Evidence for the involvement of a gastrointestinal peptide in the regulation of glucose uptake in the mammary gland of the lactating rat

Tony PAGE*

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

1. A method of obtaining serial arterial and mammary-venous blood samples was used to identify possible factors involved in the regulation of glucose uptake in the gland of the lactating rat. 2. Administration of insulin alone increased the arteriovenous glucose difference across the mammary gland of starved rats, but the time course of the recovery could not account for the restoration of arteriovenous glucose difference observed during refeeding [Page & Kuhn (1986). *Biochem. J.* **239**, 269–274]. 3. A crude extract of the gastrointestinal tract (stomach–ileum) from lactating rats enhanced the change in mammary glucose uptake observed with insulin, but only when large amounts (100 munits/rat) of insulin were used. To achieve a similar recovery of arteriovenous glucose difference using near-physiological amounts (5 munits/rat) of insulin it was necessary to sever the mammary nerves. 4. A peptide fraction (of < 10 kDa) isolated from the gut extract enhanced the effect of insulin in a similar manner to the crude extract. 5. It is suggested that in addition to insulin at least another component, probably a gut peptide, is required for the restoration of mammary glucose uptake during refeeding. An inhibitory component may also contribute to the regulation of mammary glucose extraction in the lactating rat.

INTRODUCTION

At peak lactation in the fed rat the mammary gland is one of the main tissues metabolizing glucose, and it has been estimated that the rate of glucose consumption is about 30 mmol/day (Williamson, 1980). The fate of this glucose is documented (Katz & Wals, 1972): about 65 % is converted into lipid and 25 % into lactose, and the remainder is oxidized. Synthesis of lactose and lipid is acutely sensitive to the nutritional state of the rat (Bussmann *et al.*, 1984; Williamson *et al.*, 1983), and, in accordance with this, the extraction of glucose by the mammary gland, as monitored by arteriovenous glucose difference, is sensitive to starvation and refeeding (Page & Kuhn, 1986).

Overnight starvation leads to the complete inhibition of lipid synthesis and almost total inhibition of lactose synthesis and glucose uptake; and refeeding is associated with a rapid recovery of lipid synthesis and glucose extraction, although lactose synthesis appears to be delayed (Munday & Williamson, 1981; Bussmann *et al.*, 1984; Mercer & Williamson, 1986; Page & Kuhn, 1986).

The physiological basis for the control of mammary glucose metabolism during feeding and starvation in the rat is poorly understood. A requirement for insulin has been recognized since fed rats treated with streptozotocin show a markedly decreased arteriovenous glucose difference (Page & Kuhn, 1986) and a decreased rate of mammary lipogenesis (Bussmann *et al.*, 1984). Moreover, administration of insulin to starved rats results in an increase of lipogenesis and glucose extraction (Jones *et al.*, 1984; Page & Kuhn, 1986). The mammary gland is also sensitive to infusions of insulin (Jones *et al.*, 1984;

Burnol *et al.*, 1987). In addition, refeeding is associated with a 'spike' of insulin at 15–20 min just before the recovery of lipogenesis (Mercer & Williamson, 1986) and glucose extraction (Page & Kuhn, 1986).

The apparent speed with which mammary glucose metabolism increases during refeeding makes the use of isotopic measurements unsuitable. The arteriovenous glucose difference method, however, offers an almost instantaneous measurement and has been shown to provide a useful index of the rapid changes in mammary glucose uptake (Page & Kuhn, 1986). The present paper shows that it is possible to collect several mammary arteriovenous blood samples at short intervals from individual rats. In the present study this technique has been used to investigate factors which may be important in the regulation of glucose uptake by the mammary gland during starvation and refeeding.

MATERIALS AND METHODS

Animals

Primiparous rats (Wistar strain) were used at 14–17 days of lactation, having been maintained on a 14 h-light/10 h-dark cycle (light on at 06:00 h). Most experiments were performed on overnight-starved rats and conducted between 09:00 and 12:00 h.

Sampling method

Rats were anaesthetized with sodium pentobarbitone (Sagatal; 60 mg/kg body wt.). A cannula of polythene tubing (inner diam. 0.58 mm, outer diam. 0.96 mm; Portex, Hythe, Kent, U.K.) was inserted into the left carotid artery, and the right main abdominal–inguinal

Abbreviation used: PBS, phosphate-buffered saline.

* Present address St. Edmund's College, Cambridge CB3 0BN, U.K.

mammary vein was exposed. Simultaneous arterial and venous blood samples (about 0.1 ml) were collected by syringe from the cannula and by making a small cut in the vein and then drawing the escaping blood into a heparinized capillary tube. A piece of surgical gauze was then placed on the cut to staunch the flow. The blood samples were immediately transferred to chilled heparinized microfuge tubes and centrifuged to separate the plasma, which was assayed for glucose with a glucose oxidase kit (Boehringer). Between sampling of the arterial blood, the cannula was filled with saline (0.9% NaCl) containing heparin (10 units/ml), and this was removed before collection of blood samples. Throughout the experiments the rats were kept on a warmed table and with a lamp overhead.

Treatment of rats

Starved rats were prepared as described above, and simultaneous arterial and venous blood samples were collected before and at intervals up to 30 min after an intravenous injection of 5, 25, 50 or 100 munits of insulin (Wellcome Laboratories, Beckenham, Kent, U.K.) or phosphate-buffered saline (PBS). In the initial trial experiments, arteriovenous blood samples were collected from fed or starved rats at 5 min intervals up to 30 min. For experiments in which gut extract was included, the extract (1–2 ml) or similar volume of PBS was injected intraperitoneally 10 min before administration of the insulin.

In studies in which the mammary nerves were severed, the animals were prepared as before, except that nerves supplying the inguinal and abdominal mammary glands were cut at a point about 1 cm from where the nerves entered the glands, and the cut nerve ends were separated. Arteriovenous blood samples were then collected at 5 min intervals up to 20 min before injection of the gut extract intraperitoneally and of 5 munits of insulin intravenously; when testing the purified peptide fraction, the sample (about 1.5–10 nmol) was injected with insulin (0.5–5 munits).

Preparation of gut extract

The gastrointestinal tract, comprising the stomach, duodenum and ileum, was excised from three or four fed lactating rats. Food material was removed by flushing the lumen with ice-cold saline before placing the tissue in chilled acid/ethanol (0.1 M-HCl in 75% ethanol; approx. 10 g of tissue per 100 ml) and cutting it into small pieces. The mixture was stirred for 24 h at 5 °C. Next it was centrifuged at 30 000 g_{av} for 30 min. The supernatant was decanted, and most of the ethanol was removed by evaporation under vacuum at 60 °C. The resulting mixture was freeze-dried and redissolved in about 50 ml of PBS. This gave a solution containing about 30 mg of protein/ml, which was divided into batches, and stored frozen until required.

Isolation of peptide fraction from extract

The crude gut extract was centrifuged at 150 000 g_{av} for 30 min and passed through an ultrafiltration unit fitted with a filter to exclude molecules larger than 10 kDa. The filtrate was subjected to h.p.l.c. on a reverse-phase C-18 column (5 μ m pore size). Components were eluted with a linear gradient of 0–60% acetonitrile in water (with 0.1% trifluoroacetic acid) over 30 min and were monitored by absorbance at 230 nm.

Amino acid analysis

Material from the active fraction was hydrolysed (6 M-HCl, 24 h) and subjected to amino acid analysis (using an LKB 4400 autoanalyser).

RESULTS AND DISCUSSION

Serial sampling

Initial studies established that the mammary arteriovenous glucose difference was apparently unaffected by the procedure used to obtain several mammary arteriovenous blood samples from individual rats. Fig. 1 shows that by using this technique reasonably constant values for mammary arteriovenous glucose difference were obtained with both fed and starved rats during the 30 min study. In addition, the values corresponded to those found when single measurements were made on fed and starved rats, about 60% and 5% respectively (Page & Kuhn, 1986). Since blood-flow rates were not assessed in these experiments, the technique is open to the criticism that the repeated sampling might have produced changes in blood flow. However, such changes would be expected to alter significantly mammary glucose extraction, but the relative constancy of the arteriovenous glucose differences shown in Fig. 1 clearly suggested that any changes in blood flow which might have occurred were not sufficient to affect substantially the arteriovenous glucose difference in either fed or starved rats. Arterial plasma [glucose] also appeared to be little affected by the technique, although in starved rats it was slightly elevated.

Fig. 1. Effect of serial sampling on mammary arteriovenous glucose difference and arterial plasma [glucose] of fed (●) and 18 h-starved (○) lactating rats

Values are means $\pm$ S.E.M. for six rats.

Fig. 2. Change in mammary arteriovenous glucose difference and arterial plasma [glucose] with insulin

Various amounts of insulin were injected intravenously into starved rats: 100 munits (●, $n = 4$); 50 munits (○, $n = 4$); 25 munits (▲, $n = 6$); 5 munits (△, $n = 3$); and saline (■, $n = 6$). Values are means $\pm$ S.E.M. Arteriovenous glucose differences significantly greater than control group are shown: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Mean values for refeed rats are also shown (----) (data from Page & Kuhn, 1986).

Effect of exogenous insulin

Insulin has been shown to restore mammary glucose extraction in starved rats (Page & Kuhn, 1986). Therefore, experiments were performed to determine whether this observation could be repeated by using the serial sampling technique, and consequently to establish a time course for the change in mammary glucose uptake with insulin administration. Fig. 2 shows that insulin administered to starved rats produced a significant ($P < 0.01$ and $P < 0.001$) increase in the mammary arteriovenous glucose difference by 30 min (26–43 %, compared with 3 % for the control group), and the mean values appeared to show an association with the amount of insulin, although the mean value obtained for 5 munits was slightly greater than that for 25 munits. These results not only confirmed the previous findings but also illustrated that the sampling technique could be used to show a recovery in mammary arteriovenous glucose difference.

The second important feature of these results was the time course of the restoration of mammary glucose

extraction. At 10 and 15 min after the injection of insulin only 5 and 100 munits of insulin produced a significant ($P < 0.01$ and $P < 0.05$) increase in the arteriovenous glucose difference (to about 20 %, compared with control). However, by 20 min there were significant ($P < 0.01$ and $P < 0.001$) increases, to between 23 % (5 munits) and 34 % (100 munits), with all rats given insulin. For comparison, the data of Page & Kuhn (1986) for 0, 15 and 30 min refeed values of mammary arteriovenous glucose difference are also shown in Fig. 2. Clearly, in all cases where rats were given insulin the time course of recovery of mammary glucose extraction was not as rapid as that expected with natural refeeding. In particular, 15 min refeeding has been shown to produce a mean value of 41 % arteriovenous glucose difference, whereas even with 100 munits of insulin the value reached only 26 %. Thus insulin was apparently unable to restore completely the mammary arteriovenous glucose difference of starved rats, even with large amounts (50 and 100 munits) which produced considerable hypoglycaemia. Although the hypoglycaemia is likely to result in an increase in catecholamine concentrations, these were not measured, because some preliminary findings have indicated that the infusion of adrenaline into fed lactating rats does not markedly affect the mammary arteriovenous glucose difference (T. Page, unpublished work). Further experiments using the euglycaemic-clamp technique would be more definitive, since this would overcome such changes produced by the hypoglycaemia in the present studies.

These results might be partly attributable to limitations of the sampling technique, but they suggested that insulin alone could not completely and rapidly restore the mammary glucose uptake after refeeding. The results compare with the findings of other workers (Bussmann *et al.*, 1984; Jones *et al.*, 1984), who noted that insulin can produce an increase in lipogenesis and lactose synthesis but did not restore them as effectively as refeeding.

Effect of crude gut extract with insulin

The close association observed between mammary glucose metabolism and the nutritional state of the mother in rats implicates the possible involvement of components released by the gastrointestinal tract. Fig. 3 shows that when a crude extract of the gastrointestinal tract of lactating rats was administered with 100 munits of insulin, the restoration of glucose extraction by the mammary gland resembled that seen with refeeding. A significantly ($P < 0.01$) greater arteriovenous glucose difference compared with that for insulin alone was seen after 1 min. This difference remained significant until 30 min, by which time the mean arteriovenous glucose difference for rats given insulin alone had increased to 43 %, but this was still less than the 55 % for rats treated with gut extract and insulin. The gut extract alone appeared to have little effect on the arteriovenous glucose difference, or on the arterial plasma [glucose].

These experiments suggested that one or more components of the gut extract in the presence of insulin could be involved in the physiological restoration of mammary glucose extraction during refeeding. However, in these experiments 100 munits of insulin was used, since it alone produced a large increase in the arteriovenous glucose difference. Such amounts of insulin are not within the physiological range, and resulted in severe hypoglycaemia. The possibility that lipogenesis was inhibited

Fig. 3. Change in mammary arteriovenous glucose difference and arterial plasma [glucose] of 18 h-starved lactating rats given 100 munits of insulin intravenously (at zero time) 10 min after intraperitoneal administration of PBS (●, $n = 8$) or gut extract (▲, $n = 8$), or given gut extract alone (△, $n = 8$) or PBS alone (○, $n = 6$)

Values are means $\pm$ S.E.M. Arteriovenous glucose differences significantly greater than for 100 munits of insulin + PBS are shown: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

by the hypoglycaemia seems unlikely, since in the study in which gut extract was administered with 100 munits of insulin there was a similar decrease in plasma glucose to that observed with insulin alone, but a markedly enhanced increase in the change in mammary arteriovenous glucose difference also occurred, implying that the hypoglycaemia was not affecting the restoration of mammary glucose metabolism. Also, in studies with fed lactating rats it has been shown that insulin-induced hypoglycaemia did not alter the rate of lipogenesis (Bussmann *et al.*, 1984). Attempts to reproduce the effect of the gut extract with insulin using near-physiological amounts (5 munits) of insulin failed to show any difference between rats treated with and without gut extract (results not shown). This was interpreted as an inhibition occurring either by a component of the gut extract or within the animal, and the use of large amounts of insulin could overcome this effect.

Effect of gut extract and 5 munits of insulin in rats with cut mammary nerves

Cutting the mammary nerves before administration of 5 munits of insulin had little effect on the change in glucose extraction (see Fig. 4); an increase to a mean of

Fig. 4. Change in mammary arteriovenous glucose difference and arterial plasma [glucose] with insulin and gut extract in starved rats when mammary nerves are cut

The mammary nerves were cut 20 min before the intravenous administration of 5 munits of insulin alone (○, $n = 5$) or with gut extract intraperitoneally (●, $n = 4$) at zero time. Values are means $\pm$ S.E.M. and significantly different arteriovenous glucose differences are shown: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

about 35% was still observed and compared with that found in 'intact' rats given 5 munits of insulin (see Fig. 2). However, when gut extract was given with 5 munits of insulin the arteriovenous glucose difference increased immediately ($P < 0.05$) at 5 min and reached a mean of 50% by 10 min ($P < 0.001$), remaining at about this value up to 30 min despite an apparent decrease to about 38% at 15 min. In these circumstances no significant changes were observed with the arterial plasma [glucose] between the two groups of rats. The results also indicated that cutting the mammary nerves had little effect on the arteriovenous glucose difference, since no apparent change occurred during the 20 min between cutting the nerves and administering the insulin alone or with gut extract.

Effect of a peptide fraction purified from the gut extract

Cutting the mammary nerves before testing the extract for activity was used as an assay procedure for the purification of the activity of the gut extract to enhance glucose extraction in the presence of insulin. Fig. 5 shows that when one of the fractions recovered from h.p.l.c. separation was administered with near-physiological amounts (0.5–5 munits) of insulin it produced a marked enhancement of the increase in arteriovenous glucose difference compared with that for the insulin alone; this did not occur with other fractions similarly tested. A

Fig. 5. Change in mammary arteriovenous glucose difference and arterial plasma [glucose] with insulin and isolated gut peptide fraction in starved rats with cut mammary nerves

The mammary nerves were cut 20 min before the intravenous injection of 0.5–5 munits of insulin alone (○, $n = 6$) or with the gut peptide fraction (about 1.5–10 nmol) (●, $n = 6$) at zero time. Values are means $\pm$ S.E.M., and significantly different arteriovenous glucose differences are shown: ** $P < 0.01$; *** $P < 0.001$.

high level of statistical significance ($P < 0.001$) was found at 5 min and continued until 25 min. By this time the arteriovenous glucose difference was about 50%, although by 30 min the value had decreased to 39% and was not significantly different from that for insulin alone. No difference in the arterial plasma [glucose] was observed between the two groups of rats.

Amino acid composition of 'active' fraction

The amino acid composition of the 'active' fraction recovered as a single sharp peak from the h.p.l.c. separation is shown in Table 1. This analysis indicated that the fraction contained a peptide component with a molecular mass in the region of 5 kDa, and containing most of the amino acids. On the basis of this analysis it appears that the 'active' component may be a unique peptide, since the amino acid composition differs from that of known gut peptides.

Conclusions

It has been shown that a straightforward technique for sampling the mammary arteriovenous blood of a lactating rat can be used to investigate the change in mammary glucose extraction and to attempt to identify the physiological signals leading to these changes, which may be occurring during refeeding. The results of these

Table 1. Amino acid composition of isolated gut fraction

A sample (50 μ l) of the 'active' gut fraction obtained by h.p.l.c. separation was analysed for its amino acid content as described in the Materials and methods section. The empirical number of residues of each amino acid was calculated by dividing the amount by the value for histidine.

Amino acid	Amount (nmol/sample)	Empirical no. of residues
Ala	2.13	4
Arg	1.06	2
Asp/Asn	1.23	2
Glu/Gln	4.67	9
Gly	3.79	7
His	0.55	1
Ile	0.85	2
Leu	1.92	4
Met	2.00	4
Lys	1.89	3
Phe	0.79	1
Pro	2.30	4
Ser	1.65	3
Thr	0.98	2
Tyr	0.33	1
Val	1.28	2

studies indicate that insulin alone is not sufficient to evoke the full restoration of mammary glucose uptake and that at least a second component, probably a peptide, released from the gastrointestinal tract is required to act synergistically with insulin. In addition, an inhibitory factor may be present, which requires suppression before a full restoration of glucose extraction can occur. These experiments point to a neural origin of this inhibitory factor, which contrasts with the findings of Hebb & Linzell (1970), who could find no evidence that nerves penetrated the mammary secretory cells. However, that group was staining for noradrenaline, whereas a neural peptide may be involved in this case.

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