Regulation by insulin of glucose metabolism in mammary gland of anaesthetized lactating rats

Stimulation of phosphofructokinase-1 by fructose 2,6-bisphosphate and activation of acetyl-CoA carboxylase

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The effect of insulin on glucose metabolism in mammary gland was studied by the euglycaemic/hyperinsulinaemic-clamp technique. Measurement of metabolite concentrations and enzyme activities in the mammary gland suggests two sites of action of insulin: phosphofructokinase-1 and acetyl-coA carboxylase. The increase in phosphofructokinase-1 activity could be linked to the 2-fold increase in fructose 2,6-bisphosphate concentration, since no change in maximal activity and in sensitivity of the enzyme toward fructose 6-phosphate was detected *in vitro*.

INTRODUCTION

During lactation the mammary gland progressively acquires an intense biosynthetic capacity. At peak lactation, the fraction of glucose taken up by the mammary gland to ensure milk synthesis represents a large percentage of the total glucose turnover rate in well-fed rats (Williamson, 1980). It has been shown that the mammary-gland activity is dependent on a continuous supply of substrates, and that it falls rapidly during starvation (Carrick & Kuhn, 1978; Jones & Williamson, 1984). Furthermore, the rat mammary gland is extremely sensitive to insulin (Jones *et al.*, 1984; Burnol *et al.*, 1987), and is also implicated in the switch-on of lipogenesis which occurs when lactating rats are refed after a fasting period (Mercer & Williamson, 1986). Thus the effects of insulin on glucose metabolism in mammary gland are clearly established, but the potential sites of action remain to be determined. It has been suggested that during the starved-refed transition an important site of regulation of mammary-gland lipogenesis might be at the level of phosphofructokinase (PFK) (Mercer & Williamson, 1987). Since during refeeding there is a concomitant increase in glucose availability and in plasma insulin concentration (Mercer & Williamson, 1986), it is difficult to differentiate between these two potential regulatory factors in the stimulation of glucose metabolism in the mammary gland. The aim of the present work was to study *in vivo* the intracellular steps of glucose metabolism which are stimulated by insulin itself in the mammary gland. The euglycaemic/hyperinsulinaemic-clamp technique, coupled with the measurement of metabolite concentrations and enzyme activities, allows investigation of the short-term effect of a physiological increase in plasma insulin concentration on the metabolism of glucose in the mammary gland, without altering the amount of glucose supplied to the gland.

MATERIALS AND METHODS

Animals

Female Wistar rats were mated in the laboratory. They were housed at 23 °C with light from 07:00 to 19:00 h, and had free access to water and chow pellets (energy percentages: carbohydrate 65%, fat 11%, protein 24%). At delivery the size of each litter was adjusted to 10 pups. Lactating rats were studied at 11–13 days *post partum*. The experiments were performed at 10:00 h, i.e. in the post-absorptive state (Burnol *et al.*, 1983).

Euglycaemic/hyperinsulinaemic clamp

Euglycaemic/hyperinsulinaemic clamps were performed as previously described in anaesthetized rats (Burnol *et al.*, 1983). Briefly, insulin (pig, from Novo Industri, Copenhagen, Denmark) was infused at the constant rate of 0.4 unit/h per kg body wt. into a saphenous vein; blood glucose concentration was determined every 5 min, and an exogenous glucose infusion was adjusted to maintain euglycaemia. Usually 40 min was necessary to reach a plateau of blood glucose concentration and exogenous glucose infusion rate. This steady state was maintained for 30–40 min.

Mammary-gland sampling

Mammary glands were sampled after a 40 min period of anaesthesia for lactating rats studied in the basal state, and at the end of the euglycaemic clamp for lactating rats studied under insulin stimulation, i.e. after 1 h of hyperinsulinaemia. The abdominal mammary glands were dissected without occlusion of the blood supply, and then quickly freeze-clamped and powdered in liquid N₂. The tissue samples were stored at –80 °C until the measurement of metabolites and enzyme activities.

Metabolite analysis

The following metabolites were determined enzymically in neutralized HClO₄ extracts of the mammary glands: glucose, glucose 6-phosphate and fructose

Abbreviations used: PFK, phosphofructokinase.

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6-phosphate (Slein, 1963); pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate (Czok & Eckert, 1963); dihydroxyacetone phosphate (Bücher & Hohorst, 1963); fructose 1,6-bisphosphate (Michal & Beutler, 1963); citrate (Dagley, 1963); acetyl-CoA (Pearson, 1965). Malonyl-CoA concentrations were measured by a radioisotopic assay (McGarry *et al.*, 1978). Fructose 2,6-bisphosphate concentrations were determined in neutralized NaOH extracts of the mammary glands, by the procedure of Van Schaftingen *et al.* (1982).

Concentrations were expressed in nmol/g wet wt. and were not corrected for extracellular water. Indeed, it was considered that the milk content of the mammary gland had not been modified during the short length of the clamp studies.

Acetyl-CoA carboxylase assay

Acetyl-coA carboxylase activity was determined as described by Halestrap & Denton (1973), with some modifications. Mammary gland (200 mg) was homogenized in 800 μ l of 0.3 M-mannitol/0.1 M-Tris/2 mM-EDTA/2 mM-EGTA/50 mM-NaF/1 mM-dithiothreitol buffer (pH 7.4) containing 10 mg of fatty-acid-free bovine serum albumin/ml, and centrifuged twice at 13000 *g* for 15 min at 4 °C. The supernatant was passed through a Sephadex G-25 column. The incubation buffer contained 50 mM-Tris, 0.5 mM-EDTA, 10 mM-MgCl₂, 10 mg of fatty-acid-free bovine serum albumin/ml, 6 mM-ATP, 1 mM-dithiothreitol, 50 mM-NaF, 0.1 mM-acetyl-CoA and 15 mM-NaH¹⁴CO₃. The incubation was performed at 37 °C for 2 min on 25 μ l samples of supernatants, in a final volume of 0.5 ml. The reaction was linear for 3 min for the range of protein concentrations used (6–8 mg/ml). In addition, in preliminary experiments it was also established that pyruvate carboxylase activity represents less than 10% of acetyl-CoA carboxylase activity in the assay. For each experiment a blank without acetyl-CoA was performed. The total activity of the enzyme was determined under the same conditions, after a 20 min preincubation of the supernatants in the presence of 25 mM-potassium citrate. The reaction was ended by addition of 100 μ l of 5 M-HCl. After evaporation to dryness, the residue was redissolved in 0.5 ml of water and counted for radioactivity in a liquid-scintillation spectrophotometer (Kontron Beta-matic, Velizy, France).

Phosphofructokinase-1 assay

PFK-1 activity was measured spectrophotometrically, as described by Zammit (1979) and Ward & Kuhn (1985), by measuring the decrease in NADH absorbance at 340 nm. Briefly, mammary gland (60 mg) was homogenized in 1.5 ml of an ice-cold medium containing 0.25 M-sucrose, 5 mM-Tris, 1 mM-EDTA, 1 mM-dithiothreitol and 0.1 mM-ATP, pH 8.2. The homogenate was centrifuged twice at 13000 *g* for 15 min at 4 °C. Originally, samples were run through a Sephadex G-25 column. However, since no difference in *K_m* or maximal activity of PFK was observed with or without this treatment in either group of rats, the Sephadex G-25 step was omitted afterwards. The reaction medium contained 50 mM-imidazole, 5 mM-KCl, 0.1 mM-MgCl₂, 0.15 mM-NADH, 0.2 mM- or 1 mM-ATP, 0.15 mM-AMP, 0.75 mM-ADP, aldolase (450 munits/ml), glycerol-3-phosphate dehydrogenase (850 munits/ml) and triose-phosphate

Fig. 1. Effect of hyperinsulinaemia on mammary-gland metabolite concentrations in lactating rats

Values of concentrations are given as percentages of those in the basal state, and are means $\pm$ S.E.M. for 6–14 determinations. The concentrations of the metabolites in the mammary gland of lactating rats in the basal state were (in μ mol/g wet wt.): glucose (Glu) 1.0 ± 0.1 ; glucose 6-phosphate (G6P) 0.12 ± 0.07 ; fructose 6-phosphate (F6P) 0.022 ± 0.002 ; fructose 1,6-bisphosphate (F1,6P₂) 0.018 ± 0.002 ; dihydroxyacetone phosphate (DHAP) 0.027 ± 0.002 ; 3-phosphoglycerate (3PG) 0.088 ± 0.008 ; 2-phosphoglycerate (2PG) 0.022 ± 0.002 ; phosphoenolpyruvate (PEP) 0.06 ± 0.01 ; pyruvate (Pyr) 0.060 ± 0.007 ; lactate (Lact) 1.7 ± 0.3 .

isomerase (25 units/ml) at pH 7.1. All assays were performed in a final volume of 1 ml, at 28 °C. The reaction was started by addition of fructose 6-phosphate.

Statistical analysis

Results are presented as means $\pm$ S.E.M. Statistical differences were tested by Student's unpaired *t* test.

RESULTS AND DISCUSSION

The euglycaemic/hyperinsulinaemic clamp is a valuable technique to study *in vivo* the effects of insulin itself on the different intracellular metabolic pathways, because it allows study of short-term effects of a physiological hyperinsulinaemia without altering the concentration of glucose reaching the organs. It should be noted that the clamp technique does not allow study of very short-term effects of insulin, in the range of a few minutes, but the effects of the hormone after 1 h of hyperinsulinaemia can be studied. Blood glucose and plasma insulin concentrations were respectively 0.82 ± 0.03 and 0.78 ± 0.02 mg/ml, and 41 ± 8 and 225 ± 17 μ units/ml, in lactating rats studied in the basal state (*n* = 7) and during the euglycaemic/hyperinsulinaemic clamp (*n* = 7).

The concentrations of glycolytic metabolites in the mammary gland during the clamp are presented in Fig. 1, as percentages of the values obtained in the basal state. Concerning glycolysis, four steps can be considered as being potentially regulatory: glucose transport and reactions catalysed by hexokinase, PFK-1 and pyruvate kinase. In a previous study (Burnol *et al.*, 1987), a hyperinsulinaemia similar to that in the present study (a

Table 1. Concentrations of mammary-gland metabolites in lactating rats in the basal state and during the euglycaemic clamp

Values are means $\pm$ S.E.M. for six to eight determinations. Significance of differences was determined by Student's *t* test: **P* < 0.05, ***P* < 0.001 compared with basal state.

Mammary-gland metabolite (nmol/g wet wt.)	State	
	Basal	Clamp
Fructose 2,6-bisphosphate	1.3 $\pm$ 0.1	2.8 $\pm$ 0.2**
Citrate	151 $\pm$ 6	138 $\pm$ 21
Acetyl-CoA	19.4 $\pm$ 2.6	12.6 $\pm$ 0.8*
Malonyl-CoA	0.6 $\pm$ 0.1	2.6 $\pm$ 0.7*

4-fold increase over basal value), was shown to induce a 7-fold increase in glucose transport and phosphorylation. A direct stimulatory effect of insulin on glucose transport in lactating rat mammary gland has previously been reported, but in starved rats (Threadgold & Kuhn, 1984). It is difficult to infer from the present study whether insulin acts directly on the glucose-transport system or indirectly through the decrease of intracellular glucose concentration (Fig. 1), which should enhance inwards net flux, since glucose and glucose 6-phosphate concentrations are respectively decreased by 75% (*P* < 0.001) and 72% (*P* < 0.001) during the clamp. Nevertheless, the decreased intracellular glucose concentration suggests that glucose transport is not the major rate-limiting step for glucose metabolism in the mammary gland.

The increase in glucose phosphorylation under insulin stimulation (Burnol *et al.*, 1987), despite a lower intracellular glucose concentration, is probably brought about by the large decrease in glucose 6-phosphate (Fig. 1), a negative allosteric inhibitor of the hexokinase reaction. The decrease in glucose 6-phosphate in the presence of a higher phosphorylation flux also indicates that insulin has activated removal of glucose 6-phosphate. Fructose 6-phosphate concentration varies inversely with the glycolytic flux during the clamp (Fig. 1), strongly suggesting that PFK-1 has been activated by the hyperinsulinaemia. This is reinforced by the fact that the ratio [fructose 1,6-bisphosphate]/[fructose 6-phosphate] is increased 2-fold during the clamp [0.9 ± 0.1 (*n* = 13) in the basal state, to 1.8 ± 0.3 (*n* = 13) during the clamp; *P* < 0.05]. Maximal activity of PFK-1 measured in the presence of 1 mM-ATP and 1 mM-fructose 6-phosphate shows no change during the clamp [basal, 4.4 ± 0.3 (*n* = 6), clamp, 4.2 ± 0.4 (*n* = 7) μ mol/min per g wet wt.]. In addition, the sensitivity of PFK-1 to fructose 6-phosphate is similar in mammary gland of lactating rats studied in the basal state and during the clamp in the presence of 1 mM- or 0.2 mM-ATP (results not shown). It has been reported that during the starved/refeeding transition the switch-on of lipogenesis in the mammary gland of lactating rats was associated with a decrease in the [glucose 6-phosphate]/[fructose 1,6-bisphosphate] in the gland, suggesting an increase in PFK-1 activity (Mercer & Williamson, 1987). PFK-1 activity is known to be modulated by numerous negative (citrate, ATP) and positive (AMP, fructose 2,6-bisphosphate 6-phosphogluconate) effectors. The concentration of citrate

Table 2. Acetyl-CoA carboxylase activity in mammary gland of lactating rats in the basal state and during the euglycaemic/hyperinsulinaemic clamp

Values are means $\pm$ S.E.M. for six to eight determinations. **P* < 0.05 for significant difference compared with basal state.

	Acetyl-CoA carboxylase activity (munits/mg of protein)		
	Initial	Total	Initial/total
	Basal	Clamp	
Basal	1.0 $\pm$ 0.2	10.1 $\pm$ 1.5	0.08 $\pm$ 0.01
Clamp	2.3 $\pm$ 0.4*	13.8 $\pm$ 1.2	0.16 $\pm$ 0.02*

does not change during the hyperinsulinaemic clamp (Table 1). However, it should be noted that citrate is secreted in the milk, and that the concentration measured has not been corrected for extracellular water. This can mask a potential small modification of intracellular citrate content. It has previously been shown that ATP concentrations do not vary in mammary gland of fed, starved or refeed lactating rats (Jones *et al.*, 1984; Williamson *et al.*, 1985; Mercer & Williamson, 1987). The present study shows that the concentration of fructose 2,6-bisphosphate, the most potent activator of PFK-1, is increased 2-fold (*P* < 0.01) in mammary gland during the euglycaemic/hyperinsulinaemic clamp (Table 1). The concentration of 6-phosphogluconate in the mammary gland does not change during the transition to refeeding (Mercer & Williamson, 1987), but no information is actually available about the effect of insulin on AMP concentrations in the mammary gland. The present work indicates that the control of the activity of PFK-1 in the mammary gland may be dependent on the concentration of fructose 2,6-bisphosphate, as it has been described in other tissues (for review see Hue & Rider, 1987). It is possible to correct the concentrations of fructose 2,6-bisphosphate found in the present study for extracellular water, by using the value of 0.442 g of intracellular water/g of mammary gland (Threadgold & Kuhn, 1984). In such conditions, the intracellular concentrations of fructose 2,6-bisphosphate in the basal state (3 nmol/g) and during insulin stimulation (6.3 nmol/g) are within the range of concentrations that have a strong stimulatory effect on PFK-1 activity (Ward & Kuhn, 1985). The fructose 6-phosphate concentration decreases during the hyperinsulinaemic clamp, and thus cannot induce an increase in phosphofructokinase-2 (PFK-2) activity. The rise in fructose 2,6-bisphosphate concentration during the hyperinsulinaemic clamp may thus be explained by a stimulation by insulin of PFK-2 activity. The activation by insulin of PFK-1 seems thus to be secondary to an activation of PFK-2.

Another potential site of action of insulin on glycolysis is pyruvate kinase. However, in the mammary gland pyruvate kinase is of the M type (A. Kahn, personal communication), and as such is not subject to marked co-operative interaction with phosphoenolpyruvate, stimulation by fructose 1,6-bisphosphate, or regulation by phosphorylation/dephosphorylation processes. That

pyruvate kinase is not a preferential site of action of insulin in the mammary gland is corroborated by the fact that the ratio [pyruvate]/[phosphoenolpyruvate] is not significantly altered during the clamp [basal, 1.1 ± 0.2 ($n = 6$), clamp 0.8 ± 0.1 ($n = 7$)]. Insulin might also modulate pyruvate dehydrogenase activity. Another site of action of insulin has been suggested in previous studies showing an increase in the activity of pyruvate dehydrogenase by insulin in the mammary gland of lactating rats (Baxter & Coore, 1978; Kilgour & Vernon, 1987).

During the euglycaemic/hyperinsulinaemic clamp the rate of lipogenesis is enhanced 3-fold in mammary gland of lactating rats (Burnol *et al.*, 1983). This suggests another possible site of action of insulin on mammary-gland metabolism, at the level of acetyl-CoA carboxylase. The 40% decrease in acetyl-CoA concentration, together with the 4.5-fold increase in malonyl-CoA concentration during the euglycaemic/hyperinsulinaemic clamp (Table 1), suggest that acetyl-CoA carboxylase activity is increased by insulin. In fact, measurement of acetyl-CoA carboxylase activity shows a 2-fold increase in the initial activity during the clamp, without change in total activity (Table 2). These results are in agreement with previous studies reporting an increase in the initial activity of acetyl-CoA carboxylase in mammary gland of starved lactating rats injected with insulin and glucose (McNeillie & Zammit, 1982) or refed for 2.5 h (Munday & Williamson, 1982). It has been shown that during short-term refeeding the regulation of acetyl-CoA carboxylase activity is likely to occur preferentially by phosphorylation/dephosphorylation mechanisms dependent on insulin concentration, rather than by changes in the enzyme content (Munday & Hardie, 1986). This suggests that during the euglycaemic/hyperinsulinaemic clamp the increase in acetyl-CoA carboxylase activity may also occur via a decrease in the phosphorylation state of the enzyme, but this remains to be established.

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