Insulin activation of lipogenesis in isolated mammary acini from lactating rats fed on a high-fat diet

Evidence that acetyl-CoA carboxylase is a site of action

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1. Feeding lactating rats on high-fat cheese crackers in addition to laboratory chow increased the dietary intake of fat from 2 to 20% of the total weight of food eaten and decreased mammary-gland lipogenesis *in vivo* by approx. 50%. 2. This lipogenic inhibition was also observed in isolated mammary acini, where it was accompanied by decreased glucose uptake. These inhibitions were completely reversed by incubation with insulin. 3. Insulin had no effect on the rate of glucose transport into acini, nor on pyruvate dehydrogenase activity as estimated by the accumulation of pyruvate and lactate, suggesting that these are not the sites of lipogenic inhibition. 4. Insulin stimulated the incorporation of [1-¹⁴C]acetate into lipid in acini from high-fat-fed rats. 5. In the presence of α -cyano-hydroxycinnamate, a potent inhibitor of mitochondrial pyruvate transport, and with glucose as the sole substrate, neither [1-¹⁴C]glucose incorporation into lipid nor glucose uptake were stimulated by insulin. Insulin did stimulate the incorporation of [1-¹⁴C]acetate into lipid in the presence of α -cyano-hydroxycinnamate, and this was accompanied by an increase in glucose uptake by the acini. This indicated that increased glucose uptake was secondary to the stimulation of lipogenesis by insulin, which therefore must occur via activation of a step in the pathway distal to mitochondrial pyruvate transport. 6. Insulin stimulated acetyl-CoA carboxylase activity measured in crude extracts of acini from high-fat-fed rats, restoring it to values close to those of chow-fed controls. 7. The effects of insulin on acetyl-CoA carboxylase activity and lipogenesis were not antagonized by adrenaline or dibutyl cyclic AMP.

INTRODUCTION

High-fat diets have profound inhibitory effects on lipogenesis in both liver and white adipose tissue of non-lactating rats (Lavau *et al.*, 1979; Romsos & Leveille, 1974) and mice (York, 1975; Romsos *et al.*, 1978; Cawthorne & Cornish, 1979). A high-fat diet given throughout lactation similarly decreases the rate of lipogenesis *in vivo* in the mammary gland of mice (Romsos *et al.*, 1978) and rats (Agius *et al.*, 1980; Grigor & Warren, 1980). This inhibition of lipogenesis by dietary fat persists in rat mammary-gland preparations *in vitro*, as measured by the incorporation into lipid of [¹⁴C]acetate in tissue slices (Coniglio & Bridges, 1966) and [¹⁴C]glucose in isolated acini (Agius *et al.*, 1980). It has been demonstrated in our laboratory that the inhibition of mammary-gland lipogenesis by high-fat feeding can be relieved by insulin, either administered to the rat *in vivo* (Agius *et al.*, 1981) or added to the incubation medium of isolated acini *in vitro* (Agius *et al.*, 1980).

Possible sites in the pathway from glucose to fatty acid in the lactating rat mammary gland at which this lipogenic inhibition and re-activation may occur include glucose transport (Threadgold *et al.*, 1982; Busmann *et al.*, 1984), hexokinase and phosphofructokinase (Martin & Baldwin, 1971; Williamson *et al.*, 1975; Sochor *et al.*, 1984; Jones *et al.*, 1984), pyruvate dehydrogenase (Baxter & Coore, 1978) and acetyl-CoA carboxylase (McNeillie & Zammit, 1982; Munday & Williamson,

1982). In response to high-fat feeding, the fatty acid composition of the milk lipid resembles that of the diet (Garton, 1963; Coniglio & Bridges, 1966; Grigor & Warren, 1980) as the lactating mammary gland switches from fatty acids synthesized *de novo* to those of dietary origin. The inhibition of lipogenesis is therefore more likely to occur within the pathway that is committed to the synthesis of fatty acids. Acetyl-CoA carboxylase catalyses the first and rate-limiting step in this pathway, and is known to be regulated both allosterically (citrate activates and long-chain fatty acyl-CoA inhibits) and by reversible phosphorylation (for reviews see Hardie, 1980; Hardie *et al.*, 1984). The following work presents evidence that the activation of this enzyme by insulin is the important event in the re-activation of lipogenesis in isolated mammary acini from rats fed on a palatable high-fat diet consisting of chow and cheese crackers.

EXPERIMENTAL

Materials

Radioactive compounds. D-[1-¹⁴C]Glucose, [1-¹⁴C]lactate (sodium salt), NaH¹⁴CO₃, 2-deoxy-D-[1-³H]glucose and ³H₂O were obtained from Amersham International, Amersham, Bucks., U.K.

Biochemicals. All enzymes, including collagenase (grade V) and coenzymes, were obtained from Boehringer Corp. (London), Lewes, Sussex, U.K. Highly purified

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insulin for experiments *in vitro* was a gift from Dr. N. Lazarus, Wellcome Research Laboratories, Beckenham, Kent, U.K.

Chemicals. Phenazine methosulphate, adrenaline and dibutyl cyclic AMP were obtained from Sigma Chemical Co., Poole, Dorset, U.K. α -Cyano-4-hydroxycinnamic acid was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Animals. Lactating (12–16 days) rats of the Wistar strain (270–380 g) with between 8 and 12 pups were used. The rats were fed *ad libitum* on Oxoid breeding diet (Oxoid, London S.E.1., U.K.) throughout pregnancy. At parturition one group of rats was allowed free access both to the chow diet and to cheese crackers with a high-fat content ('Cheddars'; McVitie's, London, U.K.). Another group of rats was given only chow throughout lactation.

Measurement of fatty acid synthesis

Rates of fatty acid synthesis *in vivo* were measured by the incorporation of $^3\text{H}_2\text{O}$ into fatty acids as described by Munday & Williamson (1981).

Rates of fatty acid synthesis in isolated acini were measured by incubating the acini as described below in the presence of 500 μCi of $^3\text{H}_2\text{O}$. The total lipid was extracted from the acini with chloroform/methanol (2:1, v/v). This was saponified and the fatty acids were extracted as described for experiments *in vivo* (Munday & Williamson, 1981). A portion of the incubation medium was counted for ^3H in order to determine the specific radioactivity of the $^3\text{H}_2\text{O}$ pool.

Experiments *in vitro* with acini

Acini were prepared from the mammary glands as described by Robinson & Williamson (1977). The acini (about 100 mg wet wt.) were incubated at 37 °C in a total volume of 4 ml of Krebs–Henseleit (1932) saline containing glucose (5 mM) and other additions where indicated. These were sodium acetate (2 mM), phenazine methosulphate (50 μM), α -cyanohydroxycinnamate (1 mM), insulin (30 munits/ml), adrenaline (10 $\mu\text{g}/\text{ml}$) and dibutyl cyclic AMP (1 mM). Other conditions of the incubation were as described by Robinson & Williamson (1977).

Determination of metabolites

At the termination of each incubation, the incubation medium was deproteinized and neutralized (Robinson & Williamson, 1977) and the following metabolites were determined by enzymic methods: glucose (Slein, 1963); L-lactate and pyruvate (Hohorst *et al.*, 1959).

Expression of results

Incubations *in vitro* were carried out for 20, 40 and 60 min, and rates of metabolite accumulation or utilization or incorporation of ^3H or ^{14}C label were calculated from the plots of the individual results. The results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry wt. of acini.

Measurements of glucose transport

Phenazine methosulphate. This is essentially the method previously described for isolated adipocytes (Taylor *et al.*, 1976). Phenazine methosulphate uncouples

the pentose phosphate pathway from lipogenesis so that the rate of oxidation of C-1 of glucose in this pathway is representative of the rate of glucose transport into the cell. Acini were incubated with D-[1- ^{14}C]glucose (5 mM) in the presence or absence of phenazine methosulphate (50 μM) in flasks with a centre well and fitted with a rubber seal. Incubations were terminated by the injection of 0.4 ml of 20% (w/v) HClO_4 into the outer compartment and 0.3 ml of 1 M-NaOH, to absorb $^{14}\text{CO}_2$, into the centre well.

2-Deoxy-D-glucose. This is a modification of the method described by Threadgold *et al.* (1982). Acini (40–50 mg) suspended in a total volume of 3.8 ml of Krebs–Henseleit (1932) saline, containing 0.3 mM-sodium pyruvate and 4.7 mM-sodium L-lactate as substrates in place of glucose, were incubated in the presence or absence of insulin (30 munits/ml) at 37 °C for 30 min. 2-Deoxy-D-[1- ^3H]glucose (0.125 $\mu\text{Ci}/\mu\text{mol}$) was added to each incubation to give a final concentration of 5 mM in a total volume of 4 ml. Incubations were arrested at 10 s intervals during a 60 s incubation at 37 °C, by the addition of ice-cold 0.9% NaCl (20 ml). The suspension was immediately filtered through a pre-weighed Millipore pre-filter (Millipore AP 2002500) and washed with a further 40 ml of ice-cold 0.9% NaCl. The time from stopping the incubation to completion of the filtration was routinely 60 s. Filter papers were dried and weighed (the weight of acini was determined from the difference) and immersed in 0.4 ml of 5% Triton X-100 in a scintillation vial overnight. The ^3H content was determined by liquid-scintillation counting and was corrected for blank incubations carried out in the absence of acini. Glucose transport was expressed as nmol of 2-deoxy-D-glucose taken up/mg dry wt. of acini, and increased linearly over the 0–60 s time course (Fig. 1).

Measurement of acetyl-CoA carboxylase activity in acini

Acini (approx. 100 mg) were incubated for 30 min as described above and the contents of the incubation flask were then rapidly centrifuged (4000 g for 1 min). The supernatant was discarded and the pellet was immediately homogenized by hand in 1 ml of extraction buffer (0.3 M-mannitol, 50 mM-Tris/HCl, pH 7.4, 50 mM-NaF, 2 mM-EDTA; for measurement of 'total activity', the NaF was omitted). This was spun in an Eppendorf centrifuge (12000 g, 1 min) and the supernatant was diluted 1:1 with extraction buffer containing 10% (w/v) bovine serum albumin. This supernatant was then assayed for acetyl-CoA carboxylase activity by the incorporation of $\text{H}^{14}\text{CO}_3^-$ into acid-stable material as described for crude tissue extracts by Munday & Hardie (1986).

RESULTS AND DISCUSSION

High-fat feeding

Over a period of 15 days lactation, rats allowed free access to the palatable 'Cheddars' cheese crackers decreased their intake of chow by approx. 70% compared with chow-fed controls (Table 1). This decrease was observed immediately on day 1 after parturition and remained fairly constant throughout the succeeding 15 days (results not shown). These rats clearly supplemented their food intake with the 'Cheddars' cheese crackers (Table 1).

Table 1. Composition of the food intake of chow-fed and high-fat-fed rats during the first 15 days of lactation

The amount of food eaten over a 24 h period by lactating rats fed on the standard chow diet or on chow diet plus high-fat cheese crackers was measured at 10:00 h each day. Results show the total weight (g) of food eaten between 0 and 15 days lactation, and the composition of this food intake in terms of the three major classes of nutrient. Each value represents the mean $\pm$ S.E.M. of the food intake of six different rats.

	Rats fed ad libitum on:	
	Chow alone	Chow + high-fat cheese crackers
Wt. of chow eaten		258.6 $\pm$ 16.4
Wt. of cheese crackers		406.4 $\pm$ 14.6
Total food intake	861.1 $\pm$ 57.5	664.9 $\pm$ 18.2
Carbohydrate from chow		132.9 $\pm$ 8.4
Carbohydrate from cheese crackers		205.6 $\pm$ 7.4
Total carbohydrate intake	442.6 $\pm$ 29.5	338.5 $\pm$ 9.3
Protein from chow		36.6 $\pm$ 2.0
Protein from cheese crackers		44.3 $\pm$ 1.6
Total protein intake	136.1 $\pm$ 9.1	80.9 $\pm$ 3.1
Fat from chow		5.2 $\pm$ 0.3
Fat from cheese crackers		132.5 $\pm$ 4.8
Total fat intake	17.2 $\pm$ 1.1	137.7 $\pm$ 4.7

The cheese crackers have a high fat composition by weight (protein 10.9% ; carbohydrate 50.6% ; fat 32.6%) compared with the standard laboratory chow (protein 15.8% ; carbohydrate 51.4% ; fat 2% ; Rolls *et al.*, 1980). Therefore, although the carbohydrate and protein intakes of these rats were slightly decreased (Table 1), there was a very large (7-fold) increase in the amount of fat consumed (Table 1). Hence in these high-fat-fed rats the proportion of dietary fat increased from 2% to 20% of the total weight of food eaten. Overall, the daily energy intake of rats fed on the high-fat diet was approx. 23% higher than that of chow-fed controls.

Fatty acid synthesis

The rate of lipogenesis in the lactating mammary gland *in vivo* was 62.7 $\pm$ 19.4 ($\pm$ S.E.M.; $n = 6$) μ mol of $^3\text{H}_2\text{O}$ incorporated/h per g of tissue in high-fat-fed rats, compared with 128.4 $\pm$ 16.8 ($n = 9$) in rats given only chow ($P < 0.005$). This inhibition (approx. 50%) is similar to that reported by Agius *et al.* (1980).

The inhibition of lipogenesis by high-fat feeding clearly persists in isolated mammary acini preparations. Rates of lipogenesis were 40% lower in acini from high-fat-fed rats (Table 6), and were completely restored to those of chow-fed control rats by incubation with insulin (Table 6). This is in agreement with measurements of [^{14}C]glucose incorporation into total lipid, previously made in this laboratory (Agius *et al.*, 1980).

Insulin and acetate metabolism

In acini from normal 24 h-starved lactating rats, where the activation state of pyruvate dehydrogenase is low, insulin stimulates the conversion of [^{14}C]acetate into lipid, and this has been interpreted as indicating activation of acetyl-CoA carboxylase (Munday & Williamson, 1981). Possible effects of the hormone on acetate metabolism in acini from high-fat-fed rats were therefore examined (Table 2). The rate of acetate oxidation in these cells was low, and this was depressed further by insulin (Table 2). In contrast, insulin increased

the conversion of [^{14}C]acetate into lipid by 163% and the utilization of glucose by 112%.

The major fate of pyruvate in acini is oxidation via pyruvate dehydrogenase or conversion into lactate. Hence measurement of the percentage of glucose utilized which accumulates in the medium as lactate and pyruvate is an indication of the activity of pyruvate dehydrogenase. Accumulation of lactate and pyruvate in the medium of acini from high-fat-fed rats was not affected by insulin, and only represented approx. 20% of the glucose utilized, compared with 16% and 65% in acini

Table 2. Effects of insulin on [^{14}C]acetate and glucose metabolism in isolated acini from lactating rats fed on a high-fat diet

Details of the experiment are described in the Experimental section. Where indicated, insulin (30 munits/ml) was added to the incubation medium. The results are expressed as μ mol/min per 100 mg defatted dry wt. of acini, and are shown as mean values $\pm$ S.E.M. for five separate experiments. Values obtained in the presence of insulin that are significantly different (by Student's *t* test) from controls are shown: ** $P < 0.005$; *** $P < 0.0005$.

	Control	Control + insulin
[^{14}C]Acetate $\rightarrow$ $^{14}\text{CO}_2$	0.068 $\pm$ 0.008	0.019 $\pm$ 0.002***
[^{14}C]Acetate $\rightarrow$ [^{14}C]lipid	0.19 $\pm$ 0.04	0.50 $\pm$ 0.03***
Glucose removal	0.78 $\pm$ 0.07	1.66 $\pm$ 0.09***
Pyruvate formation	0.10 $\pm$ 0.03	0.16 $\pm$ 0.04
Lactate formation	0.20 $\pm$ 0.03	0.60 $\pm$ 0.11**
Pyruvate and lactate formation (% of glucose uptake)	19.7 $\pm$ 2.3	23.1 $\pm$ 3.1

Table 3. Effect of α -cyanohydroxycinnamate on [1- ^{14} C]acetate incorporation into lipid in acini from lactating rats fed on a high-fat diet

Details of the experiment are described in the Experimental section. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry wt. of acini and are mean values $\pm$ S.E.M. for five separate experiments. Values that are significantly different (by Student's *t* test) from control values are shown: *** $P < 0.0005$. Values obtained in the presence of α -cyanohydroxycinnamate and insulin that are significantly different from those obtained in the presence of α -cyanohydroxycinnamate alone are shown: ††† $P < 0.0005$.

	No addition	Insulin (30 munits/ml)	α -Cyanohydroxycinnamate (1 mM)	α -Cyanohydroxycinnamate + insulin
[1- ^{14} C]Acetate $\rightarrow$ [^{14} C]lipid	0.15 $\pm$ 0.03	0.38 $\pm$ 0.05***	0.23 $\pm$ 0.07	0.48 $\pm$ 0.07***†††
[1- ^{14} C]Acetate $\rightarrow$ $^{14}\text{CO}_2$	0.063 $\pm$ 0.005	0.030 $\pm$ 0.005***	0.059 $\pm$ 0.006	0.031 $\pm$ 0.005***†††
Glucose uptake	0.76 $\pm$ 0.10	1.53 $\pm$ 0.11***	1.01 $\pm$ 0.15	1.77 $\pm$ 0.10***†††
Pyruvate formation	0.09 $\pm$ 0.03	0.15 $\pm$ 0.02	0.13 $\pm$ 0.02	0.05 $\pm$ 0.05
Lactate formation	0.20 $\pm$ 0.06	0.44 $\pm$ 0.08***	0.57 $\pm$ 0.08***	1.39 $\pm$ 0.09***†††
Pyruvate and lactate accumulation (% of glucose uptake)	18.4 $\pm$ 5.2	19.0 $\pm$ 2.9	35.5 $\pm$ 3.5***	45.8 $\pm$ 5.7***

Table 4. Effect of α -cyanohydroxycinnamate on the glucose metabolism of acini from lactating rats fed on a high-fat diet

Details of the experiment are described in the Experimental section. Results are expressed as $\mu\text{mol}/\text{min}$ per 100 mg defatted dry wt. of acini and are mean values $\pm$ S.E.M. for five separate experiments. Values that are significantly different (by Student's *t* test) from control values are shown: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

	No addition	Insulin (30 munits/ml)	α -Cyanohydroxycinnamate (1 mM)	α -Cyanohydroxycinnamate + insulin
[1- ^{14} C]Glucose $\rightarrow$ [^{14} C]lipid	0.22 $\pm$ 0.02	0.39 $\pm$ 0.01***	0.09 $\pm$ 0.02***	0.11 $\pm$ 0.02***
[1- ^{14} C]Glucose $\rightarrow$ $^{14}\text{CO}_2$	0.24 $\pm$ 0.03	0.38 $\pm$ 0.02***	0.17 $\pm$ 0.02*	0.19 $\pm$ 0.02
Glucose uptake	0.95 $\pm$ 0.09	1.39 $\pm$ 0.08**	1.18 $\pm$ 0.07*	1.27 $\pm$ 0.09*
Pyruvate formation	0.07 $\pm$ 0.01	0.09 $\pm$ 0.01	0.17 $\pm$ 0.03***	0.16 $\pm$ 0.03***
Lactate formation	0.40 $\pm$ 0.03	0.83 $\pm$ 0.11***	1.25 $\pm$ 0.11***	1.51 $\pm$ 0.10***
Pyruvate and lactate accumulation (% of glucose uptake)	26.2 $\pm$ 3.8	33.1 $\pm$ 3.7	58.8 $\pm$ 4.5***	66.3 $\pm$ 7.2***

from chow-fed and 24 h-starved rats respectively (Robinson & Williamson, 1977). This suggests that pyruvate dehydrogenase is not inactivated by high-fat feeding, as it is in 24 h starvation (Kankel & Reinauer, 1976; Baxter & Coore, 1978).

It can be concluded from this experiment that either insulin directly stimulates acetate incorporation into lipid or, by stimulating glucose utilization it indirectly increases acetate incorporation (e.g. by increased provision of NADPH or glycerol 3-phosphate, or by an increase in intracellular citrate). If the effect is direct, then the site of action of insulin is likely to be acetyl-CoA synthetase (a cytosolic enzyme in rat mammary gland; Buckley, 1974) or acetyl-CoA carboxylase.

Inhibition of pyruvate transport

To examine whether the flux of glucose into the cytosolic pool of citrate was important in the stimulation of [1- ^{14} C]acetate incorporation into lipid by insulin, the experiments were repeated in the presence of α -cyanohydroxycinnamate, a potent inhibitor of pyruvate transport into the mitochondrial matrix (Halestrap & Denton, 1974; Halestrap, 1975). The effectiveness of

α -cyanohydroxycinnamate as an inhibitor of pyruvate transport in isolated mammary acini was demonstrated by the increased accumulation of lactate and pyruvate in these cells (Tables 3 and 4) and the inhibition of lipogenesis from [1- ^{14} C]glucose (Table 4).

The presence of α -cyanohydroxycinnamate (1 mM) did not affect the rate of [1- ^{14} C]acetate incorporation into lipid, nor did it prevent the stimulation of the rate of lipogenesis from [1- ^{14} C]acetate by insulin (Table 3). This is in agreement with the observations by Halestrap & Denton (1974) in adipocytes, although this compound is reported to inhibit acetate incorporation into lipid in hepatocytes (Beynen *et al.*, 1981). Stimulation of glucose utilization by insulin was also not affected by the inhibitor in these incubations (Table 3), suggesting that increased glucose utilization was secondary to the stimulation of lipogenesis. Further evidence in support of this view was obtained with [1- ^{14} C]glucose as the sole substrate (Table 4). In these incubations, insulin increased the incorporation of [1- ^{14} C]glucose into lipid by 77% and glucose utilization by 46%. The addition of α -cyanohydroxycinnamate inhibited (60% decrease) lipogenesis from [1- ^{14} C]glucose and prevented the insulin

Table 5. Effect of insulin on glucose transport in acini from high-fat-fed lactating rats as measured by the oxidation of [1-¹⁴C]glucose to ¹⁴CO₂ in the presence of phenazine methosulphate

Acini were prepared and incubated as described in the Experimental section. Insulin at a final concentration of 30 munits/ml and phenazine methosulphate at 50 μ M were added to the incubations as indicated. Results are expressed as μ mol/min per 100 mg defatted dry wt. of acini and are mean values $\pm$ S.E.M. for five separate experiments. Values that are significantly different (by Student's *t* test) from control values are shown: ***P < 0.0005. Values in the presence of phenazine methosulphate and insulin that are significantly different (by Student's *t* test) from those in the presence of phenazine methosulphate alone are shown: ††*P* < 0.005.

	[1- ¹⁴ C]Glucose $\rightarrow$ ¹⁴ CO ₂	[1- ¹⁴ C]Glucose $\rightarrow$ [¹⁴ C]lipid
Control	0.22 $\pm$ 0.03	0.19 $\pm$ 0.02
+ Insulin	0.32 $\pm$ 0.03***	0.30 $\pm$ 0.02***
+ Phenazine methosulphate	0.75 $\pm$ 0.04***	0.037 $\pm$ 0.008***
+ Phenazine methosulphate + insulin	0.76 $\pm$ 0.05***	0.053 $\pm$ 0.011***††

stimulation of this process (Table 4). Glucose utilization was slightly increased (24%) by the addition of α -cyanohydroxycinnamate, but in the presence of this inhibitor the insulin stimulation of glucose uptake by these acini was also abolished (Table 4). The absence of an insulin effect on glucose utilization when lipogenesis is inhibited supports the postulate that the hormone activates a step in the lipogenic pathway distal to pyruvate dehydrogenase and that glucose uptake increases as a secondary consequence.

Glucose transport

An alternative explanation for the effects of insulin shown in Tables 2–4 would be stimulation of glucose transport by the hormone and inhibition of this effect by α -cyanohydroxycinnamate. Glucose transport in the presence and absence of insulin was therefore measured by using the artificial electron acceptor, phenazine methosulphate, to uncouple lipogenesis from the pentose phosphate pathway and thus bypass regulation of the latter by the cytosolic [NADP⁺]/[NADPH] ratio. In this system conversion of [1-¹⁴C]glucose into ¹⁴CO₂ provides a measure of the rate of glucose transport into the cell (Taylor *et al.*, 1976; Taylor & Halperin, 1979). As expected, in acini from the fat-fed lactating rats phenazine methosulphate increased the oxidation of [1-¹⁴C]glucose to ¹⁴CO₂ by 250% and decreased the incorporation of [1-¹⁴C]glucose into lipid by 80% (Table 5). Phenazine methosulphate also decreased the incorporation of [1-¹⁴C]acetate into lipid by 80% (results not shown), confirming its direct inhibition of lipogenesis.

In the absence of the electron acceptor, the stimulatory effects of insulin on [1-¹⁴C]glucose incorporation into lipid and [1-¹⁴C]glucose oxidation to ¹⁴CO₂ (Table 5), and on the uptake of glucose into the cell, were similar to those observed in previous experiments (Table 4). However, in the presence of phenazine methosulphate insulin did not increase ¹⁴CO₂ production from [1-¹⁴C]glucose. This suggests that, under the incubation conditions described here, insulin does not stimulate glucose transport into the isolated acini. It is noteworthy that in the presence of phenazine methosulphate, and presumably maximum rates of glucose transport, insulin causes a small but significant increase in lipogenesis (Table 5).

To confirm these observations, the effects of insulin on the uptake of 2-deoxy-D-glucose into acini from high-fat-fed lactating rats were investigated. This sugar

is taken up by acini via the same carrier-mediated process as D-glucose (Threadgold *et al.*, 1982). In these experiments, preincubation (30 min) of the acini with pyruvate and lactate (Fig. 1) did not affect the viable metabolism of the cells, since subsequent incubation (30 min) with glucose (5 mM) gave rates of glucose uptake of 0.82 ± 0.08 ($\pm$ S.E.M., *n* = 3) and 1.29 ± 0.1 (*n* = 3) μ mol/min per 100 mg defatted dry wt. of acini in the absence and presence of insulin respectively. These rates are directly comparable with those shown in Table 4.

The uptake of 2-deoxy-D-[1-³H]glucose by the acini increased linearly over the 60 s incubation, the rate being approx. 7 nmol/min per mg dry wt. of acini (Fig. 1). Insulin produced no significant change in this rate (Fig. 1), confirming the results of the phenazine methosulphate experiments. These methods of measuring glucose

Fig. 1. Effect of insulin on 2-deoxy-D-[1-³H]glucose transport in acini isolated from lactating rats fed on a high-fat diet

Experiments were carried out as described in the Experimental section. Acini were preincubated (30 min at 37 °C) with pyruvate (0.3 mM) and lactate (4.7 mM) as sole substrates in the presence (○) or absence (●) of insulin (30 munits/ml). 2-Deoxy-D-[1-³H]glucose (5 mM) was then added, and its uptake over the initial 60 s of the subsequent incubation was measured. Each point represents the mean value for acini from five separate rats, with the S.E.M. indicated by the vertical bars.

transport are both open to criticism. The first method uses phenazine methosulphate to channel glucose through the pentose phosphate pathway and assumes that the flux, measured by the formation of $^{14}\text{CO}_2$ from D-[1- ^{14}C]glucose, reflects the rate of glucose transport. However, even in the presence of phenazine methosulphate, a certain small proportion of this glucose is still converted into other products without passage through the pentose phosphate pathway, as shown by the incorporation of ^{14}C label into lipid in the present study. The more direct measurement of 2-deoxy-D-glucose uptake relies on the assumption that, once phosphorylated by hexokinase, this compound is 'trapped' within the cell. It is possible that the shock of exposure of the acini to ice-cold saline and the time taken to filter the cells from the medium could lead to loss of 2-deoxy-D-glucose 6-phosphate. Although these experimental procedures may have certain limitations and may not measure absolute rates of glucose transport, the results from each method used in the present study are in agreement and confirm that the insulin activation of lipogenesis in acini from high-fat-fed rats is not the result of an insulin stimulation of glucose transport.

Insulin and acetyl-CoA carboxylase activity in acini

In acini from high-fat-fed lactating rats incubated with glucose (5 mM), acetyl-CoA carboxylase activity, measured in crude extracts, was stimulated by insulin *in vitro* (Table 6). Insulin significantly increased the activity of the enzyme measured at physiological (0.5 mM) and saturating (10 mM) citrate concentrations, restoring them to the activity in chow-fed controls (Table 6). The hormone had no effect on enzyme activity in the cells of chow-fed controls. Insulin stimulated acetyl-CoA carboxylase activity in acini from high-fat-fed rats without affecting the 'total enzyme activity', i.e. the activity after incubation in the presence of Mg^{2+} and citrate (Munday & Williamson, 1982; Munday & Hardie, 1986). This was 34.8 ± 7.3 ($\pm \text{S.E.M.}$, $n = 6$) and

35.7 ± 6.3 ($n = 6$) nmol of $\text{H}^{14}\text{CO}_3^-$ incorporated/min per mg of homogenate protein for high-fat-fed control and insulin-treated acini respectively.

A similar stimulation of acetyl-CoA carboxylase activity was observed in incubations in the presence of glucose (5 mM) and acetate (2 mM), and addition of α -cyanohydroxycinnamate (1 mM) did not prevent this stimulation (results not shown). This suggests that the effect was due to direct hormone action rather than an indirect effect on carbon flux into the lipogenic pathway.

Effects of adrenaline and cyclic AMP

We decided to examine whether the stimulatory effects of insulin on lipogenesis and acetyl-CoA carboxylase activity in acini from rats fed on a high-fat diet could be antagonized by adrenaline or cyclic AMP. In white adipose tissue from non-lactating rats, adrenaline inhibits insulin-stimulated fatty acid synthesis *in vitro* (Smith & Saggerson, 1978), which involves a phosphorylation and inactivation of acetyl-CoA carboxylase that is mediated by cyclic-AMP-dependent protein kinase (Witters *et al.*, 1983; Holland *et al.*, 1985). Despite the presence of β -adrenergic receptors (Clegg & Mullaney, 1985; Lavandero *et al.*, 1985) and cyclic-AMP-dependent protein kinase (Burchell *et al.*, 1978) in the lactating mammary gland, neither adrenaline nor dibutyl cyclic AMP had any significant effect on the insulin stimulation of fatty acid synthesis or acetyl-CoA carboxylase activity in acini from high-fat-fed rats (Table 6).

It was interesting that, in the absence of insulin, adrenaline produced a significant increase in fatty acid synthesis (Table 6). It has previously been reported that adrenaline (in the presence of a phosphodiesterase inhibitor) increases lipogenesis in mammary-gland slices (Plucinski & Baldwin, 1982). In the present experiments, adrenaline also increased acetyl-CoA carboxylase activity (Table 6), and since these effects were not mimicked by dibutyl cyclic AMP, it is tempting to speculate that adrenaline may be acting through some other receptor

Table 6. Fatty acid synthesis and acetyl-CoA carboxylase activity in isolated mammary acini

Mammary acini were prepared from chow-fed and high-fat-fed lactating rats and incubated as described in the Experimental section with other additions as indicated below, i.e. insulin (30 munits/ml), adrenaline (10 $\mu\text{g}/\text{ml}$) and dibutyl cyclic AMP (1 mM). Acetyl-CoA carboxylase activity in crude acini extracts and rates of fatty acid synthesis were measured as described in the Experimental section. Values are means $\pm$ S.E.M. for the numbers of observations in parentheses. Values that differ significantly (by Student's *t* test) from those for chow-fed controls are shown: * $P < 0.05$; ** $P < 0.005$. Values for acini from high-fat-fed rats that differ significantly (by Student's *t* test) from high-fat-fed controls are shown: † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.005$.

Acini from:	Fatty acid synthesis (μmol of $^3\text{H}_2\text{O}/\text{min}$ per 100 mg dry defatted wt. of acini)	Acetyl-CoA carboxylase activity (nmol of $\text{H}^{14}\text{CO}_3^-$ incorporated/min per mg of homogenate protein)	
		0.5 mM-citrate	10 mM-citrate
Chow-fed rats			
Control	0.54 ± 0.06 (4)	3.42 ± 0.63 (4)	19.44 ± 3.19 (4)
+ Insulin (30 munits/ml)	0.52 ± 0.03 (4)	2.92 ± 0.59 (4)	17.25 ± 1.57 (4)
High-fat-fed rats			
Control	0.33 ± 0.03 (7)**	1.59 ± 0.12 (6)**	10.68 ± 1.29 (6)*
+ Insulin (30 munits/ml)	0.58 ± 0.09 (7)††	3.42 ± 0.51 (6)††	16.41 ± 1.63 (6)†
+ Adrenaline	0.47 ± 0.06 (7)†	2.56 ± 0.52 (6)	14.50 ± 2.24 (6)
+ Dibutyl cyclic AMP	0.35 ± 0.04 (7)*	1.92 ± 0.58 (6)*	9.69 ± 1.32 (6)**
+ Insulin + adrenaline	0.50 ± 0.06 (7)††	3.44 ± 0.41 (6)††	17.02 ± 2.29 (6)†
+ Insulin + dibutyl cyclic AMP	0.58 ± 0.07 (7)†††	3.05 ± 0.23 (6)†††	16.05 ± 1.23 (6)†

mechanism. However, preliminary experiments with the α -agonist phenylephrine have not produced the same activations in these acini. These results contribute to the mounting evidence that, in the lactating rat mammary gland, hormones which normally raise cyclic AMP concentrations in cells are not counter-regulatory to the effects of insulin on fatty acid synthesis (Plucinski & Baldwin, 1982; Williamson *et al.*, 1983; Jones *et al.*, 1984; Bussmann *et al.*, 1984; Robson *et al.*, 1984; Clegg *et al.*, 1986).

Conclusions

These results clearly demonstrate an important role for acetyl-CoA carboxylase in mediating the inhibition of mammary-gland lipogenesis in response to feeding a high-fat diet throughout lactation. It is also evident that in isolated acini from mammary glands of high-fat-fed rats the re-activation of this enzyme, rather than any other step, is the significant event in the re-activation of lipogenesis by insulin. Acetyl-CoA carboxylase is known to have regulatory function in the lactating mammary gland during the starved-refed transition, where it is controlled by changes in its phosphorylation state (Munday & Hardie, 1986). Further investigations into the role of phosphorylation in the inactivation of mammary acetyl-CoA carboxylase in response to high-fat feeding, and into the signal responsible for these changes, are required.

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