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lactation and they weighed between 287 and 370 g (329 ± 14 g). The litter size varied between eight and ten pups. Rats in which the pups had been weaned for 7 days had the pups removed after 10 days lactation. For convenience these rats are referred to as 7-day weaned rats. The mothers weighed 281 ± 11 g. Rats in which the pups had been weaned for 1–2 days had pups removed after 9–10 days lactation and weighed 314 ± 6 g. Again, these rats are referred to, simply, as 1–2-day weaned rats. Hepatocytes were prepared simultaneously from one rat in each group. Sterility was maintained during hepatocyte preparation [23] and the cells were cultured for 24 h in the presence of [^{3}H]oleate (0.75 mM) and in the presence or absence of insulin as previously described [24]. In some cases, non-radioactive oleate was present during the first 24 h of culture. In these experiments, after 24 h, the medium was removed and fresh medium containing [^{3}H]oleate (0.75 mM, 0.98×10^6 d.p.m./μmol) was added. The cells were cultured for a further 24 h. At the end of each period of culture, the medium was removed and the cells were harvested.

Preparation of VLDL and measurement of TAG and apolipoprotein B (apoB)

VLDL secreted into the medium by the cells during each 24 h culture period was obtained by ultracentrifugation at a density of 1.006 as previously described [23]. The total lipid fractions of the VLDL and of the cell pellets were obtained as described earlier [25]. The mass of TAG in the extracts was assayed [26] using a kit from Boehringer-Mannheim (Triglycerides GPO-PAP). Where required, ^{3}H-labelled TAG was isolated from the total lipid extract by TLC and the band corresponding to TAG was isolated [27]. The amount of TAG synthesized exclusively from extracellular [^{3}H]oleate was calculated from the specific radioactivity of the [^{3}H]oleate in the medium (0.98×10^6 d.p.m./μmol). ApoB was measured using a non-competitive ELISA assay [28]. Rat plasma VLDL was used to prepare the standard curve for this assay, and anti-human apoB antiserum was used as the primary antibody. Cellular protein was measured by the method of Lowry et al. [29]. Oleate bound to BSA (essentially fatty acid free) was prepared as previously described [23]. Ketone bodies were measured in the medium using the method of Williamson et al. [30].

Statistical methods

Values are expressed as the means ± S.E.M. of five independent experiments each containing one animal from each group except for the 1–2-day weaned group, in which there were four animals. 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) using the SPSS program for MS Windows.

RESULTS

During the first 24 h of cell culture in the absence of insulin there was no significant difference in the output of VLDL TAG in the hepatocytes from the three groups of rats (see legend to Figure 1). However, although addition of increasing concentrations of insulin suppressed VLDL TAG output in hepatocytes from rats in each of the three physiological states, this effect was less pronounced in cells from the lactating and 1–2-day weaned animals compared with that in those from the 7-day weaned animals (Figure 1). In the presence of insulin, therefore, the

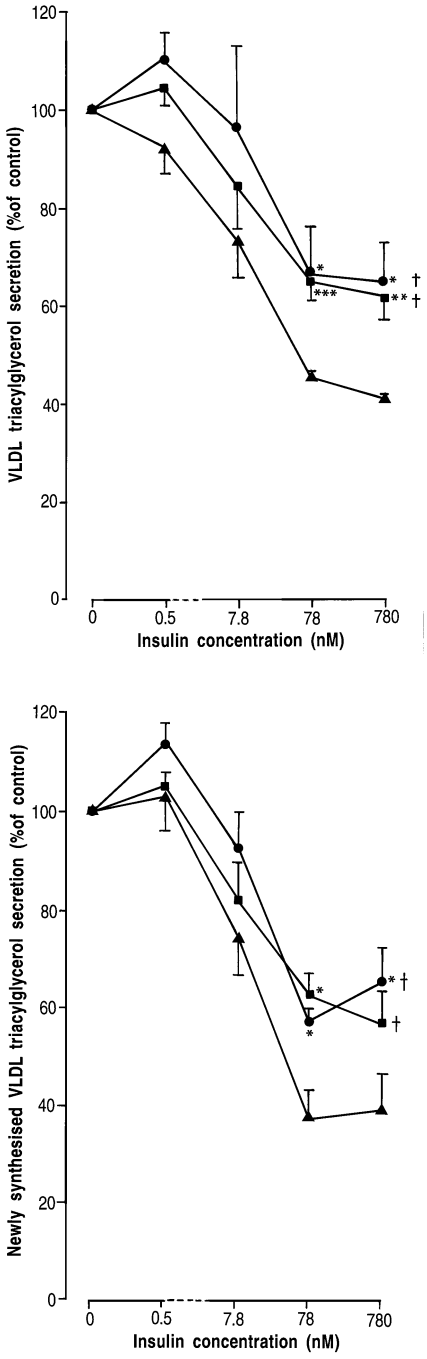

Figure 1 Effect of insulin on the secretion of VLDL total TAG (top) and TAG newly synthesized from [^{3}H]oleate (bottom)

Hepatocytes from lactating (●), 7-day weaned (▲) and 1–2-day weaned (■) rats were cultured for 24 h in the absence or presence of increasing concentrations of insulin. In all cases [^{3}H]oleate was present at an initial concentration of 0.75 mM. The total mass of VLDL TAG was measured enzymically. Newly synthesized VLDL TAG was determined on the basis of its radioactivity and the specific radioactivity of [^{3}H]oleate in the medium. Each point represents the mean ± S.E.M. of five (lactating and 7-day weaned) or four (1–2-day weaned) individual hepatocyte preparations. Values marked *, ** and *** are significantly different ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively) from the corresponding values in the preparation from 7-day weaned rats. Lines marked † are significantly different from that described for the hepatocytes from 7-day weaned rats using repeated-measures ANOVA. In the absence of insulin, the outputs of VLDL total TAG were 200 ± 39, 224 ± 33 and 211 ± 38 μg/24 h per mg of cell protein for the cells from the lactating, 7-day weaned and 1–2-day weaned rats respectively. The corresponding values for newly synthesized VLDL TAG were 107.2 ± 5.8, 104.0 ± 10.8 and 87.2 ± 8.1 nmol/24 h per mg of cell protein respectively.

Table 1 Effect of lactation on the relative suppression of VLDL TAG output by insulin during the second day of culture

Hepatocytes were cultured for 24 h in the presence of 0.75 mM oleate. Insulin was not present during this period. The medium was removed, the cells were washed and fresh medium was added, again with 0.75 mM oleate. During this period insulin was either absent or present at the concentration shown. Each value represents the mean ± S.E.M. of five animals in each group. The values marked * are significantly different from the corresponding value observed in the lactating group. The values obtained at zero insulin concentration have been arbitrarily standardized at 100%. The absolute values in the absence of insulin were 193 ± 38 μg of VLDL TAG/mg of protein for lactating rats and 193 ± 30 μg of VLDL TAG/mg of protein for 7-day weaned rats.

	VLDL TAG output (%)		
Insulin concentration (nM)...	0	78	780
Lactating rats	100	28 ± 4	31 ± 3
7-day weaned rats	100	17 ± 3*	16 ± 5*

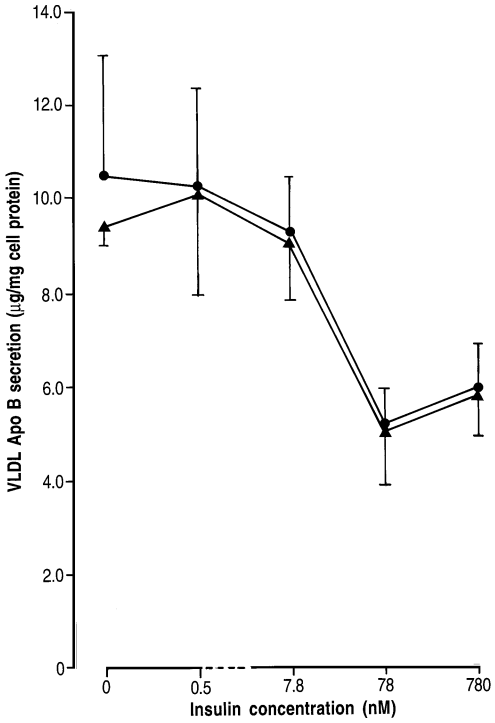

Figure 2 Response of VLDL apoB secretion to insulin

For details, see the legend to Figure 1. There was no significant difference in the response of apoB secretion in the hepatocytes from the lactating (●) and the 7-day weaned (▲) rats at any concentration of insulin.

output of VLDL TAG was higher in the hepatocytes from the lactating group (ANOVA, $P < 0.01$) and the 1–2-day weaned group ($P < 0.01$) than in hepatocytes from the 7-day weaned animals. The higher sensitivity of hepatocytes in the latter group persisted during the second 24 h period in culture (Table 1). Insulin inhibited the secretion of VLDL TAG to a greater extent when added on the second day of culture than when added on the first day. This was the case irrespective of whether the cells were derived from the lactating or the 7-day weaned animals. Similar patterns were apparent for the effects of insulin on the secretion of labelled TAG newly synthesized from exogenous oleate

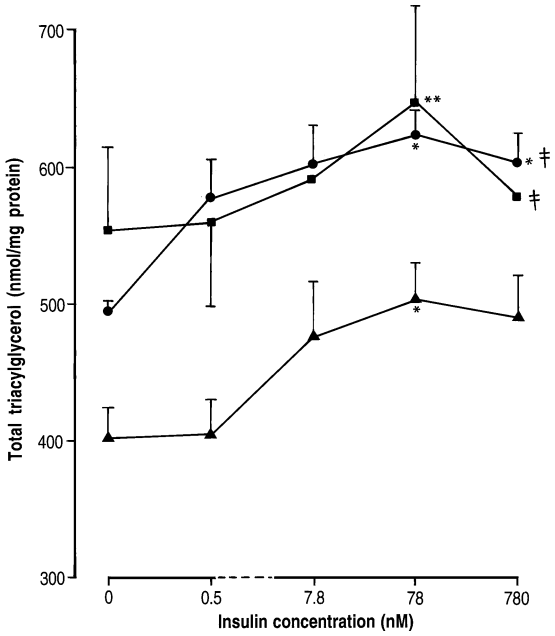

Figure 3 Response of TAG production to insulin

For details, see the legend to Figure 1. At the end of the 24 h culture period the mass of cellular TAG was added to that of the secreted VLDL for each hepatocyte type at each concentration of insulin. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding value obtained in the absence of insulin. The S.E.M.s for 7.8 nM and 780 nM insulin in the hepatocytes from the 1–2-day weaned rats were ± 68 and ± 77 respectively. Lines marked ‡ for the lactating and 1–2-day weaned rats are significantly different ($P < 0.001$) from that for the 7-day weaned rats by repeated-measures ANOVA.

(Figure 1). Once again, although the outputs of TAG in hepatocytes from the three groups were broadly similar when insulin was absent from the medium, insulin was more effective at suppressing newly synthesized TAG secretion in hepatocytes from the 7-day weaned animals than in those from the lactating animals (ANOVA, $P < 0.05$) or from the 1–2-day weaned group ($P < 0.05$).

Despite the differing responses of VLDL TAG secretion to insulin in the lactating compared with the 7-day weaned group, the responses of VLDL apoB in the two groups were almost identical (Figure 2). Thus there was no effect of lactation on the sensitivity of apoB secretion to suppression by insulin.

The total TAG produced by the cell is the sum of the VLDL TAG secreted and that remaining within the cell at the end of the 24 h culture period. This quantity was greater in the hepatocytes from the lactating animals compared with those from the 7-day weaned rats (Figure 3) and in both cases there was an increase in TAG production in the presence of insulin. After 1–2 days of weaning, the pattern of TAG production had not changed significantly from that shown by the cells from the lactating animals (Figure 3).

The overall patterns of ketone-body production in the cells from animals in the three physiological states were almost the mirror images of their respective patterns of TAG synthesis. Thus in the absence of insulin, ketone-body production was suppressed in the cells from the lactating animals and from the 1–2-day weaned animals compared with those from the 7-day weaned animals (Figure 4). In all cases insulin suppressed ketone-body production. The opposing effects of lactation on hepatic ketogenesis and TAG production became more apparent when these parameters were plotted as a ratio of their rates of

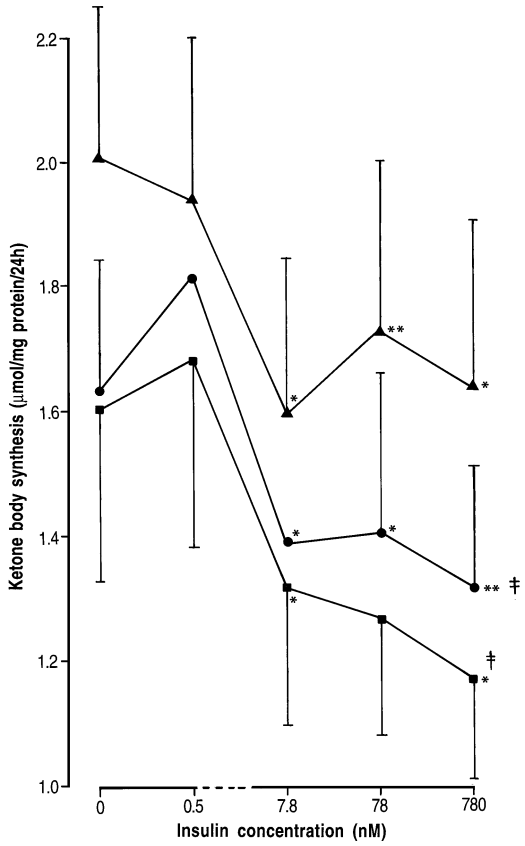

Figure 4 Response of ketone-body synthesis to insulin

For details, see the legend to Figure 1. At the end of the 24 h culture period, the output of ketone bodies was determined on an aliquot of the culture medium. Each measurement represents the sum of acetoacetate and β-hydroxybutyrate concentrations. Values marked * and ** are significantly different ($P < 0.05$ and $P < 0.01$ respectively) from the corresponding value in the absence of insulin. Lines marked ‡ for the lactating and 1–2-day weaned rats are significantly different ($P < 0.001$) from the corresponding line for the 7-day weaned by repeated-measures ANOVA.

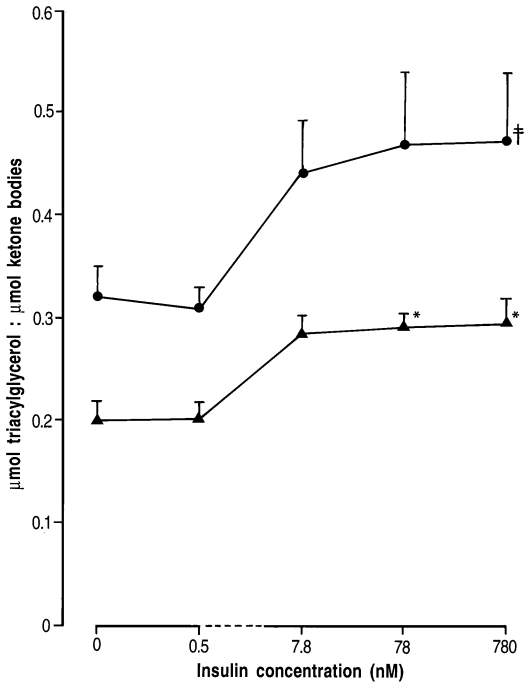

Figure 5 Effect of insulin on relative synthesis of TAG and ketone bodies in hepatocytes from lactating and 7-day weaned rats

For details, see the legends to Figures 1, 3 and 4. Values marked * are significantly different from the corresponding value in the absence of insulin ($P < 0.05$). The line marked ‡ for the lactating animals is significantly different ($P < 0.001$) from the corresponding line for the 7-day weaned animals using repeated-measures ANOVA. Symbols: ●, lactating animals; ▲, weaned animals.

formation. These data are presented in Figure 5 and show that, in the absence of insulin, there was a 50 % increase in flux of fatty acids into TAG relative to ketone bodies in the cells from the lactating animals compared with those from the 7-day weaned rats. In both cases insulin increased the relative flux into TAG. Nevertheless, at each concentration of insulin, the original difference in relative flux in the hepatocytes from lactating and weaned animals persisted.

DISCUSSION

Physiological states associated with a high rate of hepatic lipogenesis *de novo* are usually linked with an increased output of hepatic VLDL (for a review, see [15]). In the present case, however, despite an increased hepatic lipogenesis during lactation [8–10] and an increased TAG production (Figure 3), hepatocytes from lactating animals cultured in the absence of insulin showed no increase in VLDL TAG or apoB output compared with that observed in hepatocytes from 7-day weaned animals (Figures 1 and 2). It might be argued, of course, that cultured hepatocytes from lactating animals do not reflect the metabolic characteristics of the liver *in vivo*. However, the decreased rate of ketogenesis and the increased rate of TAG production in the cultured cells from lactating animals observed in the present work are consistent with changes in enzymic activities in intact livers [9]. Lipid metabolism in hepatocytes from lactating sheep also behaves in a similar manner to that observed in the intact liver [31]. It seems, therefore, that, when expressed per mg of protein, the basal level of VLDL output in the hepatocytes from the lactating animals is no different from that in hepatocytes from the 7-day weaned rats. Because of liver hypertrophy during lactation [32] it is, nevertheless, probable that the total hepatic output of VLDL is increased in lactation. In the present work, however, the major difference in VLDL output between hepatocytes from lactating and 7-day weaned animals arose from differences in response to insulin. Thus when insulin was added to the medium of the cultured cells, there was a greater decrease in the secretion of VLDL TAG in the hepatocytes from the 7-day weaned than in those from the lactating animals (Figure 1). When insulin was present, therefore, cells from the lactating animals secreted more VLDL TAG than those from the 7-day weaned animals and the same was true of TAG newly synthesized from exogenous [^{3}H]oleate during the culture period (Figure 1).

If the above results can be interpreted in terms of whole-body physiology, they might suggest that when plasma insulin levels increase, for instance, in the post-prandial state, insulin is less effective in suppressing hepatic VLDL TAG output when the animals are lactating. This would ensure an increased flux of hepatic VLDL TAG to the lactating mammary gland at a time when VLDL secretion is normally suppressed [33]. That acquisition of normal sensitivity of VLDL secretion to insulin occurs only slowly after lactation ceases is suggested by the finding that after 1–2 days of weaning, VLDL secretion remained

relatively high in the presence of insulin (Figure 1). Since plasma insulin levels increase 3-fold on weaning [3,8,34] the continued insensitivity of the liver in this respect may be required to ensure adequate hepatic VLDL secretion under these conditions to replenish adipose tissue TAG stores depleted during lactation. Despite the different response of VLDL TAG secretion to insulin, the response of apoB secretion was virtually identical in hepatocytes from the lactating and 7-day weaned animals (Figure 2). It would appear, therefore, that compared with the 7-day weaned dams, in the lactating animals, insulin gives rise to the formation of larger VLDL particles.

The decreased rate of ketogenesis in hepatocytes from the lactating animals (Figure 4) is consistent with a physiological requirement to maximize the rate of hepatic TAG production. *In vivo*, this effect would be reinforced by an increased flux of exogenous fatty acids to the liver in the lactating rat [14]. In the present work insulin further increased the flux of fatty acids entering the TAG pathway at the expense of fatty acid oxidation. The resulting re-direction of substrate flux was reflected by an insulin-dependent increase in the TAG/ketone-body ratio (Figure 5). The effect of insulin occurred in hepatocytes from both the lactating and the weaned animals but the relative production of TAG remained significantly higher in the hepatocytes from lactating rats at all concentrations of insulin. Although in the present work, we did not measure CO_2 production from oleate, Whitelaw and Williamson [35] have shown that changes in total fatty acid oxidation are adequately reflected by changes in ketogenesis in hepatocytes from lactating and non-lactating animals.

Metabolic and hormonal factors which, in the lactating animal, desensitize the liver to the effects of insulin on the secretion of VLDL appear to be imprinted in the hepatocyte. This is evidenced by the decreased response to insulin even after periods as long as 48 h in a chemically defined medium (Table 1). The delay in acquiring normal insulin sensitivity may reflect a low turnover rate of a gene product(s) which is responsible, *in vivo*, for the original hepatic insulin resistance. By contrast, hepatocytes from Zucker fatty rats, which are initially also insulin-resistant, rapidly acquire normal insulin sensitivity during culture [24].

Concluding remarks

The onset of lactation is accompanied by major metabolic changes in the mother which are required to sustain the needs of milk production in the mammary gland [1]. A major component of this metabolic adaptation involves a redistribution of the increased nutrient-energy intake resulting from the hyperphagia of lactation. Thus, TAG is diverted away from adipose tissue into the mammary gland. Since insulin is normally responsible for mediating adipose tissue lipid storage, part of the strategy by which this redistribution is achieved involves a desensitization of adipose tissue to the metabolic effect of insulin (for a review, see [1]). One of the consequences is an increased mobilization and release of lipids from adipose tissue for uptake by the lactating mammary gland. After food consumption, insulin may also promote lipid storage in liver by suppressing TAG release as VLDL [15]. The present results show that changes in the response of the liver to insulin during lactation may potentiate the secretion of VLDL TAG which would then augment the total quantity of plasma lipid available for milk production by the mammary gland. This effect at the hepatic level would be facilitated by an increased synthesis of TAG, an effect already shown to result, at least in part, from a decreased rate of ketogenesis [7,9,35]. Support for this redirection of fatty acid flux is also provided in the present work (Figure 5). In summary, our results suggest that the increased hepatic TAG production previously observed in livers of lactating rats is used to fuel an increase in the secretion of VLDL which results from the decreased ability of insulin to suppress VLDL output. The increased flux of hepatic lipids into the blood plasma reinforces the increased lipid flux arising from a similar desensitization of adipose tissue. The overall effect of these changes is to increase the quantity of lipid available for milk production by the lactating mammary gland.

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