The regulation of lipogenesis *in vivo* in the lactating mammary gland of the rat during the starved–refed transition

Studies with acarbose, a glucosidase inhibitor

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1. Depression of carbohydrate digestion by oral administration of acarbose, a glucosidase inhibitor, led to a 75% inhibition of the re-activation of lipogenesis *in vivo* in the mammary gland of 18 h-starved lactating rats refed with 5 g of chow diet. Rates of [$1\text{-}^{14}\text{C}$]glucose incorporation *in vitro* into lipid and CO_2 in mammary-gland acini isolated from refed animals were elevated compared with acini from starved rats, but acarbose treatment completely prevented this stimulation. 2. Gastric intubation of glucose led to a large stimulation of lipogenesis in the mammary gland of starved lactating rats, similar to that induced by refeeding with chow diet; this was dependent on the amount of glucose given and the time elapsed between glucose administration and injection of $^3\text{H}_2\text{O}$ for the measurement of lipogenesis. 3. The switch-on of lipogenesis in the mammary gland of starved lactating rats, by refeeding or by intubation of glucose, was associated with a decrease in the ratio of [glucose 6-phosphate]/[fructose 1,6-bisphosphate] in the gland, indicative of an increase in phosphofructokinase activity. A time-course study revealed that the ratio decreased rapidly over the first 30 min of chow refeeding, after which a large surge in lipogenesis was seen. Acarbose, given 25 min after the onset of refeeding, led to a stepwise increase in the ratio, in parallel with the observed decrease in lipogenic activity. 4. It is concluded that the control of lipogenesis in the mammary gland is closely linked to the availability of dietary carbohydrate. An important site of regulation of lipogenesis in the gland appears to be at the level of phosphofructokinase. A possible role of insulin in the regulation of phosphofructokinase activity, and the acute modulation of insulin-sensitivity in the gland during the starved–refed transition, are discussed.

INTRODUCTION

During lactation in the rat, the mammary gland becomes the major glucose-utilizing tissue in the body, with lipogenesis being quantitatively the most important pathway in terms of glucose utilization by the gland (for review, see Williamson, 1980). A striking feature of lipogenesis in the mammary gland is its high sensitivity to dietary carbohydrate supply. Short-term starvation of lactating rats, for as little as 6 h, results in a near-full suppression of lipogenesis in the gland (Jones *et al.*, 1984), which can be rapidly re-activated, within 90 min (Williamson *et al.*, 1985; Mercer & Williamson, 1986), by refeeding with chow diet (Munday & Williamson, 1981; Busmann *et al.*, 1984; Williamson *et al.*, 1985). The signals involved in these rapid changes in mammary-gland lipogenesis are not fully understood, although insulin appears to play a key role (Robinson & Williamson, 1977; Robinson *et al.*, 1978; Burnol *et al.*, 1983; Busmann *et al.*, 1984; Jones *et al.*, 1984; Mercer & Williamson, 1986; Page & Kuhn, 1986).

Recent work has suggested that the insulin-sensitivity of the mammary gland may be acutely increased during limited refeeding (5 g of chow diet to 18 h-starved lactating rats; Mercer & Williamson, 1986); furthermore, the temporal pattern of re-activation of lipogenesis in this refeeding model suggests that the control of mammary-gland lipogenesis by insulin is very precisely tuned to the availability of substrate (Mercer & Williamson, 1986). The aim of the present study was to investigate this hypothesis, by the use of acarbose, a

glucosidase inhibitor which retards carbohydrate digestion (Puls *et al.*, 1984). The importance of substrate supply was further investigated by oral administration of graded amounts of glucose.

EXPERIMENTAL

Materials

Rats. Primiparous lactating Wistar rats (11–16 days of lactation) were maintained at an ambient temperature of $22 \pm 2^\circ\text{C}$ under a 12 h-light/12 h-dark cycle (light from 07:30 h) and were fed *ad libitum* on a low-fat/high-carbohydrate chow diet (52% carbohydrate, 21% protein, 4% fat; residue non-digestible material; Special Diet Services, Witham, Essex, U.K.).

Biochemicals. All enzymes, including collagenase (grade II), and coenzymes were from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Acarbose was generously given by Dr. W. Puls, Bayer A.G., Wuppertal, Germany.

Radioactive compounds. $^3\text{H}_2\text{O}$ and D-[1- ^{14}C]glucose were obtained from Amersham International, Amersham, Bucks., U.K.

Studies *in vivo*

Treatments of rats. Experiments were initiated between 09:00 h and 10:00 h. All the animals were starved for 18 h before this. Throughout the entire experimental

Table 1. Effects of natural refeeding, acarbose and oral glucose on rates of lipogenesis *in vivo* in mammary gland and liver of starved lactating rats

All groups of lactating rats were starved overnight (18 h) before the experiments. For further details see the Experimental section. The results are means $\pm$ s.e.m., expressed as μmol of $^3\text{H}_2\text{O}$ incorporated into saponified lipid/h per g fresh wt. of tissue and with the numbers of observations shown in parentheses. Values that are significantly different by Student's *t* test from those for the refed group are indicated by: **P* < 0.05; ****P* < 0.001. Values that are significantly different by the Student's *t* test from those for the starved group are indicated by: †*P* < 0.005; ††*P* < 0.01; †††*P* < 0.001.

Treatment of rats	Rates of lipogenesis <i>in vivo</i> (μmol of $^3\text{H}_2\text{O}$ incorporated into saponified lipid/h per g fresh wt. of tissue)	
	Mammary gland	Liver
Starved (18 h)	3.7 $\pm$ 0.6 (9)***	5.5 $\pm$ 0.7 (8)
Refed (90 min)	73.0 $\pm$ 6.7 (13)†††	6.6 $\pm$ 0.5 (13)
Refed (90 min) + acarbose	17.7 $\pm$ 2.9 (14)***, †††	6.4 $\pm$ 0.8 (14)
Oral glucose (6 mmol; 75 min)	60.3 $\pm$ 7.8 (9)†††	7.9 $\pm$ 0.8 (8)†
Oral glucose (3 mmol; 75 min)	28.9 $\pm$ 7.9 (4)***, †††	5.8 $\pm$ 0.5 (4)
Oral glucose (1.5 mmol; 75 min)	15.4 $\pm$ 2.8 (4)***, ††	3.8 $\pm$ 0.2 (4)***, †
Oral glucose (6 mmol; 3 h)	5.9 $\pm$ 1.6 (4)***	3.5 $\pm$ 0.3 (4)***, †
Refed (90 min) + acarbose + glucose (6 mmol; 75 min)	102.6 $\pm$ 14.2 (6)†††	8.6 $\pm$ 1.8 (6)
Refed (90 min) + acarbose + glucose (1.5 mmol; 75 min)	48.3 $\pm$ 6.4 (6)*, †††	5.8 $\pm$ 0.8 (5)

procedures the rats were kept in their individual home cages with their pups present. The experimental groups in the first experiment were as follows: (a) starved; injected with $^3\text{H}_2\text{O}$ intraperitoneally; (b) chow-refed; starved rats were given 5 g of pelleted chow diet (which they consumed within 10–20 min), and $^3\text{H}_2\text{O}$ was injected 30 min after the start of refeeding; (c) chow-refed plus acarbose; 1 ml of water containing 5 mg of acarbose was administered by gastric intubation 25 min after start of refeeding (5 g chow), and $^3\text{H}_2\text{O}$ was injected at 30 min; (d) glucose load; starved rats were given 3 ml of a glucose solution (containing 6 mmol, 3 mmol or 1.5 mmol) by gastric intubation, and injected with $^3\text{H}_2\text{O}$ 15 min later; (e) 6 mmol glucose load 2 h before injection of $^3\text{H}_2\text{O}$; (f) refed plus acarbose plus 6 mmol of glucose (glucose given 15 min before $^3\text{H}_2\text{O}$); as in (c); (g) refed plus acarbose plus 1.5 mmol of glucose; as in (f). The intubation of solutions was performed without anaesthesia and with the minimum of stress. In each experimental group the average number of pups per dam was similar (average nine), as was the mean litter weight (approx. 175 g).

Measurement of lipogenesis. Lipogenesis was measured *in vivo* with $^3\text{H}_2\text{O}$ as previously described (Robinson *et al.*, 1978). The rats were injected intraperitoneally with 2.5 mCi (0.25 ml) of $^3\text{H}_2\text{O}$, and tissue was removed 1 h later; 5 min before tissue removal the rats were anaesthetized with pentobarbital (60 mg/kg body wt.). At 60 min the animals were dissected and the tissues freeze-clamped for metabolite determinations (see below). Duplicate samples of mammary gland and liver were then taken for saponification and extraction of fatty acids (Stansbie *et al.*, 1976) to measure the incorporation of ^3H into saponified lipid *in vivo*.

In the second experiment, the time-course study, the lactating rats were fitted with polyethylene cannulae in the right carotid artery and left jugular vein, 2 days

before the experiment was performed (for details see Mercer & Williamson, 1986). In these animals, lipogenesis was measured over 30 min periods during refeeding, with the $^3\text{H}_2\text{O}$ being introduced via the jugular-vein cannulae, and blood samples removed from the carotid artery at 3, 5, 10, 15 and 25 min post-infusion. Half the rats received acarbose (5 mg in 1 ml); the others received 1 ml of water by gastric intubation, 25 min after the start of refeeding. The animals were conscious throughout these experiments until 3–5 min before tissue removal for freeze-clamping.

Tissue and blood metabolites. Mammary-gland and liver samples (1–2 g) were rapidly removed from anaesthetized animals and freeze-clamped between liquid- N_2 -cooled light-alloy tongs (Wollenberger *et al.*, 1960). The samples were then stored in liquid N_2 until the metabolites were determined in the neutralized tissue extract (Robinson & Williamson, 1977). Arterial blood was collected from the abdominal aorta into heparinized syringes. A sample of this whole blood (0.2 ml) was taken for metabolite measurements and added to 2 ml of 6% (w/v) HClO_4 and treated as described by Robinson & Williamson (1977). Plasma was separated by centrifugation at 3000 rev./min for 10 min and used for determination of the specific radioactivity of $^3\text{H}_2\text{O}$ and plasma insulin concentrations.

Gut metabolites. The gastrointestinal tract, consisting of the stomach, small intestine and large intestine, was removed from the anaesthetized animals. The whole gut, including the gut contents, was then transferred to 150 ml of ice-cold 3% HClO_4 and homogenized for 3–4 min in a Waring blender. A portion of the homogenate was centrifuged, and the supernatant neutralized for the determination of glucose and lactate concentrations.

Fig. 1. Temporal changes in mammary-gland lipogenesis and in gastrointestinal and blood concentrations of glucose and lactate during refeeding, with and without acarbose treatment of starved lactating rats

Starved lactating rats (18 h) were refed with 5 g of chow diet, which they consumed within 20 min. Acarbose was administered by gastric intubation at 25 min. Results are expressed as mean values $\pm$ S.E.M. for at least four animals per time point. A significant difference between refed and refed plus acarbose-treated animals at a given time point as indicated by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Studies *in vitro*

Experiments with acini. Mammary-gland acini were prepared as described by Robinson & Williamson (1977). Approx. 100 mg of acini (wet wt.) were incubated in a total volume of 4 ml of Krebs–Henseleit (1932) saline containing $[1-^{14}\text{C}]$ glucose (5 mM). Measurements were carried out in the basal state (no insulin added) and in the presence of 30 munits of insulin/ml. For further details see Robinson & Williamson (1977).

Determination of metabolites. The neutralized extracts of freeze-clamped mammary tissues were filtered through 0.45 μm -pore-size type HA Millipore filters (Millipore SA, Molsheim, France) to remove fat globules before measurements of metabolites. The following metabolites were determined enzymically: glucose (Slein, 1963), L-lactate (Hohorst, 1963), glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963), glycogen (Keppler & Decker, 1974), dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, fructose 1,6-bisphosphate (Michal & Beutler, 1974), 6-phosphogluconate (Haid, 1974), pyruvate (Hohorst, 1963), 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

Effect of natural refeeding, acarbose and oral glucose on rates of lipogenesis *in vivo* in the mammary gland of starved lactating rats

Refeeding 5 g of chow diet to the 18 h-starved lactating rats led to a 20-fold increase in the rate of lipogenesis in the mammary gland within a period of 90 min (Table 1; see also Williamson *et al.*, 1985; Mercer & Williamson, 1986). However, intubation of acarbose, to decrease carbohydrate digestion (Puls *et al.*, 1984; Fig. 1b), resulted in a 75% suppression of the normal lipogenic switch-on in the mammary gland of chow-refed animals. That the effect of acarbose is limited to carbohydrate digestion in the intestine is indicated by the large lipogenic re-activation in the glands of refed acarbose-treated rats given oral glucose loads (Table 1).

Interestingly, a normal activation of lipogenesis in the gland occurred in the animals intubated with 6 mmol of glucose at 75 min before death (Table 1). Gastric intubation of 3 mmol and 1.5 mmol of glucose produced a graded response in mammary-gland lipogenic rates, to values almost exactly 50% and 25% respectively of the 6 mmol value (Table 1). This re-activation of the lipogenic activity of the mammary gland of starved lactating rats by 6 mmol of oral glucose contrasts markedly with the findings, and implications, of previous studies (Munday & Williamson, 1981; Bussmann *et al.*,

Table 2. Effects of natural refeeding, acarbose and oral glucose on hepatic glycogen of starved lactating rats

All groups of lactating rats were starved overnight (18 h) before the experiments. For further details see the Experimental section. The results are mean values $\pm$ S.E.M., expressed as $\mu\text{mol/g}$ fresh wt. of liver with the numbers of observations in parentheses. Values that are significantly different by Student's *t* test from those for the refed group are indicated by: **P* < 0.05; ****P* < 0.005. Values that are significantly different by the Student's *t* test from those for the starved group are indicated by: †*P* < 0.05; ††*P* < 0.01; †††*P* < 0.001.

Treatments of rats	Glycogen ($\mu\text{mol/g}$ fresh wt. of liver)
Starved (18 h)	9.8 $\pm$ 5.6 (5)***
Refed (90 min)	44.0 $\pm$ 5.3 (8)†††
Refed (90 min) + acarbose	30.4 $\pm$ 5.3 (9)†
Oral glucose (6 mmol; 75 min)	32.0 $\pm$ 5.3 (5)†
Oral glucose (3 mmol; 75 min)	15.5 $\pm$ 2.9 (4)***
Oral glucose (1.5 mmol; 75 min)	10.6 $\pm$ 5.6 (2)***
Oral glucose (6 mmol; 3 h)	34.5 $\pm$ 4.0 (4)††
Refed (90 min) + acarbose + glucose (6 mmol; 75 min)	58.7 $\pm$ 14.2 (4)†
Refed (90 min) + acarbose + glucose (1.5 mmol; 75 min)	22.0 $\pm$ 4.9 (4)*

1984). The reason for this discrepancy appears to lie in the precise time course between glucose administration and measurement of lipogenic rate; although 6 mmol of oral glucose produced a large increase in lipogenic rate within 75 min, by 3 h this response had gone, and the lipogenic rate in the mammary gland had returned to that in starvation (Table 1).

Hepatic lipogenesis and glycogen synthesis

Rates of lipogenesis in the liver of the lactating 18 h-starved rats were not responsive to 90 min refeeding (see also Williamson *et al.*, 1985), nor to oral glucose loading (Table 1), in marked contrast with the extreme

sensitivity of this process in the mammary gland. However, hepatic glycogen contents were influenced by the different treatments (Table 2). Refeeding led to a 4.5-fold increase in glycogen content, and oral glucose administration also markedly stimulated glycogen repletion in starved animals, the response being graded according to the amount of glucose administered. Interestingly, however, in rats given 6 mmol of glucose 3 h before death, glycogen concentrations remained high, at the same value as in those rats killed 75 min after the same load of glucose, whereas lipogenesis in the mammary gland had been depressed by this time. Another interesting feature is the normal rise in glycogen stores observed in the chow-refed rats treated with acarbose (Table 2), despite the failure to stimulate lipogenesis in the mammary gland of the same animals (Table 1). Several studies have indicated that, on refeeding starved lactating (Williamson *et al.*, 1985) and non-lactating (Newgard *et al.*, 1983, 1984; Sugden *et al.*, 1983) rats, the liver remains in a gluconeogenic mode for some time. The normal increase in hepatic glycogen in the refed-plus-acarbose-treated animals implies that dietary glucogenic amino acids may be important precursors of hepatic gluconeogenesis and glycogen synthesis in the lactating animal.

Glucose metabolism in isolated mammary-gland acini from starved, refed, and refed-plus-acarbose-treated rats

To support the measurements *in vivo* of mammary-gland lipogenesis, glucose metabolism was also studied *in vitro*, in acini isolated from the mammary glands of starved, refed, and refed-plus-acarbose-treated lactating rats. Refeeding in the presence of acarbose (depressed carbohydrate absorption) produced no augmentation of glucose (5 mM) metabolism in comparison with the acini from starved animals, under either basal or insulin-stimulated conditions (Table 3). Refeeding alone, however, produced elevated rates of $^{14}\text{CO}_2$ production and glucose incorporation into lipid relative to 'starved' values, as shown previously (Robinson & Williamson, 1977; Munday & Williamson, 1981; Williamson *et al.*, 1985). Thus natural refeeding, in the absence of normal

Table 3. Glucose utilization and lipogenesis *in vitro* in mammary-gland acini isolated from starved, refed and refed-plus-acarbose-treated lactating rats

The isolated acini were incubated with [1- ^{14}C]glucose (5 mM) in either the presence or the absence of insulin (30 munits/ml). For other details see the Experimental section. Results are mean values $\pm$ S.E.M., expressed as $\mu\text{mol/min}$ per 100 mg defatted dry wt. and are for five to seven individual experiments in each group. Values that differ significantly by the Student's *t* test from the corresponding values for the acini from the starved rats are indicated by: **P* < 0.05; ***P* < 0.002. A significant effect of insulin compared with the corresponding group in the absence of the hormone is indicated by: †*P* < 0.05; ††*P* < 0.01.

Measurement	Insulin . . .	Treatment of rats					
		Starved (18 h)		Refed		Refed + acarbose	
		—	+	—	+	—	+
Glucose removal		1.02 $\pm$ 0.09	1.22 $\pm$ 0.08	1.13 $\pm$ 0.09	1.30 $\pm$ 0.14	1.09 $\pm$ 0.09	1.21 $\pm$ 0.10
[1- ^{14}C]Glucose $\rightarrow$ $^{14}\text{CO}_2$		0.15 $\pm$ 0.02	0.23 $\pm$ 0.02††	0.23 $\pm$ 0.02**	0.29 $\pm$ 0.02	0.17 $\pm$ 0.02	0.20 $\pm$ 0.04
[1- ^{14}C]Glucose $\rightarrow$ ^{14}C -labelled lipid		0.15 $\pm$ 0.02	0.21 $\pm$ 0.01†	0.28 $\pm$ 0.04**	0.30 $\pm$ 0.03*	0.17 $\pm$ 0.03	0.21 $\pm$ 0.03
Lactate formation		0.89 $\pm$ 0.13	1.11 $\pm$ 0.13	0.85 $\pm$ 0.08	0.96 $\pm$ 0.10	0.95 $\pm$ 0.04	1.03 $\pm$ 0.06
% of glucose utilized appearing as lactate		43.5 $\pm$ 4.7	45.1 $\pm$ 3.3	37.8 $\pm$ 3.9	37.8 $\pm$ 3.9	44.9 $\pm$ 3.5	44.3 $\pm$ 3.7

Table 4. Effects of natural refeeding, acarbose and oral glucose on metabolite concentrations in freeze-clamped mammary glands of starved lactating rats

For full details see the Experimental section. The results are mean values $\pm$ s.e.m. expressed as $\mu\text{mol/g}$ fresh wt. of tissue. Values that are significantly different by Student's *t* test from the corresponding values for the refed group are indicated by: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Values that are significantly different by Student's *t* test from the corresponding values for the starved group are indicated by: †*P* < 0.05; ††*P* < 0.01; †††*P* < 0.001.

Treatment	Mammary-gland metabolite concn. ($\mu\text{mol/g}$ wet wt.)						
	Glucose	Glucose 6-phosphate	Fructose 1,6-bisphosphate	Dihydroxyacetone phosphate	Glyceraldehyde 3-phosphate	Lactate	Pyruvate
Starved (18 h)	1.56 $\pm$ 0.13* (7)	0.205 $\pm$ 0.009*** (9)	0.009 $\pm$ 0.001** (8)	0.015 $\pm$ 0.004 (9)	0.003 $\pm$ 0.002 (9)	0.66 $\pm$ 0.09* (6)	0.05 $\pm$ 0.01** (8)
Refed (90 min)	0.96 $\pm$ 0.17† (7)	0.099 $\pm$ 0.007††† (9)	0.019 $\pm$ 0.003†† (9)	0.022 $\pm$ 0.002 (9)	0.006 $\pm$ 0.001 (9)	1.89 $\pm$ 0.42† (7)	0.16 $\pm$ 0.03†† (9)
Refed (90 min) + acarbose	1.50 $\pm$ 0.09* (8)	0.157 $\pm$ 0.012***, ††† (10)	0.010 $\pm$ 0.002* (9)	0.009 $\pm$ 0.002*** (10)	0.005 $\pm$ 0.002 (10)	0.97 $\pm$ 0.12 (7)	0.09 $\pm$ 0.01† (10)
Oral glucose (6 mmol; 75 min)	1.05 $\pm$ 0.33 (6)	0.073 $\pm$ 0.013††† (6)	0.021 $\pm$ 0.004† (6)	0.020 $\pm$ 0.005 (6)	0.009 $\pm$ 0.004 (6)	2.16 $\pm$ 0.45†† (6)	0.14 $\pm$ 0.04† (5)
Oral glucose (3 mmol; 75 min)	1.59 $\pm$ 0.15* (4)	0.159 $\pm$ 0.021* (4)	0.016 $\pm$ 0.002† (4)	0.010 $\pm$ 0.003** (3)	0.004 $\pm$ 0.003 (3)	1.12 $\pm$ 0.14† (4)	0.08 $\pm$ 0.01* (4)
Oral glucose (1.5 mmol; 75 min)	1.50 $\pm$ 0.10* (4)	0.203 $\pm$ 0.018*** (4)	0.011 $\pm$ 0.002* (4)	0.011 $\pm$ 0.006 (4)	0.004 $\pm$ 0.001 (4)	0.99 $\pm$ 0.18 (4)	0.05 $\pm$ 0.01** (4)
Oral glucose (6 mmol; 3 h)	1.33 $\pm$ 0.04 (4)	0.211 $\pm$ 0.006*** (4)	0.013 $\pm$ 0.003 (4)	0.010 $\pm$ 0.002*** (4)	0.005 $\pm$ 0.003 (4)	1.34 $\pm$ 0.13†† (4)	0.09 $\pm$ 0.02 (4)
Refed (90 min) + acarbose + oral glucose (6 mmol; 75 min)	0.70 $\pm$ 0.10††† (5)	0.085 $\pm$ 0.017††† (4)	0.022 $\pm$ 0.004† (5)	0.027 $\pm$ 0.006††† (5)	0.004 $\pm$ 0.002 (5)	1.61 $\pm$ 0.35† (5)	0.07 $\pm$ 0.02* (5)
Refed (90 min) + acarbose + oral glucose (1.5 mmol)	1.11 $\pm$ 0.19 (5)	0.141 $\pm$ 0.025† (5)	0.014 $\pm$ 0.003 (5)	0.013 $\pm$ 0.002** (5)	0.006 $\pm$ 0.001 (5)	1.08 $\pm$ 0.14† (5)	0.11 $\pm$ 0.02† (5)

Fig. 2. Temporal changes in mammary-gland metabolite concentrations during refeeding with and without acarbose treatment of starved lactating rats

For details see the legend to Fig. 1 and the text. Results are expressed as mean values ($\mu\text{mol/g}$ fresh wt. of tissue) $\pm$ S.E.M. for at least four animals at each time point. A significant difference between refed and refed plus acarbose-treated animals at a given time point is indicated by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

substrate (carbohydrate) supply *in vivo*, would not appear to be sufficient to activate glucose metabolism in the mammary-gland acini *in vitro*.

Pyruvate dehydrogenase undergoes inactivation in the mammary gland on short-term starvation (Baxter & Coore, 1978; Munday & Williamson, 1981) and is presumably re-activated during refeeding. However, the time course of re-activation of pyruvate dehydrogenase during refeeding is not known, and in the present model (90 min refeeding, 5 g of chow) it would appear, from the amount of glucose utilized appearing as lactate (an indirect measure of pyruvate dehydrogenase activity), that the enzyme does not undergo major re-activation.

Mammary-gland metabolite concentrations

Glucose and glucose 6-phosphate concentrations were significantly decreased by 90 min refeeding (high lipogenic switch-on) compared with the values in the starved animals (Table 4; see also Jones *et al.*, 1984; Williamson *et al.*, 1985). Acarbose prevented this decrease in tissue glucose and glucose 6-phosphate on refeeding; generally, the metabolic profile of the mammary gland of the acarbose-treated refed rats resembled that of the starved animals, rather than that of the refed (see time-course study, below). That substrate supply is of over-riding importance in the activation of the gland is implied by the

similarity in metabolite concentrations observed between the refed animals and those intubated with 6 mmol of glucose for 75 min (which showed a high lipogenic switch-on). Notably, the fall in lipogenic rate in the gland 3 h after the 6 mmol of glucose (Table 1) was accompanied by a significant increase in glucose 6-phosphate, indicating the high sensitivity of the tissue to rapid changes in dietary carbohydrate availability. In view of the inverse relationship between mammary tissue glucose 6-phosphate concentration and lipogenic rate, it is unlikely that glucose transport is a primary site of regulation. Rather, the increase in glucose 6-phosphate in the gland on starvation, and the decrease on refeeding or glucose administration, points to regulation of glucose flux at a step beyond glucose 6-phosphate.

In addition to the negative relationship observed between glucose 6-phosphate concentration and lipogenic rate in the mammary gland, a positive correlation was also found between lipogenesis and fructose 1,6-bisphosphate concentration (Table 4). This inverse relationship between glucose 6-phosphate and fructose 1,6-bisphosphate suggests an important point of metabolic regulation at the level of phosphofructokinase [see below and Jones *et al.* (1984)].

Lactate and pyruvate concentrations in the mammary gland rose substantially (more than 3-fold) on refeeding,

Table 5. Relationship between the rate of lipogenesis and [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio in mammary glands of starved and refed lactating rats

Data for lipogenesis are taken from Table 1 and for the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio from Table 4. The results are given as mean values $\pm$ S.E.M., with the numbers of observations shown in parentheses. Values that are statistically different by Student's *t* test from those for refed rats are indicated by: **P* < 0.05; ****P* < 0.001. Values that are statistically different by Student's *t* test from those for starved rats are indicated by: †*P* < 0.05; †††*P* < 0.001.

Treatment of rats	Mammary-gland lipogenesis <i>in vivo</i> (μ mol/h per g)	[Glucose 6-phosphate] [fructose 1,6-bisphosphate] ratio
Starved (18 h)	3.7 $\pm$ 0.6 (9)***	23.0 $\pm$ 3.8 (8)***
Refed (90 min)	73.0 $\pm$ 6.7 (13)†††	6.0 $\pm$ 0.8 (9)†††
Refed (90 min) + acarbose	17.7 $\pm$ 2.9 (14)***,†††	19.0 $\pm$ 4.8 (9)*
Oral glucose (6 mmol; 75 min)	60.3 $\pm$ 7.8 (9)†††	4.4 $\pm$ 1.0 (6)†††
Oral glucose (3 mmol; 75 min)	28.9 $\pm$ 7.9 (4)***,†††	10.2 $\pm$ 1.9 (4)†
Oral glucose (1.5 mmol; 75 min)	15.4 $\pm$ 2.8 (4)***,†††	2.3 $\pm$ 5.7 (4)*
Oral glucose (6 mmol; 3 h)	5.9 $\pm$ 1.6 (4)***	22.5 $\pm$ 9.8 (4)
Refed (90 min) + acarbose + glucose (6 mmol; 75 min)	102.6 $\pm$ 14.2 (6)†††	5.0 $\pm$ 0.9 (5)†††
Refed (90 min) + acarbose + glucose (1.5 mmol; 75 min)	48.3 $\pm$ 6.4 (6)*,†††	13.4 $\pm$ 4.7 (5)

and this increase was considerably less in the presence of acarbose (Table 4). Oral intubation of glucose produced an increase in both lactate and pyruvate concentrations in the gland (Table 4). This increase in lactate and pyruvate in the gland during refeeding supports the evidence *in vitro* that pyruvate dehydrogenase does not undergo appreciable re-activation within 90 min of refeeding (see Table 3). Thus during short-term refeeding it would appear that glucose is taken up by the mammary gland in large amounts, perhaps in excess of normal (fed) requirements, in order to compensate for the relative inactivity of pyruvate dehydrogenase, and hence the 'loss' of glucose as lactate (or pyruvate), which presumably is released from the tissue to be reconverted into glucose in the liver (see Williamson *et al.*, 1985) and returned to the gland. This view is supported by the temporal changes in blood and gland lactate concentrations over the refeeding period (see below, and Figs. 1 and 2).

Time course of changes in mammary-gland lipogenesis and metabolite concentrations during refeeding; the effects of acarbose

In order to relate the observed changes in metabolite concentrations directly to the regulation of lipogenesis, a time-course study was performed on starved/refed lactating rats (Figs. 1 and 2).

Lipogenic rate (measured over 30 min periods) showed a sharp increase after the first 30 min of refeeding (Fig. 1a), and this was significantly inhibited by acarbose (given at 25 min). Between 60 and 90 min, acarbose-treated animals exhibited lipogenic rates similar to the 'starved' values. Lipogenesis had also fallen substantially in the refed rats by this stage, underlining the sensitivity of the process in the gland to the availability and supply of substrate.

The free glucose content of the gastrointestinal tract rose rapidly on refeeding, but was significantly decreased after acarbose intubation (Fig. 1b). In contrast, gut lactate concentrations, which also increased significantly on refeeding, were not influenced by acarbose (Fig. 1c).

Blood glucose concentration was unaffected by acarbose treatment (Fig. 1d), whereas acarbose tended to decrease the blood lactate (Fig. 1e). Over the first 30 min of refeeding, lactate in the blood rose by some 97% (*P* < 0.002) compared with the starved value. In the mammary gland, glucose concentration fell, and lactate rose, during refeeding, and these changes were essentially reversed by acarbose administration (Figs. 2a and 2b).

Glucose 6-phosphate was significantly decreased in the mammary gland by 30 min of refeeding (*P* < 0.0001; Fig. 2c). Acarbose led to a significant increase in glucose 6-phosphate by 60 min (*P* < 0.05) compared with the 60 min-refed value. The changes in fructose 1,6-bisphosphate concentration in the gland were temporally similar to, but the opposite of, the changes in glucose 6-phosphate (Fig. 2d), again with acarbose largely reversing them. No significant temporal changes in dihydroxyacetone phosphate and glyceraldehyde 3-phosphate concentrations were found (results not shown).

Phosphofructokinase as a site of regulation of the activation of lipogenesis in the mammary gland

As indicated above, the inverse relationship between mammary-gland glucose 6-phosphate and fructose 1,6-bisphosphate concentrations observed in the present study suggests an important role of phosphofructokinase in the modulation of glucose flux and lipogenesis in the mammary gland during starvation and short-term refeeding. Table 5 demonstrates this more clearly, and shows for each experimental group in Expt. 1 the rate of lipogenesis *in vivo* in the gland, and the corresponding [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio. The time course of changes in the ratio, from Expt. 2, is shown in Fig. 2(e). A large fall in the ratio was observed by 30 min of refeeding (indicative of a rapid increase in phosphofructokinase activity), and this occurred immediately before the large surge in lipogenic activity in the gland (Fig. 1a). Acarbose led to a stepwise increase in the ratio, i.e. decreased phosphofructokinase activity, in

Table 6. Blood ketone bodies and mammary-gland citrate and 6-phosphogluconate concentrations in fed, starved, refed, and refed-plus-acarbose-treated lactating rats

For full details see the Experimental section. Metabolite concentrations are mean values $\pm$ S.E.M., expressed as $\mu\text{mol/g}$ fresh wt. of tissue for the mammary gland and $\mu\text{mol/ml}$ of whole blood. Values that are significantly different by Student's *t* test from those for the fed group are indicated by: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Values that are significantly different by Student's *t* test from those for the starved group are indicated by: †*P* < 0.05; ††*P* < 0.01; †††*P* < 0.001.

Metabolite	Treatment of rats . . .	Concn.			
		Fed	Starved	Refed	Refed + acarbose
6-Phosphogluconate		0.0409 $\pm$ 0.0035†† (4)	0.0135 $\pm$ 0.0017** (5)	0.0150 $\pm$ 0.0011*** (5)	—
Citrate		0.271 $\pm$ 0.059† (3)	0.586 $\pm$ 0.053* (4)	0.329 $\pm$ 0.022†† (5)	0.355 $\pm$ 0.039†† (8)
Acetoacetate		0.139 $\pm$ 0.014 (4)	0.163 $\pm$ 0.029 (7)	0.113 $\pm$ 0.023 (8)	0.117 $\pm$ 0.019 (8)
3-Hydroxybutyrate		0.069 $\pm$ 0.040†† (4)	0.292 $\pm$ 0.045** (7)	0.062 $\pm$ 0.018††† (8)	0.083 $\pm$ 0.014††† (8)
Total ketone bodies		0.208 $\pm$ 0.048† (4)	0.455 $\pm$ 0.067* (7)	0.175 $\pm$ 0.023†† (8)	0.187 $\pm$ 0.023†† (8)

keeping with the observed changes in lipogenic rate (Fig. 1a).

Overall, these results imply a tight coupling between phosphofructokinase activity and glucose flux/lipogenesis. A key question, however, is the intracellular signal(s) leading to these rapid changes in the activity of phosphofructokinase. In other tissues, several allosteric regulators of the enzyme have been suggested, with particular emphasis on fructose 2,6-bisphosphate (see Hers & Van Schaftingen, 1982). However, the concentration of fructose 2,6-bisphosphate in the mammary gland does not change substantially on starvation or refeeding (Ward & Kuhn, 1985).

Citrate (an inhibitor: Randle *et al.*, 1964) and 6-phosphogluconate (an activator: Sommercorn & Freedland, 1982; Sommercorn *et al.*, 1984) are known to modulate phosphofructokinase activity in other tissues of the rat, with citrate concentrations being linked to changes in ketone-body metabolism. In the present study 6-phosphogluconate concentrations in the mammary gland were significantly altered by nutritional status, being some 3-fold higher in the fed state than in the starved state (Table 6). However, the concentration of this metabolite did not rise within 90 min of refeeding with chow diet. Thus, although this intermediate may play a regulatory role in phosphofructokinase activity in the mammary gland in the fed/starved transition, it cannot account for the apparent rise in enzyme activity during refeeding.

Citrate concentrations in the gland rose significantly on starvation, in conjunction with the expected increase in circulating ketone bodies (Table 6; see also Robinson & Williamson, 1977; Jones *et al.*, 1984). Refeeding with chow diet led to a fall (within 90 min) in blood ketone-body concentrations, and a significant decrease in mammary-gland citrate concentration, compared with starved values. Thus the observed changes in the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio in the present study (Table 5) could be brought about, in part, by the influence of citrate on phosphofructokinase activity (Zammit, 1979), via changes in ketone-body

metabolism. However, citrate cannot be the sole regulator of phosphofructokinase activity in the mammary gland, because blood ketone bodies and mammary-gland citrate concentrations were also significantly decreased below starvation values in refed rats treated with acarbose (Table 6), yet these animals showed no decrease of [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio compared with the starved group (indicating a lack of activation of phosphofructokinase), and accordingly lipogenic activity remained low (Table 5). Changes in citrate concentrations alone are not sufficient, therefore, to explain the rapid modulation of phosphofructokinase activity and lipogenesis during short-term refeeding of starved lactating rats. Previous work has shown no change in ATP concentrations in the mammary gland of fed, starved, and refed lactating rats (Jones *et al.*, 1984; Williamson *et al.*, 1985), and similarly, in the present study, no significant changes in mammary-gland ATP concentrations were found (results not shown). Thus the physiological regulation of phosphofructokinase in the lactating rat mammary gland during refeeding remains unclear.

Role of insulin in the regulation of mammary-gland lipogenesis

Short-term insulin deficiency inhibits phosphofructokinase in the intestine (Kellett *et al.*, 1984) and it may be, therefore, that the hormone regulates the activity of the enzyme in the mammary gland. In the present refeeding model (5 g of chow diet) the surge in lipogenesis in the mammary gland is preceded by a transitory hyperinsulinaemia (Mercer & Williamson, 1986). However, the physiological significance of this initial insulin surge is unclear, since suppression of insulin release acutely (either by streptozotocin or by somatostatin) after the initial surge still blocks the switch-on of lipogenesis in the mammary gland (Mercer & Williamson, 1986). This suggests that the continuing secretion of insulin is of major importance in the activation of lipogenesis over the 30–90 min period, despite the relatively low circulating insulin concentrations at this time. Since blood

flow is not substantially increased to the gland during this period (Mercer & Williamson, 1986), it would appear that the insulin-sensitivity of the gland is enhanced in some way by refeeding (Mercer & Williamson, 1986; Page & Kuhn, 1986).

In the present study acarbose was given at 25 min, i.e. after the peak of the insulin surge (Mercer & Williamson, 1986), yet produced a large inhibition of lipogenesis and a rise in the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio. Plasma insulin concentrations were significantly decreased by acarbose; 10 min after administration the value was 9.5 ± 5.6 munits/ml, compared with 32.5 ± 5.6 for the refeed controls at the same time point ($P < 0.05$). Overall, the total insulin response during the 30–60 min period (calculated from the area under the insulin curve) was 9.4 ± 2.1 munits/ml in the acarbose group and 43.9 ± 14.3 munits/ml ($P < 0.05$) in the refeed group. A further decrease occurred in both groups over the 60–90 min period, commensurate with the decline in mammary-gland lipogenic activity (Fig. 1a). The area under the curve during the initial 30 min of refeeding, when little re-activation of lipogenic activity took place, was 170.2 ± 23.4 munits/ml, i.e. some 4-fold higher than during the 30–60 min period, when the major lipogenic surge is found (Fig. 1a). These data emphasize our view that there is an acute increase in the sensitivity of the gland to the stimulatory effects of insulin on lipogenesis within about 30 min of refeeding (Mercer & Williamson, 1986). Thus the relatively small, but nonetheless significant, decrease in circulating insulin after acarbose treatment may well explain the subsequent poor lipogenic switch-on and the apparent rapid inactivation of phosphofructokinase (Figs. 1a and 2e). This view is supported by the observation that suppression of insulin secretion over this 30–90 min period by streptozotocin not only blocks the lipogenic re-activation (Mercer & Williamson, 1986) but also increases the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] ratio to near starved values (S. W. Mercer, unpublished work). This apparent rapid modulation of phosphofructokinase activity may be regulated either directly by insulin, or indirectly via insulin-induced changes in as yet unidentified allosteric regulator.

In conclusion, the present work provides evidence of an extremely close relationship between substrate supply and mammary-gland lipogenic activity. Insulin appears to play a key role in this link, perhaps via rapid changes in the insulin-sensitivity of the gland.

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