Rapid inhibition by intragastric triolein of the re-activation of glucose utilization and lipogenesis in the mammary gland during the starved–refed transition in lactating rats

Evidence for a direct effect of oral lipid on mammary tissue

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INTRODUCTION

During lactation in the rat, lipogenesis in the mammary gland is very closely related to nutritional status. Short-term starvation inactivates lipogenesis in the gland (Jones et al., 1984), and refeeding with chow diet rapidly re-activates this process (Munday & Williamson, 1981; Williamson et al., 1983; Bussmann et al., 1984; Mercer & Williamson, 1986). The re-activation of lipogenesis in the gland during refeeding follows a transitory rise in circulating insulin concentration during the initial 30 min of refeeding, which may be linked to an acute increase in the insulin-sensitivity of the tissue (Mercer & Williamson, 1986; Page & Kuhn, 1986). The extreme sensitivity of this lipogenic switch-on to substrate supply is demonstrated by the suppression of lipogenesis in the mammary gland by acarbose, a glucosidase inhibitor, which delays carbohydrate digestion in the gut, thus decreasing glucose availability during the refeeding period (Mercer & Williamson, 1987).

Previous studies have indicated that lipogenesis in the mammary gland of fed lactating rats is suppressed by both acute (Agius & Williamson, 1980; Bussmann et al., 1984) and chronic (Agius et al., 1980; Grigor & Warren, 1980; Munday & Williamson, 1987) feeding of fat, although the mechanisms for the inhibition of lipogenesis may be different. The aim of the present study was to examine whether or not administration of triacylglycerol would suppress the switch-on of lipogenesis in the mammary gland during the chow-refeeding of starved lactating rats. The temporal changes in lipogenic activity, glucose uptake, lactate output and metabolite concentrations in the gland were assessed to obtain information on the short-term intracellular regulation of lipogenesis in the gland by dietary lipid. Changes in plasma insulin and blood metabolite concentrations were also measured, because small changes in plasma insulin concentration during the 30–90 min period of refeeding appear to have crucial effects on the glucose metabolism of the gland (Mercer & Williamson, 1986, 1987).

MATERIALS AND METHODS

Rats

Primiparous lactating rats (Wistar strain, 11–14 days of lactation) were maintained at an environmental temperature of 22 ± 1 °C with a 12 h-light/12 h-dark cycle (light from 07:30 h).

Biochemicals

All enzymes and coenzymes were from Boehringer Corp. (London), Lewes, Sussex, U.K. Triton WR1339 (Tyoxap) was obtained from Sigma.

Radioactive compounds

\(^{3}\)H\(_{2}\)O was obtained from Amersham International, Amersham, Bucks., U.K.

Treatment of rats

The animals were starved overnight (18 h) and used between 09:00 and 10:00 h next day. The rats were refed with 5 g of chow diet (52% carbohydrate, 21% protein, 4% fat; residue non-digestible material; Special Diet
services, Witham, Essex, U.K.) which they consumed within 20 min. At 30 min, half the rats received orally 2 ml of triolein (65% pure, Sigma) by gastric intubation. The control refed animals were intubated with 2 ml of paraffin oil. Intubations were performed without anaesthesia, but with a minimum of stress. Throughout the experiments the rats were kept in their home cages with their pups present and suckling. In each group the number of pups per dam was similar (8–12).

Measurement of lipogenesis

Lipogenesis was measured in vivo with $^3$H$_2$O as previously described (Robinson et al., 1978). In the first experiment the rats were injected intraperitoneally with 3 mCi (0.3 ml) of $^3$H$_2$O at 30 min after the onset of refeeding (at the time of gastric intubation), and killed 1 h later. In the second experiment, the time-course study, lipogenesis was measured over 30 min periods after injection of $^3$H$_2$O.

At 5 min before removal of tissue, the animals were anaesthetized with Nembutal (60 mg/kg body wt., in water), and mammary tissue from the right inguinal gland was freeze-clamped (Wollenberger et al., 1960) for metabolite determinations. Venous blood was collected from the left (intact) gland, and arterial blood from the abdominal aorta. Duplicate samples of mammary gland were then taken for saponification and extraction of fatty acids (Stansbie et al., 1976) to measure the rate of lipogenesis in vivo.

Blood sampling from conscious rats

In the second experiment, the rats were fitted with a polyethylene cannula in the left carotid artery 2 days before experiment (see Jones et al., 1984; Mercer & Williamson, 1986). Blood samples were taken from the conscious animals during the experiment (0.3–0.35 ml samples, total removed < 1.5 ml) and replaced immediately with an equivalent volume of 0.9% NaCl. Another group of rats (triolein-intubated) were fitted with cannulae in the right jugular vein to allow the injection of 1.0 ml of 10% (w/v) Triton WR1339 at 30 min after the start of refeeding.

Tissue, blood and gut metabolites

Tissue metabolites were determined in freeze-clamped samples, as described previously (Mercer & Williamson, 1987). Whole blood was heparinized on collection, and added to 6% (w/v) HClO$_4$ (Robinson & Williamson, 1977a). Plasma was collected and frozen. The whole gut (stomach, small intestine and large intestine) was removed from the anaesthetized animals at the end of the experiment, and homogenized in cold 3% HClO$_4$, as described by Mercer & Williamson (1987).

Determination of metabolites

Whole-blood, gut and tissue metabolites were determined on the neutralized extracts as described previously (Mercer & Williamson, 1987). The metabolites were determined by standard enzymatic methods: glucose (Slein, 1963), l-lactate and pyruvate (Hohorst, 1963), glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963), dihydroxyacetone phosphate, glycerdehyde 3-phosphate, fructose 1,6-bisphosphate (Michal & Beutler, 1974), ketone bodies (Williamson et al., 1962) and citrate (Dagley, 1963).

Insulin

Plasma insulin was determined by radioimmunoassay with a rat insulin standard (Albano et al., 1972).

RESULTS AND DISCUSSION

In the first experiment (conscious rats, but not cannulated), refeeding chow diet to 18 h-starved lactating rats (followed by intubation of paraffin oil) caused a large re-activation of lipogenesis in the mammary gland in vivo over the first 90 min, but this was markedly (60%) suppressed if triolein was intubated (Table 1). The switch-on of lipogenesis in the control rats was similar to that previously reported from this laboratory in refed rats without paraffin-oil intubation (Mercer & Williamson, 1987); thus the presence of a non-metabolizable oil in the gut appears not to affect appreciably hydrolysis or absorption of dietary carbohydrate. Similarly, Bussmann et al. (1984) found that intubation of paraffin oil did not depress lipogenesis in mammary tissue of fed lactating rats, whereas olive oil had a marked effect.

At 90 min there were significant increases in [glucose] and [glucose 6-phosphate] in mammary gland of the triolein-treated refed rats compared with the paraffin-oil-intubated refed rats (Table 1). In contrast, neither the hepatic rates of lipogenesis nor the hepatic [glucose],

Table 1. Effect of oral triolein on lipogenesis and metabolite concentrations in the mammary gland of starved-refed lactating rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Rats . . . Starved (18 h)</th>
<th>Refed +paraffin oil</th>
<th>Refed + triolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipogenesis ($\mu$mol of $^3$H$_2$O incorporated/h per g)</td>
<td>2.9 ± 0.9 (6)</td>
<td>83.6 ± 8.7 (10)</td>
<td>33.5 ± 7.2 (13)</td>
</tr>
<tr>
<td>Tissue metabolites ($\mu$mol/g wet.)</td>
<td></td>
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</tr>
<tr>
<td>Glucose</td>
<td>1.76 ± 0.190 (7)</td>
<td>1.04 ± 0.095 (11)</td>
<td>1.45 ± 0.059 (12)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.202 ± 0.021 (7)</td>
<td>0.091 ± 0.009 (11)</td>
<td>0.203 ± 0.015 (13)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.35 ± 0.332 (7)</td>
<td>2.62 ± 0.315 (10)</td>
<td>1.76 ± 0.303 (13)</td>
</tr>
<tr>
<td>ATP</td>
<td>1.04 ± 0.173 (7)</td>
<td>1.09 ± 0.164 (11)</td>
<td>1.05 ± 0.112 (13)</td>
</tr>
</tbody>
</table>
Oral fat and mammary-gland lipogenesis

did not significantly alter this pattern. Similarly, blood [glucose] was not influenced by triolein administration (Fig. 2b), nor was the blood [lactate] (Fig. 2c). Blood [acetoacetate] and [3-hydroxybutyrate] decreased rapidly during the initial 30 min, and triolein had no effect over the 30–60 min period (Figs. 2d and 2e). However, by 60 min the blood [acetoacetate] was significantly elevated in the refed + triolein group (Fig. 2d), whereas there was no significant increase in [3-hydroxybutyrate] (Fig. 2e).

Mammary-gland lipogenesis

Lipogenesis was measured in vivo over 30 min periods, and the results are shown in Fig. 3. Little re-activation occurred over the first 30 min, despite the marked hyperinsulinaemia (Fig. 2a; see also Mercer & Williamson, 1986, 1987). Over the next 30 min (30–60 min) a large rise in lipogenic rate occurred in both groups, and there was no inhibitory influence of triolein at this stage. However, lipogenesis was dramatically decreased in the triolein-intubated rats over the 60–90 min period, i.e. 30 min after triolein intubation. Thus the inhibition of lipogenesis by triolein reported in Table 1, measured over 1 h (30–90 min), occurs solely as a result of a marked suppression over the latter 30 min of the experimental period. This pattern of inactivation of lipogenesis by triolein differs from that observed with acarbose (Mercer & Williamson, 1987), which, with an identical experimental protocol, caused an almost immediate decrease in plasma insulin and depressed lipogenesis in the mammary gland over the first 30 min after oral administration. However, whereas the locus of action of acarbose is the gut, where it decreases carbohydrate availability, the site of action of triolein appears to be at the mammary gland itself (see below). Preliminary work on cannulated animals indicates that, after oral [14C]triolein administration, there is a 30 min time lag before the radioactive lipid begins to appear in the circulation (S. W. Mercer, unpublished work), which explains why there is no immediate effect of the fat meal on lipogenesis in the mammary gland. This lag presumably represents the time taken for hydrolysis of the triolein, re-esterification of fatty acids, packaging as chylomicrons and subsequent transport via the lymphatic system to the peripheral blood. At 90 min the plasma triacylglycerol in the paraffin-oil-intubated refed rats was 72.7 ± 10.1 mg/100 ml (S.E.M.; n = 5), and 147.6 ± 22.2 mg/100 ml (S.E.M.; n = 10; P < 0.02) in the triolein-intubated rats.

Arteriovenous differences across the mammary gland

The time course of glucose utilization by the mammary gland during refeeding and triolein ingestion was also assessed directly in the second experiment, by measuring the arteriovenous differences across the gland; such measurements may serve as an ‘instantaneous monitor’ of glucose utilization (Page & Kuhn, 1986). As shown in Fig. 4(a), refeeding with chow increased glucose extraction by the gland by 30 min, after which glucose uptake remained constant in the refed controls. However, when lactate production is taken into consideration (Fig. 4c), it is clear that some of the glucose taken up by the gland at 30 min of refeeding is returned to the circulation as lactate; in terms of net C3-unit utilization by the tissue (Fig. 4b), there was a temporal increase, the 60 min and 90 min values being significantly higher than the 30 min value (P < 0.05).

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Triolein administration resulted in a significant decrease in mammary-gland glucose uptake by 90 min, but had no significant effect on the arteriovenous differences for lactate. Net C₃-unit uptake was also significantly decreased by triolein at 90 min, in agreement with the observed suppression of lipogenesis.

These data thus provide direct evidence of a rapid suppression of glucose utilization in the mammary gland by oral triolein. However, the changes in lipogenesis measured in vivo during refeeding and after triolein loading are rather larger than the changes observed in glucose utilization measured by arteriovenous difference. Since blood flow to the gland does not appear to change substantially during refeeding (Jones & Williamson, 1984; Mercer & Williamson, 1986), some reservation must exist as to whether measurements of arteriovenous differences in anaesthetised animals can be considered to provide results quantitatively similar to those in conscious rats (see also Page & Kuhn, 1986).

**Mammary-gland metabolite concentrations**

Refeeding starved lactating rats has previously been shown to result in a rapid decrease in mammary-gland [glucose] and [glucose 6-phosphate] (Mercer & Williamson, 1987), and this was confirmed in the present study (Fig. 5). By 30 min of refeeding of chow diet, there was a significant decrease in [glucose] (Fig. 5a) and [glucose 6-phosphate] (Fig. 5d) in the gland (P < 0.05), and a significant increase in [lactate] (Fig. 5b) and [fructose 1,6-bisphosphate] (P < 0.05) (Fig. 5e). Glucose and glucose 6-phosphate remained low in the refed controls at 60 and 90 min, but triolein intubation resulted in a significant increase in [glucose 6-phosphate] at 60 min, and a further increase at 90 min. In contrast, [fructose 1,6-bisphosphate] in the gland was decreased by triolein at both 60 min and 90 min (Fig. 5e). This cross-over in [glucose 6-phosphate]/[fructose 1,6-bisphosphate] points to regulation of glucose metabolism at the level of phospho-
fructokinase, similar to our previous findings with acarbose (Mercer & Williamson, 1987).

Lactate concentration in the gland was not influenced by triolein loading (Fig. 5b). There was, in both groups, a decrease in the mammary-tissue [lactate] at 60 min and 90 min, relative to the high 30 min value. This agrees with the data from the arteriovenous measurements, which indicated that production of lactate by the gland was greatest at 30 min, decreasing by 60 min and 90 min (Fig. 4c). [Pyruvate] in the mammary gland changed in a similar fashion to [lactate], with a rise at 30 min followed by a decrease, with no effect of triolein (Fig. 5c). These findings collectively indicate that pyruvate dehydrogenase is activated to some degree between 30 and 90 min of refeeding, but that triolein inhibition of lipogenesis does not involve changes in pyruvate dehydrogenase activity.

Of the other metabolites measured, i.e. ATP, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, no significant temporal changes were observed (results not shown).

Evidence for a direct effect on the mammary gland

The above results show that dietary triacylglycerol can rapidly inhibit the switch-on of lipogenesis in the mammary gland that occurs when starved lactating rats are refeed with a high-carbohydrate/low-fat chow meal. A key question, however, is whether this effect of oral triolein is due to a direct action of the triacylglycerol (or non-esterified fatty acid and glycerol released by action of lipoprotein lipase) on the mammary gland, or an indirect one. As we have shown, triolein treatment has no effect on plasma insulin concentrations, on gut [glucose] or [lactate] or on blood [glucose], which would tend to point towards a direct mode of action. To test this hypothesis, we have investigated the effect of blocking lipoprotein lipase activity with Triton WR1339 (Scanu, 1965) on the ability of oral triolein to suppress the lipogenic switch-on in the mammary gland during chow.
Fig. 5. Temporal changes in mammary-gland metabolite concentrations in starved-refed lactating rats given oral triolein

For details see the legend to Fig. 1 and the text. Triolein (○) or paraffin oil (●) was administered by gastric intubation at 30 min. Results are expressed as mean values (μmol/g fresh wt. of tissue) ± S.E.M. for five to ten animals at each time point. A significant difference between the refed plus paraffin oil and the refed plus triolein-treated rats at a given time point is indicated by: *P < 0.05; **P < 0.02; ***P < 0.001.

refeeding. The plasma triacylglycerol at 90 min was 147.6 ± 22.2 mg/100 ml (S.E.M.; n = 10) in the rats intubated with triolein and 885.0 ± 85.0 mg/100 ml (S.E.M.; n = 5; P < 0.001) in those injected with Triton WR1339. The inhibition of lipogenesis in the gland by triolein during the 60–90 min period after chow refeeding was prevented by inhibition of lipoprotein lipase activity with Triton WR1339 (Table 2). This result is strong evidence that dietary lipid acutely inhibits lipogenesis in the tissue via a direct mechanism requiring the uptake of the plasma triacylglycerol as non-esterified fatty acids. Administration of glycerol (the other product of lipoprotein lipase action) to lactating rats (blood concn. 3 mM) did not inhibit lipogenesis in mammary gland (Agius & Williamson, 1980).

Phosphofructokinase as a site of regulation

The present data (Fig. 5) and our previous work (Mercer & Williamson, 1987) implicate phosphofructokinase activity in the rapid modulation of lipogenesis in the mammary gland during the starvation–refed transition. This can be best seen in Fig. 5(f), which shows the ratio [glucose 6-phosphate]/[fructose 1,6-bisphosphate] during the course of the experimental period. The changes in ratio can be interpreted to indicate that phosphofructokinase is rapidly activated by refeeding, and inactivated
Table 2. Effects of Triton WR1339 on blood ketone bodies, mammary-gland lipogenesis and mammary-gland [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio and [citrate] in refed lactating rats given oral triolein

For full details see the Materials and methods section. Lipogenesis was measured in vivo with $^3$H$_2$O over the 60–90 min period, and the results are expressed as $\mu$mol of $^3$H$_2$O incorporated/h per g wet wt. Triton WR1339 was injected at 30 min via an indwelling cannula in the right jugular vein. Mammary-gland metabolites are expressed as $\mu$mol/g wet wt. Blood ketone bodies are $\mu$mol/ml of whole blood. Values that are significantly different by Student’s t test from the refed + paraffin oil group are indicated by: *$P < 0.05$; **$P < 0.001$. A significant difference between the refed + triolein and the Triton-treated rats is indicated by: †$P < 0.05$; ††$P < 0.001$.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Rats . . .</th>
<th>Refed + paraffin oil</th>
<th>Refed + triolein</th>
<th>Refed + triolein + Triton WR1339</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio</td>
<td>56.9±12.8 (5)</td>
<td>12.8±3.9 (9)*</td>
<td>40.0±9.2 (6)†</td>
<td></td>
</tr>
<tr>
<td>[Citrate]</td>
<td>4.0±0.7 (9)</td>
<td>20.8±5.8 (8)*</td>
<td>5.0±0.7 (5)†</td>
<td></td>
</tr>
<tr>
<td>[Citrate]</td>
<td>0.194±0.015 (8)</td>
<td>0.275±0.009 (7)**</td>
<td>0.209±0.012 (5)††</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Acetoacetate]</td>
<td>0.140±0.023 (9)</td>
<td>0.227±0.038 (9)</td>
<td>0.255±0.033 (6)*</td>
<td></td>
</tr>
<tr>
<td>[3-Hydroxybutyrate]</td>
<td>0.157±0.042 (9)</td>
<td>0.282±0.041 (9)*</td>
<td>0.094±0.056 (6)†</td>
<td></td>
</tr>
<tr>
<td>[Total ketone bodies]</td>
<td>0.296±0.043 (9)</td>
<td>0.508±0.069 (9)*</td>
<td>0.349±0.056 (6)</td>
<td></td>
</tr>
</tbody>
</table>

by triolein loading, with a significant effect of triolein (given at 30 min) by 60 min. It is important to note that this inhibition of phosphofructokinase by triolein is detectable immediately before the inhibition of lipogenesis in the gland (Fig. 3), which suggests that phosphofructokinase may play a key role in the short-term regulation of glucose flux/ lipogenesis in the gland by dietary lipid. This contention is further supported by the fact that the prevention by Triton WR1339 of the triolein-induced inhibition of lipogenesis was associated with no increase in the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio (Table 2).

How then does oral triolein, acting via a direct effect on the mammary gland itself, regulate the activity of phosphofructokinase? A candidate for allosteric regulation of phosphofructokinase is citrate, which has been found to inhibit phosphofructokinase in mammary tissue (Zammit, 1979) and in other tissues (Randle et al., 1964). In such tissues, fatty acid-induced inactivation of glycolytic flux is mediated by changes in cytosolic [citrate], which potentiates the inhibition of phosphofructokinase by ATP. As shown in Table 2, [citrate] in the mammary gland was significantly ($P < 0.01$) elevated by triolein loading, and Triton WR1339, which prevented the inhibitory effect of triolein on lipogenesis and phosphofructokinase activity in the gland, also abolished this rise in [citrate]. The increase in [citrate] after triolein intubation may occur because of the increase in fatty acid uptake by the gland, and/or the rise in circulating [acetoacetate] over the 60–90 min period (Fig. 2d). The latter possibility seems less likely, because Triton WR1339 had no significant effect on blood [acetoacetate] or [total ketone bodies] (Table 2). Indeed, the general importance of ketone bodies in the control of glucose metabolism in the mammary gland is still controversial; whereas Robinson & Williamson (1977b) found an appreciable decrease in glucose uptake by the mammary gland in vivo after intravenous administration of acetoacetate to fed lactating rats, Page & Kuhn (1986) found no effect. In vitro, physiological concentrations of acetoacetate have been found to inhibit glucose utilization by mammary-gland acini (Robinson & Williamson, 1977a).

The concentration of fructose 2,6-bisphosphate, a potential activator of phosphofructokinase in mammary gland (Sochor et al., 1984; Ward & Kuhn, 1985), was not measured in the present experiments. However, the changes in [fructose 2,6-bisphosphate] on transition from the starved to the refed state or during short-term starvation (6 h) are modest (50%); Ward & Kuhn, 1985), compared with the 10-fold change in lipogenesis in these situations. Perhaps more important, Ward & Kuhn (1985) concluded from kinetic studies that phosphofructokinase was virtually saturated with fructose 2,6-bisphosphate in vivo.

In conclusion, the present study provides evidence of the extreme rapidity with which the mammary gland responds to changes in food availability and composition of nutrient supply. Phosphofructokinase appears to play an important role in the modulation of lipogenesis in the gland during both activation by chow refeeding and inhibition by oral triolein. The present evidence suggests that the activity of pyruvate dehydrogenase is not acutely regulated by increased availability of lipid in rat mammary tissue. Further work is required to elucidate the precise time course of the changes in activity of other key lipogenic enzymes, such as acetyl-CoA carboxylase, which has been implicated in the chronic response of the gland to dietary lipid (Munday & Williamson, 1987).

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REFERENCES

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