Biochem. J. (1976) **156**, 333–337
Printed in Great Britain

Control of Rat Mammary-Gland Pyruvate Dehydrogenase by Insulin and Prolactin

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(*Received* 19 *December* 1975)

Withdrawal of prolactin or of insulin from the circulation of lactating rats leads, within 3h, to increased inactivation by phosphorylation of mammary-gland pyruvate dehydrogenase. Prolactin may act by priming the tissue to respond directly to normal concentrations of circulating insulin and by this means be responsible for the increased activation of the enzyme during the course of normal lactation.

We have shown (Field & Coore, 1975) that lactating-rat mammary-gland pyruvate dehydrogenase (EC 1.2.4.1) is inactivated *in vivo* by 24h withdrawal of prolactin caused by injection of 2-bromo-α-ergocryptine. The inactivation was due to increased phosphorylation of the enzyme, presumably similarly to the phosphorylation control of homologous mammalian enzymes (Linn *et al.*, 1969). At 12 or 21 days of lactation, but not at 4 days of lactation, a prolactin injection, given at the same time as 2-bromo-α-ergocryptine, prevented the effect of the latter. We have now investigated further the situation at 4 days of lactation to discover any factors, additional to prolactin, that might be involved in the control of the enzyme. Our results indicate that the concentration of circulating plasma insulin may be such a factor. Since it was possible that altered feeding habits of the lactating rats (or of the pups) due to the injection of 2-bromo-α-ergocryptine may have contributed to the results of experiments extending over 24h, we have also tested the effects of short-term (3h) withdrawal of insulin or prolactin.

Experimental

2-Bromo-α-ergocryptine, prolactin and streptozotocin were the generous gifts of, respectively, Sandoz, Basle, Switzerland, N.I.H., Bethesda, MD, U.S.A., and Upjohn Co., Kalamazoo, MI, U.S.A.

Insulin was obtained from Weddel Pharmaceuticals, London E.C.1, U.K., and Burroughs Wellcome and Co., London N.W.1, U.K., and cortisol was from BDH Chemicals, Poole, Dorset, U.K. Sources of other materials used, rats, operative procedure, methods of tissue extraction, assay of pyruvate dehydrogenase and glutamate dehydrogenase were described by Coore & Field (1974). One unit of enzyme activity implies 1 μmol of product formed/min at 30°C, except for pyruvate dehydrogenase phosphatase, where 1 unit of phosphatase activity formed 1 unit of pyruvate dehydrogenase activity in 5min at 30°C. 2-Bromo-α-ergocryptine, dissolved in 0.2ml of 40% (v/v) ethanol, and prolactin, dissolved in 0.2ml of 154mM-NaCl, pH10, were both injected subcutaneously. Streptozotocin was dissolved in 0.2ml of 10mM-sodium acetate buffer, pH4.5, and immediately injected intraperitoneally. Soluble and protamine–zinc–insulin were injected subcutaneously. Freeze-clamped gland (obtained from an animal under halothane anaesthesia) was powdered under liquid N_2, and 300–500mg of frozen powder was extracted into either 2ml of ice-cold 25mM-triethanolamine/7mM-mercaptoethanol, pH 7.0, or into 2ml of ice-cold 20mM-triethanolamine/7mM-mercaptoethanol/5mM-EDTA, pH7.0. The EDTA extract was assayed directly to yield 'initial' pyruvate dehydrogenase activity. The extract without EDTA was incubated at 30°C in the presence of more than 0.5 unit of pyruvate dehydrogenase phosphatase/ml and 10mM-$MgCl_2$, and then assayed at successive time-intervals (<15min) to yield maximal activity, 'total' pyruvate dehydrogenase activity. It was necessary to add 5mM-EDTA to the extraction medium to guarantee chelation of Mg^{2+} and Ca^{2+} in mammary tissue and hence a true value of 'initial' pyruvate dehydrogenase activity. Determination of 'total' enzymic activity by phosphatase activation in such a medium would involve adding excess of Mg^{2+}, which would have caused a fall in pH of the buffer used in these and earlier studies.

Blood was collected from the tail vein into chilled tubes, containing EDTA, and immediately centrifuged. Glucose assay was by using a glucose oxidase kit (Boehringer Corp., Bilton House, 54/58 Uxbridge Road, London W5 2TZ, U.K.), and in a few instances a rough estimate was made by using Dextrostix (Ames Co., Stoke Poges, Slough, Bucks., U.K.). Non-esterified fatty acids were measured by the method of Itaya & Ui (1965). Normally, blood was sampled when dissection of the mammary gland was complete immediately before freeze-clamping and about 15min after anaesthetic induction.

Results and Discussion

Efficiency of enzyme extraction

It was important to compare the efficiency of enzyme extractions in media with and without EDTA. Fot the mitochondrial enzyme glutamate dehydrogenase the apparent activity in the medium containing EDTA, was 1.25 ± 0.09 (7) units/g wet wt., which did not differ significantly from the activity in the medium without EDTA 1.19 ± 0.04 (7) units/g wet wt. For pyruvate dehydrogenase the activity in the medium without EDTA before activation was usually slightly greater than in the medium with EDTA.

Effects of 2-bromo-α-ergocryptine injection in 4-day-lactating rats

Series A experiments in Table 1 show that 24h after injection of the drug the 'initial' pyruvate dehydrogenase activity was considerably decreased, but the 'total' enzyme activity was not significantly affected. Prolactin injection at the same time as 2-bromo-α-ergocryptine injection was without effect on either parameter. Increasing the dose of 2-bromo-α-ergocryptine to 2mg did not further increase its effect (results not shown). Injection of 6i.u. of soluble insulin 22h after 2-bromo-α-ergocryptine injection not only prevented the effect of the drug but increased both 'initial' and 'total' enzyme activity above control values, an effect which was not seen after insulin injection alone (line 4, Series B experiments). An increase in total enzyme activity 2h after hormonal treatment is an unexpected and at the moment inexplicable observation which deserves further careful investigation. This does not obscure the immediate conclusion that soluble insulin injected after 2-bromo-α-ergocryptine completely prevented the effect of the latter. A combination of prolactin and long-acting protamine–zinc–insulin also decreased the effect of 2-bromo-α-ergocryptamine when all three substances were injected at the same time.

The increases in plasma glucose and non-esterified fatty acid concentrations after injection of 2-bromo-α-ergocryptine in Series B experiments may indicate some inhibition of insulin secretion, although the effect was not great and there are no similar reports in the literature. Since prolactin replacement was adequate to prevent the effect of 2-bromo-α-ergocryptine in middle and late lactation (Table 2, and results given by Field & Coore, 1975), one might conclude that either any effect of the drug on insulin secretion is seen only in early lactation or at 4 days there is a greater requirement for insulin than later in lactation.

Cortisol is known to be important in the development of rat mammary gland during pregnancy (Turkington, 1972), but in these experiments cortisol, given either with or without prolactin, did not prevent the effects of 2-bromo-α-ergocryptine on mammary-gland pyruvate dehydrogenase, and in fact might have further diminished 'initial' enzyme activity.

Effects of short-term (3h) deprivation of prolactin or insulin

Table 2 shows that injection of 2-bromo-α-ergocryptine into early- (4-day) or mid-lactating (12-day) animals led to significant decreases in 'initial' pyruvate dehydrogenase activity, which was only reversible by prolactin in the latter instances. Even more drastic decreases in 'initial' enzyme activity were seen 3h after streptozotocin injections, when all the rats showed elevated blood glucose and non-esterified fatty acid concentrations, as would be expected after grossly impaired insulin secretion. The effect of streptozotocin was completely reversed by insulin given at the same time and partially reversed by insulin given 2h later (12-day lactating rats).

Physiological control of lactating mammary-gland pyruvate dehydrogenase

An earlier report (Coore & Field, 1974) documented the marked changes in 'initial' and 'total' mammary-gland pyruvate dehydrogenase activity during the course of lactation and weaning. In the light of the present results one might ask whether those physiological changes are explicable on the basis of maternal plasma concentrations of prolactin and insulin. In both cases the data are scanty, but from the reports of Amenomori *et al.* (1970) and Simpson *et al.* (1973) it appears that by 4 days of lactation prolactin concentration has reached its peak and declines during the second half of lactation to reach virtually pre-lactation concentrations by 21 days. There appears to be little systematic change in insulin concentrations in rats starved for 12h after the second day of lactation, after a decrease shortly before and after birth (Sutter-Dub *et al.*, 1974). Therefore although the rise in 'initial' and 'total' pyruvate dehydrogenase activity in the first 4 days of lactation corresponds to the rise in prolactin concentration, there is no ready explanation for the maintenance of enzyme activity during the rest of lactation (see the control values in Table 2). A possible explanation might be that insulin is the 'main' hormonal control of the enzyme, but that the tissue is 'primed' by exposure to rising concentrations of prolactin to respond to a concentration of plasma insulin to which it was previously indifferent. If we imagine the process of 'priming' to be only complete sometime after 4 days of lactation, then we can understand why abolition of the prolactin increment and a possibly small decrease in insulin concentration (at 4

Table 1. *Long-term (24h) effects of 2-bromo-α-ergocryptine injection with or without prolactin, insulin and cortisol on rat mammary-gland pyruvate dehydrogenase at 4 days of lactation*

Methods of injection, tissue extraction and enzyme assay are given in the Experimental section. Values are means ± S.E.M. with the numbers of animals in parentheses. * $P<0.05$; ** $P<0.01$ compared with appropriate control.

Expt. series	Treatment	'Initial' pyruvate dehydrogenase activity (munits/mg of DNA)	'Total' pyruvate dehydrogenase activity (munits/mg of DNA)	'Initial'/'total' pyruvate dehydrogenase activity	Plasma glucose (mM)	Plasma non-esterified fatty acids (mM)
A	Control (solvent injections only), 24h before tissue sampling	80 ± 4 (5)	125 ± 19 (5)	0.68 ± 0.08 (5)	—	—
	1mg of 2-bromo-α-ergocryptine injected 24h before tissue sampling	19** ± 2 (4)	105 ± 13 (4)	0.19** ± 0.03 (4)	—	—
	1mg of 2-bromo-α-ergocryptine and 2mg of prolactin injected 24h before tissue sampling	18** ± 3 (4)	85 ± 9 (4)	0.22** ± 0.05 (4)	—	—
B	Control (solvent injections only), 24h before tissue sampling	72 ± 6.5 (6)	141 ± 3.7 (6)	0.50 ± 0.04 (6)	7.2 ± 0.18 (12)	0.58 ± 0.02 (4)
	1mg of 2-bromo-α-ergocryptine injected 24h before tissue sampling	33** ± 3.7 (8)	126 ± 10 (8)	0.28** ± 0.05 (8)	8.5** ± 0.26 (12)	0.82** ± 0.03 (4)
	1mg of 2-bromo-α-ergocryptine injected 24h and 6i.u. of soluble insulin injected 2h before tissue sampling	133** ± 20 (8)	217** ± 24 (6)	0.56 ± 0.07 (6)	4.7* ± 1 (8)	—
	Solvent injected 24h and 6i.u. of soluble insulin injected 2h before tissue sampling	88 ± 12 (4)	148 ± 13 (4)	0.60 ± 0.04 (4)	3.5** ± 0.20 (4)	—
	1mg of 2-bromo-α-ergocryptine and 2.5mg of cortisol injected 24h before tissue sampling	13** ± 1.8 (4)	131 ± 9 (4)	0.10** ± 0.01 (4)	7.2 ± 0.43 (4)	0.77** ± 0.03 (4)
	1mg of 2-bromo-α-ergocryptine, 2.5mg of cortisol and 2mg of prolactin injected 24h before tissue sampling	30** ± 3.4 (4)	130 ± 12 (4)	0.23** ± 0.04 (4)	7.7 ± 0.41 (4)	0.41* ± 0.05 (4)
	1mg of 2-bromo-α-ergocryptine, 10i.u. of protamine–zinc–insulin and 2mg of prolactin injected 24h before tissue sampling	50* ± 6 (3)	117 ± 20 (3)	0.43 ± 0.04 (3)	8.3 ± 0.55 (3)	0.73* ± 0.06 (3)

Table 2. *Short-term (3 h) effects of 2-bromo-α-ergocryptine injection with or without prolactin and streptozotocin injections and with or without insulin on rat mammary-gland pyruvate dehydrogenase*

Methods of injection, of tissue extraction and enzyme and metabolite assay are given in the Experimental section. Values are means ± S.E.M., except when only approximate blood glucose values are available. Numbers of animals are in parentheses. Blood was sampled just before tissue sampling. In the experiment shown in the bottom row, blood was also sampled 1 h before tissue sampling and just before injection of insulin, and the results for blood glucose and non-esterified fatty acids were respectively 19.7 ± 1.6 and 0.79 ± 0.14 expressed as mM and based on three determinations. * $P < 0.05$ compared with control value for same day of lactation. † $P < 0.05$ comparing values for rats injected with prolactin and 2-bromo-α-ergocryptine with values for rats injected only with the latter. ‡ $P < 0.01$ comparing values for rats injected with insulin and streptozotocin with values for rats injected only with the latter.

Treatment	Days of lactation	'Initial' pyruvate dehydrogenase activity (munits/mg of DNA)	'Total' pyruvate dehydrogenase activity (munits/mg of DNA)	'Initial'/'total' pyruvate dehydrogenase activity	Plasma glucose (mM)	Plasma non-esterified fatty acids (mM)
Control (solvent injection)	4	57 ± 6 (5)	132 ± 18 (5)	0.47 ± 0.06 (5)	7.1 ± 0.20 (5)	—
	12	101 ± 23 (3)	197 ± 46 (3)	0.53 ± 0.08 (3)	8.7 ± 0.85 (3)	0.37 ± 0.05 (3)
	21	152 ± 17 (6)	235 ± 28 (6)	0.63 ± 0.04 (6)	—	—
1 mg of 2-bromo-α-ergocryptine injected	4	35* ± 5 (5)	152 ± 17 (5)	0.24* ± 0.03 (5)	—	—
	12	46* ± 9 (4)	222 ± 39 (4)	0.24* ± 0.09 (4)	—	—
2 mg of prolactin injected at the same time as 1 mg of 2-bromo-α-ergocryptine	4	34* ± 6 (5)	122 ± 12 (5)	0.28* ± 0.06 (5)	—	—
	12	92† ± 12 (4)	212 ± 15 (4)	0.44 ± 0.04 (4)	—	—
Streptozotocin (65 mg/kg) injected	4	9* ± 2 (4)	187* ± 18 (4)	0.05* ± 0.01 (4)	>14 (4)	—
	12	0* (4)	166 ± 16 (4)	0* ± (4)	16.6* ± 2.1 (6)	0.84* ± 0.087 (6)
	21	21* ± 7 (3)	201 ± 33 (3)	0.12* ± 0.04 (3)	>14 (4)	—
Streptozotocin (65 mg/kg) and soluble insulin (6 i.u.) injected	12	125 (2)	127 (2)	0.98 (2)	2.5 (2)	—
Streptozotocin (65 mg/kg) injected and then soluble insulin (6 i.u.) injected after 2 h	12	45*‡ ± 14 (3)	124 ± 16 (3)	0.36‡ ± 0.07 (3)	6.2‡ ± 1.4 (3)	0.36‡ ± 0.05 (3)

days of lactation) could cause enzyme changes that could only be reversed by big doses of insulin or combined replacement of both hormones. Analogous co-operative hormonal effects during development of the pregnancy mammary gland are well recognized (Turkington, 1972). The hypothesized dimunition of plasma insulin concentration due to 2-bromo-α-ergocryptine treatment will require direct confirmation, but until then the dramatic effects of insulin injection in 2-bromo-α-ergocryptine-treated animals, contrasting with its insignificant effect on normal animals, strongly suggests interference by the drug with a normal insulin-mediated control of the enzyme.

Interpretation of results of dosage of whole animals with drugs or large doses of hormones is always hampered by uncertainties as to whether direct effects on presumptive 'target' tissues are involved. For 2-bromo-α-ergocryptine it seems that direct effects are not involved, since appropriate hormone therapy can counteract the effect we have examined. In unpublished experiments we have found that mammary-gland acini, prepared by a method similar to that of Katz *et al.* (1974), may be incubated for 2h with concentrations of 2-bromo-α-ergocryptine likely to be attained *in vivo* without any apparent effect on extractable pyruvate dehydrogenase activities. We therefore suggest that the effects of the drug are indeed due to inhibition of prolactin and possibly insulin secretion.

Numerous reports attest direct affects of prolactin and insulin on the metabolism of rat mammary tissue *in vitro* (e.g. Martin & Baldwin, 1971; Hallowes *et al.*, 1973). Particularly relevant is the ability of insulin to increase glucose uptake and incorporation of glucose carbon into fatty acids (Martin & Baldwin, 1971), since pyruvate dehydrogenase may be rate-limiting for the latter process (Gumaa *et al.*, 1973). Nevertheless, since insulin injection lowered the plasma concentration of non-esterified fatty acids in these animals (Table 2) and for liver, heart and kidney supply of long-chain fatty acids diminishes 'initial' activity of pyruvate dehydrogenase (Wieland *et al.*, 1972), it may be that insulin could act indirectly on the mammary-gland enzyme via alteration of the concentration of plasma fatty acids. This does not seem likely, however, since partial reversal of the effect of 2-bromo-α-ergocryptine by protamine–zinc–insulin (Table 1) did not involve lowering of the plasma concentration of non-esterified fatty acids. Further, 3 days after weaning, when 'initial' and 'total' mammary-gland pyruvate dehydrogenase are greatly diminished (Coore & Field, 1974), concentration of plasma non-esterified fatty acids is low [0.189 ± 0.04mM (6)] and liver pyruvate dehydrogenase is correspondingly activated (M. I. Titherage, personal communication). Since rat mammary tissue is probably mainly concerned with synthesis of short-chain fatty acids and derives most of the long-chain fatty acids destined for milk fat from plasma (Mendelson & Scow, 1973; Kinsella, 1974), it would be physiologically undesirable to have an inverse relationship between long-chain fatty acid supply and short-chain fatty acid synthesis. However, it will require experiments *in vitro* to determine whether the insulin effect on mammary-gland pyruvate dehydrogenase is in fact directly exerted on mammary tissue, and if so whether it is due to the established effect of insulin on glucose uptake or is independent of the latter effect, as may be the case for adipose-tissue pyruvate dehydrogenase (Coore *et al.*, 1971).

B. F. held a Research Fellowship from the Wellcome Trust during the course of this work. We are also grateful to the Trust for defraying costs of animals, chemicals and some equipment. We received skilful technical assistance from Mr. S. G. Woods.

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