Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 433-440

REDUCTION OF MITOCHONDRIAL PYRUVATE DEHYDROGENASE PHOSPHATASE ACTIVITY IN LACTATING RAT MAMMARY GLAND FOLLOWING STARVATION OR INSULIN DEPRIVATION

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Received January 18,1979

Summary: The initial rates of activation of inactivated pyruvate dehydrogenase from lactating rat mammary gland and from pig heart were employed to assay pyruvate dehydrogenase phosphatase activity in mammary gland mitochondrial extracts. 24 h-starvation or 3 h-deprivation of insulin diminished phosphatase activity compared to fed controls. Refeeding and insulin treatment of 24 h starved animals restored in 1 h control levels of phosphatase activity.

INTRODUCTION

It is established for all mammalian tissues examined that the overall activity of mitochondrial pyruvate dehydrogenase complex (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) is controlled by reversible phosphorylation of the α subunit. Phosphorylation is catalysed by an $MgATP^{2-}$ requiring kinase and dephosphorylation by an Mg^{2+}, Ca^{2+} dependent phosphatase (reviewed - 1). Generally, starvation and experimental diabetes cause an increased 'steady-state' level of phosphorylation in vivo which persists in the appropriately extracted complex (1). This effect may be partly due to a change in behaviour of the PDH_a kinase not due to known effectors (2,3,4). Our report (4) also suggested a persistent dimunition of PDH_b phosphatase activity associated with the complex extracted from starved lactating rat mammary gland mitochondria compared to that associated with the enzyme complex from fed controls. We have now

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Abbreviations: PDH_b and PDH_a = phosphorylated (inactive) and dephosphorylated (active) forms of the pyruvate dehydrogenase complexes respectively.
$PDH_T = PDH_a + PDH_b$
EGTA = Ethyleneglycol-bis(β-amino-ethylether)N,N'-tetracetic acid.

0006-291X/79/060433-08$01.00/0

confirmed this suggestion, extended the study to acutely diabetic animals and showed that the effect of starvation is rapidly reversible in vivo.

MATERIALS & METHODS

Rats and sources of most materials were as earlier described (4).

A23187 was from Eli Lilly Laboratories, Indianapolis, U.S.A.

Mammary gland mitochondria were made as earlier described (5) with indicated modifications. The 'preparation' medium used in this procedure contained 250 mM-sucrose, 5 mM-HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid), 5 mM-EGTA, bovine serum albumin (2% w/v), pH 7.4. The mitochondrial pellet from a single gland (1-3 units of PDH_T activity) was extracted by 2 bursts of 5 s sonication at 4°C in 1-3 ml of 'extraction medium' containing 30 mM-triethanolamine-HCl, pH 7, 1 mM-dithiothreitol, 0.1% lubrol and 5% (v/v) rat serum. The lysate was centrifuged for 5 min at 14,000 g.av. and the pellet discarded. Centrifugation of the supernatant for 90 min at 180,000 g.av. and 4°C yielded a further supernatant which contained PDH_b phosphatase activity but no PDH_T. There was no difference in degradative ability towards PDH_a in supernatants from mitochondria of starved compared to fed animals.

Mammary gland PDH_b The pellet resulting from 180,000 g.av. centrifugation of the mitochondrial lysate from the gland of a 24 h starved animal was suspended in 300 µl of 'extraction medium' (minus serum). This suspension contained about 2 units/ml of PDH_T activity but no intrinsic PDH_b phosphatase activity. The ratio of PDH_a/PDH_T activities was 0.25 ± 0.01 (30).

Pig heart PDH_b Pyruvate dehydrogenase complex was prepared by a simplification of a published procedure involving polyethylene glycol and iso-electric precipitation (6). The iso-electric precipitate was suspended in 60 mM-triethanolamine-HCl, 1 mM-dithiothreitol, pH 7 to a final concentration of 6 units/ml PDH_T activity. PDH_b was formed by incubation with 2 mM-ATP, 0.1 mM-EGTA, 0.1 mM-$MgCl_2$ for 10 min at 30°C. PDH_b phosphatase and ATP were removed by two successive centrifugations at 180,000 g.av. for 90 min at 4°C. The pellet from the last centrifugation was redissolved in the triethanolamine/dithiothreitol medium to a PDH_T activity of 6 units/ml and a ratio of PDH_a/PDH_T activities of 0.25. The solution was stored at -196°C in small aliquots.

Protein assays were done by the method of Lowry (7) using bovine serum albumin as standard.

Calcium in mitochondrial pellets was assayed by atomic absorption spectroscopy after dissolution of about 1 mg mitochondrial protein in 1 ml of 0.07 M-nitric acid, lanthanum chloride (10 mg/l) and 2.5 mM-EDTA.

Enzyme assays and units The method of assay of PDH_a activity was described earlier (8). PDH_T activity in any extract was the maximum PDH_a activity after incubation with excess pig heart PDH_b phosphatase and 10 mM-$MgCl_2$, 0.1 mM-$CaCl_2$. The concentration of PDH_b is expressed as the difference between PDH_T and PDH_a activities per unit volume.

A unit of PDH_b phosphatase activity is defined as forming 1 unit of PDH_a activity per min during the initial linear phase of an incubation of PDH_b with known concentrations of Mg^{2+} and Ca^{2+}. It was convenient to express the PDH_b phosphatase activity of an extract in relation to PDH_T activity in the extract.

Assays of PDH phosphatase

Experiment 1, Table 1 Varying volumes of the preparation of pig heart PDH_b were mixed with the 180,000 g.av. supernatant of mitochondrial lysates to give total volumes of 50-400 µl. After 2 min equilibration at 30°C a sample was removed for assay and $MgCl_2$ and $CaCl_2$ added to give final concentrations of 10 mM and 0.1 mM respectively. Samples were removed after 30 sec, 1 min and 5 min and immediately assayed for PDH_a activity. The dilution (x 50 - 100) in the curvette inhibited further activation (4). The rate of increase of PDH_a activity during the first 60 s was used in calculating PDH_b phosphatase activity.

Experiment 2, Table 1 500 µl of the 180,000 g.av. supernatants of mitochondrial lysates were mixed with 150 µl of the suspension of mammary gland PDH_b. PDH_T activity in the mixture was 0.53 ± .06 (12) units/ml. After 2 min equilibration at 30°C a sample was removed for assay and $MgCl_2$ (6.5 µmoles) and $CaCl_2$ (0.065 µmoles) were added. Samples were removed at 2.5, 5, 10, 15 min for assay. The rate of rise of PDH_a activity in the first 5 min was linear and was used to calculate PDH_b phosphatase activity. Values for the amounts of EGTA carried over (from the 'preparation medium') and the sum of calcium in the mitochondrial pellet (58 ng atoms/mg protein) and added calcium were substituted in a published formula (9) to yield the value for free calcium given in Table 1.

Experiment 3, Table 1 EGTA was omitted from the last mitochondrial wash before sonication and it was assumed that there was negligible carry-over of EGTA when PDH_b phosphatase activity was tested in a similar manner to that for experiment 2 except that samples were taken at 15 s, 30 s, 60 s. The rise of PDH_a activity in the first 30 s was used in calculating PDH_b phosphatase activity.

Experiment 4, Table 1 Mitochondria from the gland of a single animal were suspended in 48 ml of 'preparation medium' without bovine serum albumin but containing A23187 (2 µg/ml) and incubated for 10 min at 30°C. After centrifugation for 10 min at 8,000 g.av. and 4°C the mitochondrial pellet was suspended in 1 ml of 30 mM-triethanolamine, 7 mM-mercaptoethanol, A23187 (2 µg/ml), 5 mM-EGTA, 2 mM-ATP, pH 6.5 for 10 min at 30°C. The suspension was then transferred to 48 ml of ice-cold 'preparation medium' (without EGTA) and recentrifuged at 20,000 g.av. for 1 min. Finally, the mitochondria were suspended in 2 ml of 30 mM-triethanolamine, 7 mM-mercaptoethanol, oligomycin (0.5 µg/ml), pH 7.0 at 30°C. A sample of the suspension was removed for assay and then A23187, $MgCl_2$ and $CaCl_2$ were added to final concentrations of 2 µg/ml, 10 mM and 0.5 mM respectively. Further samples were removed at 2.5, 5, 10, 15 min. All samples were assayed immediately on addition to curvettes containing normal assay components to which 0.1% Triton-X-100 had been added to lyse the mitochondria (10). It was checked that there had been no loss of PDH_T activity from the mitochondria during the previous manipulations. The PDH_T activity in the final incubation was 1.8 ± 0.3 (8) units/ml. At zero time of this final incubation ratios of PDH_a/PDH_T activities in mitochondria from fed and starved animals were 0.56 ± .03 (4) and .04 ± .01 (4) respectively. The rise of PDH_a activity during the first 5 min following addition of Mg^{2+} and Ca^{2+} was linear and was used to calculate PDH_b phosphatase activity.

Table 1 PDH_b phosphatase activity of mitochondria of mammary glands from fed or 24 h starved lactating rats

Experiment No.	Source of PDH_b	Concn. of PDH_b in units/ml	Concn. of free Mg^{2+} in moles/L	Concn. of free Ca^{2+} in moles/L	PDH_b phosphatase in m units/unit PDH_T	
					mitochondria from fed rats	mitochondria from starved rats
1	Pig heart	0.7-3	10^{-2}	10^{-4}	210 ± 17 (10)	99 ± 10 (10)
2	Mammary gland, lysed mitochondrial extracts from starved rats	0.4	10^{-2}	10^{-7}	58 ± 5 (5)	14 ± 2 (7)
3	Mammary gland, lysed mitochondrial extracts from starved rats	0.5	10^{-2}	10^{-4}	108 ± 12 (4)	35 ± 2 (4)
4	Mammary gland, unlysed mitochondria	[50-100]	[10^{-2}]	[5×10^{-4}]	85 ± 7 (4)	31 ± 8 (4)

Definitions of units of PDH_b and of PDH_b phosphatase activity and experimental procedures were given in Materials & Methods. Data are given as means ± S.E.M. with no. of observations in parenthesis.

Values for PDH_b phosphatase activity from fed and starved animals differed significantly in all experiments at $p < 0.001$ by Students 't' test.

RESULTS & DISCUSSION

Table 1, experiments 1, 2, 3 show a large dimunition of PDH_b phosphatase activity in mitochondrial extracts from starved animals whether assayed on PDH_b from mammary gland or from pig heart and at 10^{-4} or 10^{-7} M. free calcium. The concentration of pig heart PDH_b required for half-maximal rates of activation by the extracts was about 0.160 units/ml and did not differ appreciably between extracts from 2 fed and 2 starved animals.

In experiment 4, Table 1 we aimed to study the action of PDH_b phosphatase on its endogenous substrate at physiological concentrations but at controlled free Mg^{2+} and Ca^{2+} concentrations. The mitochondria were uncoupled and permeable to small molecules due to hypotonity of the incubation medium (11). Sufficient PDH_b was provided by the preincubation with ATP which was then removed by washing. Access of Mg^{2+} and Ca^{2+} to the matrix was achieved by A23187 (12). The estimates of concentrations of PDH_b, free Mg^{2+} and Ca^{2+} assume free access of all species to matrix water and to suspension medium and we have placed these estimates in brackets to indicate the uncertainty of this assumption. Various uncontrolled intramitochondrial factors including 'multi-site phosphorylation' of the α subunit of the complex (13) may contribute to the results but nevertheless the similarity of values derived from this experiment with those from experiment 3 is noteworthy.

In all these experiments we based our measurements of phosphatase activity on the earliest linear portion of the time course of activation of PDH_b. In some experiments this period was as brief as 30 s and, in fact, after this 'burst' of activity the subsequent rate of rise of PDH_a activity often did not differ between incubations containing phosphatase extracts from fed or starved animals. In the presence of 10^{-7} M free Ca^{2+} the initial phase lasted 5 min. These observations may indicate a qualitative difference between fed and starved animal tissues in the nature of the phosphatase/α subunit interaction.

Table 2 Percentage of PDH_a compared to PDH_T activities and PDH_b phosphatase activities in lactating rat mammary glands following various treatments

Treatment of rats	100 x PDH_a/PDH_T activities in freeze-clamped gland extracts	100 x PDH_a/PDH_T activities in lysed mitochondrial extracts	PDH_b phosphatase activity in lysed mitochondrial extracts in m units/unit PDH_T
Fed	39.1 ± 1.7 (9)	87.2 ± 2.2 (5)	58 ± 5 (5)
24 h starved	9.9 ± 0.6*** (7)	10.8 ± 0.7*** (10)	14 ± 2*** (7)
24 h starved and injected 1 h before with 400 µg soluble insulin intraperitoneally and 6 mmoles of glucose subcutaneously	50.1 ± 5.1 (4)	51.9 ± 1.9*** (7)	36 ± 4** (5)
As above plus gastric intubation of 2 ml of cows' milk	-	75.8 ± 3.8* (4)	63 ± 6 (4)
Streptozotocin, 65 mg/kg in 10 mM-sodium acetate pH 4.5, injected intraperitoneally	1.6 ± 1.6*** (5)	36.5 ± 4.9*** (7)	38 ± 5* (4)
2-bromo-α-ergocryptine, 1 mg in 40% ethanol injected subcutaneously 24 h before	21.5 ± 3.0*** (11)	74.8 ± 3.8* (4)	47 ± 3 (4)

Methods of assay of PDH_a, PDH_T and PDH_b phosphatase (similar to experiment 2, Table 1) were given in Materials & Methods. Values of PDH_T activity in gland and mitochondrial extracts were respectively 11.4 ± 0.3 and 56.5 ± 1.9 m units/mg protein and did not differ between treatments.

Data are given as means ± S.E.M. with no. of observations in parenthesis.

*, **, *** are respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$ for differences from fed control according to Students 't' test.

Table 2 shows that injections of insulin and glucose and refeeding with 2 ml of cows milk reversed in 1 h the effect of 24 h starvation on mitochondrial PDH_b phosphatase activity. It therefore seemed likely that lowered plasma insulin during 24 h starvation of the lactating rats (14) may have contributed to the effect of starvation on PDH_b phosphatase. Table 2 shows a significant reduction of mitochondrial PDH_b phosphatase activity 3 h after injection of streptozotocin (which reduces plasma insulin (15)). Streptozotocin also reduces plasma prolactin (14) but 2-bromo-α-ergocryptine treatment which reduces plasma prolactin but not insulin (14) was without effect on the phosphatase activity. It may be noted that treatment with either streptozotocin or 2-bromo-α-ergocryptine diminished the ratios of PDH_a/PDH_T activities (Table 2). Therefore, increases in 'steady state phosphorylation' of mammary gland pyruvate dehydrogenase are not necessarily associated with changes in the activity of its associated phosphatase. Another feature of Table 2 is that the ratio of PDH_a/PDH_T activities in mitochondria immediately after preparation varied with different treatments of the donor animals. During the hour-long preparation of mitochondria it is likely that ATP/ADP ratios fell to near zero and the PDH_a kinase would have been inactivated. The variable rise in the ratio of PDH_a/PDH_T activities in mitochondria above the value of this ratio in freeze-clamped glands from which the mitochondria were derived may partly reflect differences in the endogenous PDH_b phosphatase activity.

There is disagreement as to whether starvation or insulin deprivation diminish adipose tissue mitochondrial PDH_b phosphatase (16,17,18). It is likely that in many tissues phosphatase activity in vivo is conditioned by free Mg^{2+} and Ca^{2+} concentration (1) and 'multi-site phosphorylation' of its substrate (13). It appears, however, that at least in mammary gland, there is an additional mode of regulation of the phosphatase which persists when the above factors are controlled.

ACKNOWLEDGEMENTS

M.A.B. holds a research studentship from the M.R.C.

We are grateful for technical assistance from Mrs. C. Davis and for calcium determinations by Mr. D.A. Rae of the Minerals Engineering Department, Birmingham University.

Some of the costs of these investigations were met by a grant from the M.R.C.

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