Defect in signal transduction at the level of the plasma membrane accounts for inability of insulin to activate pyruvate dehydrogenase in white adipocytes of lactating rats

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1. The mechanism responsible for the failure of insulin to activate pyruvate dehydrogenase (PDH) in white adipose tissue *in vivo* during lactation was investigated. 2. Insulin failed to increase PDH in isolated adipocytes from lactating rats. 3. Insulin binding to plasma membranes from adipocytes was unchanged by lactation. 4. Incubation of plasma membranes plus permeabilized mitochondria from adipocytes in the presence of insulin resulted in activation of PDH when the plasma membranes were obtained from virgin rats, whereas no activation was observed when plasma membranes from lactating rats were used. 5. The results show that the failure of insulin to activate PDH in adipose tissue from lactating rats is due to a failure of the signal-transduction system in the plasma membrane at steps subsequent to insulin binding to the insulin receptor.

INTRODUCTION

Lactation results in a diminished rate of fatty acid synthesis from glucose in white adipocytes in the rat (see Vernon & Flint, 1983). Glucose oxidation and acylglycerol glycerol synthesis from glucose are also decreased in white adipocytes of lactating rats (see Williamson, 1980; Vernon & Flint, 1984). These adaptations facilitate the preferential use of glucose by the mammary gland.

The concentration of serum insulin declines during lactation (Robinson *et al.*, 1978; Flint *et al.*, 1979; Burnol *et al.*, 1983), providing a mechanism for the adaptations described above; however, there is also evidence for a diminished ability of adipocytes to respond to insulin. Administration of insulin to lactating rats failed to stimulate fatty acid synthesis (Burnol *et al.*, 1983) and also failed to activate pyruvate dehydrogenase (PDH) (Kilgour & Vernon, 1987b) in white adipose tissue *in vivo*. The reason for the inability of insulin to activate these processes is unknown. Studies *in vitro* with isolated adipocytes from lactating rats indicate that there is a defect at the level of the adipocyte (Burnol *et al.*, 1986). However, insulin binding to adipocytes is not altered by lactation (Flint *et al.*, 1979).

The use of reconstituted subcellular systems has suggested that insulin promotes the release of substance(s), possibly a glycan-phosphoinositide (Saltiel & Cuatrecasas, 1986) or a glycopospholipid (Kelly *et al.*, 1987; Mato *et al.*, 1987), from the plasma membrane which activates PDH and some other enzymes (see Jarett & Kiechle, 1984; Cheng & Larner, 1985; Saltiel, 1987). Using a reconstituted plasma-membrane/permeabilized-mitochondrial system, we now show that lactation results in the failure of adipocyte plasma membranes to release this putative second messenger.

EXPERIMENTAL

Animals

Wistar rats (A. Tuck and Son, Rayleigh, Essex, U.K.) were fed *ad libitum* on Labsure irradiated CRM diet (Labsure, Poole, Dorset, U.K.) and were maintained on a 08:00–20:00 h light/dark cycle. They were mated from about 10 weeks of age, and the number of pups per mother was adjusted to eight within 24 h after birth. Rats were used between days 12 and 14 of lactation; all rats, including virgin rats, were 13–16 weeks old at time of use.

Studies with isolated adipocytes

Parametrial adipose tissue was removed from rats under pentobarbital anaesthesia (60 mg/kg body wt., intraperitoneally) and adipocytes were prepared in bicarbonate-buffered medium containing 5 mM-glucose and 40 mg of dialysed fatty-acid-free bovine serum albumin (Sigma)/ml, and gassed with O₂/CO₂ (19:1) as described previously (Kilgour & Vernon, 1987a). Samples (300 µl) of the packed cell suspensions were incubated in the presence of insulin in a final volume of 1.3 ml. Incubations were carried out at 37 °C in stoppered tubes and shaken at 80 oscillations/min for 20 min, after which cells were separated and assayed for PDH as described previously (Kilgour & Vernon, 1987a).

Preparation of mitochondria and plasma-membrane-enriched fractions from isolated adipocytes

Subcellular fractions were prepared from isolated adipocytes as described by Seals & Czech (1981), except that the broken-cell suspension was obtained by vigorously vortex-mixing the cells for 1 min. The plasma membrane and mitochondrial fractions were resuspended

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a, its active state.

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in 50 mM-potassium phosphate buffer, pH 7.4, and stored at -70°C for up to 1 week before use. As freezing and thawing renders mitochondria permeable to molecules such as CoA, the preparation used is subsequently referred to as 'permeabilized mitochondria'. The protein content of the fractions was measured as described by Bradford (1976), with bovine serum albumin as a standard.

Generation and assay of PDH activator substance(s) from plasma membranes

The method of Seals & Jarett (1980) was used with some modifications. Permeabilized mitochondria (75 μg of protein) were preincubated in 50 mM-potassium phosphate buffer, pH 7.4, with 250 μM -ATP, 50 μM - MgCl_2 and 50 μM - CaCl_2 for 5 min at 37°C in a total volume of 400 μl . Plasma membranes (65 μg of protein) and insulin were then added and the incubations continued for a further 5 min in a total volume of 450 μl , after which the PDH assay was initiated by the addition of 50 μl of assay mixture, to give final concentrations of 50 mM-potassium phosphate, pH 7.4, 1 mM-dithiothreitol, 0.1 mM-CoA, 0.5 mM- NAD^+ , 0.1 mM-thiamin pyrophosphate and 0.25 mM-[1- ^{14}C]pyruvate (1 mCi/mmol; Amersham International, Amersham, Bucks., U.K.). The vial was immediately sealed with a rubber stopper from which was suspended a plastic well containing a 25 mm $\times$ 75 mm piece of filter paper (Whatman no. 1), and 250 μl of Hyamine hydroxide was then added to the well with a syringe. The assay was continued for 2 min at 37°C with gentle shaking (80 oscillations/min), then terminated by injecting 500 μl of 0.08 M-citric acid/0.04 M- Na_2HPO_4 buffer (pH 3.0). The filter paper was allowed to absorb $^{14}\text{CO}_2$ for 1 h at room temperature, after which the $^{14}\text{CO}_2$ content of the wells was measured. Controls were incubated exactly as described above, but without the addition of permeabilized mitochondria or plasma membranes.

Enzyme assays

5'-Nucleotidase activity was determined as described by Vernon *et al.* (1983), and succinate dehydrogenase activity was assayed by the method of Bachmann *et al.* (1966), except that the amount of 2,6-dichloroindophenol in the assay was decreased to 10 μM .

Measurement of insulin binding to plasma membranes

Adipocyte plasma membranes were prepared as described above and resuspended in 10 mM-Tris/HCl, pH 7.4, then stored at -70°C . Plasma membranes (50 μg of protein/assay) were incubated in 10 mM-Tris/HCl, pH 7.8, containing 1% albumin, 4 mg of bacitracin/ml, 0.5 ng of ^{125}I -insulin (0.025 μCi) and a range of concentrations (0–100 ng) of unlabelled insulin at 4°C in a final volume of 400 μl . Pig insulin was iodinated by the lactoperoxidase method (Morrison & Bayse, 1970). Incubations were terminated after 16–18 h by the addition of 1 ml of ice-cold 10 mM-Tris/HCl, pH 7.8, containing 1% albumin, and centrifugation at 2000 g for 30 min at 4°C . The supernatants were removed by decanting and kept for determination of insulin degradation (Freychet *et al.*, 1972). The ^{125}I content of the pellets was determined by γ -radiation counting. All data were corrected for non-specific binding by subtracting the amount of radioactivity still bound in the presence of 10 μg of pig insulin from all other values.

Expression of results

Results are expressed as means $\pm$ S.E.M. and were analysed by Student's *t* test for unpaired observations. The numbers of observations quoted in the text, Tables and Figures represent the numbers of separate preparations used. Each preparation was made from adipose tissue obtained from three or four rats, except for the results with intact adipocytes (Fig. 1), when each individual preparation was made from a single rat.

RESULTS AND DISCUSSION

Effect of insulin *in vitro* on PDH activity in isolated adipocytes

The values for total PDH activity in isolated adipocytes from both virgin and lactating rats (see Fig. 1) were very similar to values previously reported for whole adipose tissue, expressed per 10^6 adipocytes (Kilgour & Vernon, 1987b). Both total PDH activity and PDH_a were significantly decreased in the adipocytes from lactating rats as compared with values for adipocytes from virgin rats ($P < 0.05$). However, the proportion of PDH in the active state (PDH_a) was unchanged by lactation, again in agreement with our previous observations with whole adipose tissue (Kilgour & Vernon, 1987b). Incubation of isolated adipocytes with insulin (35 pM–7 nM) for up to 1 h had no effect on total PDH activity (results not shown). However, insulin increased ($P < 0.01$) PDH_a in

Fig. 1. Effect of insulin on PDH activity *in vitro* in adipocytes from virgin and lactating rats

Adipocytes from virgin (●) or lactating (○) rats were incubated with the concentrations of insulin indicated in the presence of 5 mM-glucose and other components as given in the text at 37°C for 20 min, after which PDH activity was measured. Total PDH activity in the virgin-rat adipocytes was 45.3 ± 4.8 nmol/min per 10^6 adipocytes, and in the lactating-rat adipocytes was 22.2 ± 5.9 nmol/min per 10^6 adipocytes. Results are means $\pm$ S.E.M. for three observations.

Table 1. Protein recovery, 5'-nucleotidase activity and succinate dehydrogenase activity in mitochondrial and plasma-membrane fractions prepared from adipocytes

Mitochondrial and plasma-membrane fractions were prepared from adipocytes of virgin and 14-day-lactating rats. Specific-activity ratio is the enzyme activity per mg of protein of the mitochondrial or plasma-membrane fraction divided by the activity per mg of protein of the homogenate before fractionation. Values are means $\pm$ S.E.M. with the numbers of observations in parentheses.

Variable	Rats . . . Fraction . . .	Virgin (5)		Lactating (4)	
		Mitochondria	Plasma membranes	Mitochondria	Plasma membranes
Protein (% of homogenate protein)		5.4 $\pm$ 1.2	2.6 $\pm$ 0.2	3.1 $\pm$ 0.8	2.1 $\pm$ 0.3
5'-Nucleotidase (nmol/min per mg of protein)		2.8 $\pm$ 0.4	17.4 $\pm$ 1.7	2.3 $\pm$ 0.6	15.2 $\pm$ 1.6
(specific-activity ratio)		0.8 $\pm$ 0.1	7.0 $\pm$ 1.1	1.0 $\pm$ 0.2	7.0 $\pm$ 0.8
Succinate dehydrogenase (nmol/min per mg of protein)		16.9 $\pm$ 6.5	3.7 $\pm$ 1.4	19.9 $\pm$ 1.9	5.3 $\pm$ 0.4
(specific-activity ratio)		3.2 $\pm$ 0.5	0.7 $\pm$ 0.2	3.7 $\pm$ 0.4	1.0 $\pm$ 0.1

isolated adipocytes from virgin rats (Fig. 1). Maximal enzyme activation was achieved with 1.4 nM-insulin, which increased PDH activity by $186.1 \pm 25.7\%$ (mean $\pm$ S.E.M. for three observations): concentrations of insulin greater than 3.5 nM gave less stimulation of PDH activity (Fig. 1). Maximal activation of adipocyte PDH occurred within 20 min of incubation (results not shown). Weiss *et al.* (1974) and Sooranna & Saggerson (1979) reported that maximal activation of PDH in isolated adipocytes was achieved with 1.5 nM-insulin, although Jarrett *et al.* (1985) obtained maximal stimulation of adipocyte PDH_a with 0.7 nM-insulin.

In contrast, all concentrations of insulin used (between 35 pM and 7 nM) were without effect on PDH in adipocytes from lactating rats (Fig. 1) during incubations for up to 60 min (results not shown).

Studies with subcellular fractions prepared from rat adipocytes

To characterize the subcellular fractions, succinate dehydrogenase activity was used as a mitochondrial enzyme marker and 5'-nucleotidase activity was used as a plasma-membrane marker. The activities of these marker enzymes in the subcellular fractions (Table 1) were in good agreement with values reported in other studies in which adipose-tissue plasma membrane and mitochondrial fractions were prepared (McKeel & Jarrett, 1970; Belsham *et al.*, 1980; Begum *et al.*, 1982). The specific activity of 5'-nucleotidase was 7-fold greater in the plasma-membrane fractions than in the unfractionated adipocyte homogenates, whereas the specific activity of this enzyme in the mitochondrial fractions was similar to that in the adipocyte homogenates (Table 1). Succinate dehydrogenase activity was similar in the plasma-membrane fractions and unfractionated homogenates, but was 3–4 times greater in the mitochondrial fractions (Table 1). There was no significant difference in the protein recovery, 5'-nucleotidase activity or succinate dehydrogenase activity between the plasma-membrane or mitochondrial fractions from virgin and lactating rats (Table 1).

Similar Scatchard plots were obtained for the binding of insulin to adipocyte plasma membranes from virgin and lactating rats (Fig. 2). For the purpose of analysis,

the plots were interpreted as comprising two components. For insulin binding to plasma membranes from virgin rats, one component had a K_d for insulin of 1.1 ± 0.06 nM and one had a K_d of 7.2 ± 1.5 nM, whereas for lactating rats the two components had K_d values of 1.1 ± 0.07 and 7.7 ± 0.5 nM respectively. The number of high- and low-affinity binding sites per fg of protein were 4.9 ± 0.5 and 10.5 ± 0.5 respectively for plasma membranes from virgin rats and 4.8 ± 0.4 and 10.7 ± 0.6 for lactating rats. All results are means $\pm$ S.E.M. for four observations, and

Fig. 2. Scatchard plots of the insulin binding to adipocyte plasma membranes from virgin and lactating rats

¹²⁵I-insulin binding to plasma membranes prepared from adipocytes from virgin (○) or lactating (●) rats was measured and corrected for non-specific binding as described in the text. Each point is the mean value obtained from separate determinations with plasma membranes prepared from adipocytes of four rats.

Table 2. Effect of lactation on the activation of PDH in permeabilized mitochondria by adipocyte plasma membranes and insulin

Mitochondrial and plasma-membrane fractions were prepared from adipocytes of virgin and 14-day-lactating rats. Permeabilized mitochondria (180 μ g of protein/ml) were preincubated for 5 min with 250 μ M-ATP, and other components as given in the text. Plasma membranes (150 μ g of protein/ml) and insulin (0.7 nM) were then added, and the incubations were continued for a further 5 min, after which PDH activity was measured. Values are means $\pm$ S.E.M. for four observations; * indicates that the value is significantly different ($P < 0.05$) from the value for co-incubation of plasma membranes and mitochondria in the absence of insulin. Values for absolute changes in PDH activity and % stimulation were computed from paired observations.

Source of components			PDH activity (nmol/min per mg of mitochondrial protein)	Insulin effect	
Mitochondria	Plasma membranes	Insulin		Absolute change in PDH activity	% stimulation
Virgin rat	—	—	8.9 $\pm$ 1.4		
Virgin rat	Virgin rat	—	12.1 $\pm$ 1.7		
Virgin rat	Virgin rat	+	17.6 $\pm$ 1.1*	5.5 $\pm$ 1.0	48.3 $\pm$ 10.1
Virgin rat	Lactating rat	—	8.9 $\pm$ 1.5		
Virgin rat	Lactating rat	+	8.7 $\pm$ 1.1	−0.1 $\pm$ 0.5	—
Lactating rat	—	—	9.4 $\pm$ 1.4		
Lactating rat	Lactating rat	—	9.4 $\pm$ 1.5		
Lactating rat	Lactating rat	+	9.2 $\pm$ 1.3	−0.2 $\pm$ 0.3	—
Lactating rat	Virgin rat	—	10.5 $\pm$ 1.6		
Lactating rat	Virgin rat	+	15.2 $\pm$ 0.9*	4.7 $\pm$ 1.3	47.8 $\pm$ 18.0

values for lactating rats do not differ significantly from equivalent values for virgin rats. The K_a values measured were similar to those reported by Flint *et al.* (1979) for insulin binding to receptors on intact adipocytes. This suggests that the insulin receptors were not damaged during the isolation of plasma membranes. Non-specific binding of insulin to plasma membranes amounted to $16.7 \pm 1.3\%$ and 13.6 ± 2.1 (mean $\pm$ S.E.M. for four observations) of the total insulin bound respectively for virgin and lactating rats. When insulin binding to plasma membranes from either virgin or lactating rats was measured, less than 5% of the insulin added was degraded.

There was no significant difference in PDH activity per mg of protein between permeabilized mitochondrial fractions prepared from adipocytes from virgin and 14-day-lactating rats (Table 2). PDH activity in the plasma-membrane fractions was consistently less than 5% of that in the mitochondrial fractions (results not shown). In agreement with previous studies (Seals & Jarett, 1980; Kiechle *et al.*, 1981; Seals & Czech, 1981), incubation of permeabilized mitochondria with ATP decreased PDH_a activity (results not shown). In the present study the decrease was maximal after 4 min and persisted for at least a further 6 min (results not shown). Preincubation of permeabilized mitochondrial fractions with ATP increases the stimulation of PDH activity by insulin (Seals & Jarett, 1980; Seals & Czech, 1981), and hence such mitochondria were routinely incubated for 5 min with ATP before the addition of plasma membranes and insulin.

Co-incubation for 5 min of plasma membranes and permeabilized mitochondria from virgin rats resulted in a slight stimulation of PDH activity, in agreement with previous reports (Seals & Jarett, 1980; Begum *et al.*, 1982); this stimulation was potentiated by insulin (Fig. 3). The storage of plasma membranes and mitochondria in liquid N₂ for up to 7 days had no effect on the ability of membranes to stimulate PDH activity. Increasing concentrations of insulin produced corresponding

increases in PDH activity, until the maximum stimulation was achieved at 0.7 nM: concentrations of insulin greater than 0.7 nM produced a smaller stimulation of PDH activity. This biphasic response of PDH to increasing concentrations of insulin is in agreement with previous studies (Seals & Jarett, 1980; Seals & Czech, 1981). The maximal stimulation of PDH activity was $48.3 \pm 10.1\%$ (Table 2) and occurred after 5 min of incubation and persisted for a further 25 min (results not shown). This maximal stimulation of PDH activity is in good agreement with values reported by Begum *et al.* (1982). In contrast with the results for virgin rats, co-incubation, in the presence or absence of insulin (0.7 nM), of plasma-membrane and permeabilized mitochondrial fractions prepared from lactating rats had no effect on PDH activity (Table 2). All concentrations of insulin used (between 70 pM and 7 nM) were without effect on PDH in co-incubations of plasma membranes and permeabilized mitochondria from lactating rats (results not shown). However, co-incubation of plasma membranes from virgin rats with permeabilized mitochondria from lactating rats produced a slight increase in PDH activity, which was enhanced by insulin ($P < 0.05$), but co-incubation of plasma membranes from lactating rats with permeabilized mitochondria from virgin rats had no effect on PDH activity (Table 2). The increase in PDH activity caused by insulin was similar when plasma membranes from virgin rats were incubated with permeabilized mitochondria from virgin or lactating rats (Table 2).

A further experiment was performed with a mixture of plasma membranes from virgin and lactating rats. The presence of plasma membranes from lactating rats did not impair the ability of the membranes from virgin rats to activate PDH in the presence of 0.7 nM-insulin (results not shown), indicating that the plasma membranes from lactating rats were not releasing an inhibitor of the putative mediator.

When plasma membranes from virgin rats were incubated with insulin and then removed by centri-

Fig. 3. Stimulation of PDH activity by addition of insulin to a mixture of plasma membranes and permeabilized mitochondria from adipocytes of virgin rats

Adipocyte mitochondria (180 μ g of protein/ml), permeabilized by freezing and thawing, were preincubated with 250 μ M-ATP at pH 7.4 for 5 min at 37 °C. The incubation was continued for a further 5 min in the presence (●) or absence (○) of adipocyte plasma membranes and with the concentrations of insulin indicated, after which PDH activity was measured. The experiment was carried out three times with similar results.

fugation (50000 g for 1 min), the resulting supernatant activated PDH as well as when plasma membranes were present (results not shown), demonstrating that the putative mediator is released from the plasma membrane, as found previously (Seals & Czech, 1981; Kiechle *et al.*, 1981; Begum *et al.*, 1982). However, when plasma membranes from lactating rats were used, the supernatant had no effect on PDH activity; this could be due to an inability to release the mediator, or to a failure to produce the mediator in the plasma membrane.

General discussion

The present finding that insulin failed to activate PDH in isolated adipocytes *in vitro* from lactating rats suggests that our previous observation that insulin did not activate PDH in adipose tissue from such rats *in vivo* (Kilgour & Vernon, 1987b) was due to a defect in adipocytes rather than the presence of antagonistic hormones in the blood.

The ability to bind insulin is unimpaired by lactation in whole adipocytes (Flint *et al.*, 1979) and in isolated plasma membranes (Fig. 2), but nevertheless the present study shows that there is an impairment in the signal-transduction system at the level of the plasma membrane. The mechanism whereby insulin activates PDH is not yet known, but there is a growing body of evidence supporting the view that a second messenger is released

from the plasma membrane (e.g. Jarett & Kiechle, 1984; Cheng & Larner, 1985; Saltiel, 1987). One possibility, Ca^{2+} , has been excluded, or at least changes in Ca^{2+} cannot account for all the activation caused by insulin in adipocytes (McCormack & Denton, 1985). Another possibility, that ATPase activity of the plasma membrane hydrolysed the ATP in the medium with a concomitant activation of PDH, appears unlikely from the experiments in which the plasma membranes were removed after exposure to insulin and the supernatant was used to activate PDH. The results of the present study support the view that insulin causes the release of a mediator from the plasma membrane. Release of this mediator is impaired during lactation, but it is not clear if this is due to a failure to produce or to release the mediator. Whatever the defect, it appears to reside at the level of the membrane. The mitochondrial response mechanism appears to be unaltered by lactation, but the mitochondria used were not intact, so some alteration here cannot be excluded completely *in vivo*.

This putative mediator appears to be involved in the activation or inhibition of variety of enzyme activities by insulin, including adenylate cyclase, cyclic AMP phosphodiesterase, glycogen synthase and acetyl-CoA carboxylase, as well as PDH (see Jarett & Kiechle, 1984; Cheng & Larner, 1985; Saltiel, 1987), possibly mediating insulin-induced changes in phosphorylation status (Alemany *et al.*, 1987). However, the mediator does not appear to be involved in the stimulation of glucose transport in adipocytes by insulin (Kelly *et al.*, 1987). It is not known if the ability of insulin to alter the activity of enzymes other than PDH is modified in adipocytes during lactation, or if the ability of insulin to stimulate glucose uptake is changed. However, Burnol *et al.* (1986) reported that, whereas the ability of insulin to promote glucose oxidation and conversion into fatty acid was impaired during lactation, insulin stimulation of glucose conversion into lactate and acylglycerol was apparently unchanged. Failure to produce the mediator and hence to activate PDH, but maintenance of the ability to stimulate glucose transport, would account for these observations of Burnol *et al.* (1986).

Some nutritional and pathological states which produce insulin resistance have been shown to result in a decreased ability of plasma membranes to generate the putative insulin mediator. The ability of insulin to stimulate fatty acid synthesis and lipogenesis in hepatocytes from fasted and non-ketotic diabetic rats is decreased (Caro & Amatruda, 1982; Amatruda & Chang, 1983), and this is accompanied by a markedly decreased ability of hepatocyte plasma membranes to generate the putative insulin mediator: re-feeding of fasted rats and insulin treatment of diabetic rats restores the ability of insulin to generate the mediator (Amatruda & Chang, 1983). Adapting rats to a high-fat diet results in a decreased insulin-responsiveness of adipocytes (Ip *et al.*, 1976; Begum *et al.*, 1982) and hepatocytes (Tepperman *et al.*, 1978), and again this is accompanied by a decreased ability of insulin to stimulate the generation of the putative mediator substance from the respective plasma membranes (Begum *et al.*, 1982, 1983). Lactation is an example of a natural physiological state in which insulin resistance appears to be due to an inability to release a mediator. In common with the above states, lactation results in hypoinsulinaemia (Robinson *et al.*, 1978; Flint *et al.*, 1979; Burnol *et al.*,

1983); this suggests that insulin itself is important for maintenance of the ability of some cell types to respond to insulin.

E.K. was in receipt of an AFRC scholarship.

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Received 20 November 1987/4 February 1988; accepted 16 February 1988