Modulation of pyruvate dehydrogenase kinase activity in cultured hepatocytes by glucagon and *n*-octanoate

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The activity of pyruvate dehydrogenase kinase in extracts of mitochondria from rat hepatocytes cultured for 21 h in medium 199 was increased 2.5-fold by the presence of 55 nM-glucagon and 1 mM-sodium *n*-octanoate in the culture medium. The change was comparable with that induced *in vivo* by 48 h starvation. The potential contribution of branched-chain complex to estimates of PDH-complex activity in rat liver mitochondria has been defined.

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of animal tissues is regulated by reversible phosphorylation catalysed by mitochondrial PDH kinase and PDH phosphatase (Linn *et al.*, 1969). In rats starved for 48 h or rendered diabetic by alloxan, PDH complex is inactivated by phosphorylation in 24–48 h, leading to an up to 30-fold decrease in percentage of active form in muscles, kidney, adipose tissue and liver (for reviews see Wieland, 1983; Randle *et al.*, 1984). In heart muscle the effects of diabetes and starvation are apparently mediated by stable mechanism(s) which increase the activities of PDH kinase intrinsic to PDH complex and of a protein activator of PDH kinase, which may be separated from the complex by gel filtration or ultracentrifugation (Kerbey *et al.*, 1977; Hutson & Randle, 1978; Baxter & Coore, 1978; Kerbey & Randle, 1982; Kerbey *et al.*, 1984).

Nothing is yet known about hormones or metabolites which may initiate the longer-term stable mechanism(s) of PDH kinase activation in starved or diabetic rats, except for reversal *in vivo* by 24–48 h of carbohydrate re-feeding (starved rats) or insulin treatment (diabetic rats). We have chosen to begin to study this problem by long-term culture of hepatocytes, because they are more readily obtained in sufficient quantity than are cardiac myocytes. Changes in circulating concentrations induced by starvation or alloxan-diabetes are known to include an increase in non-esterified fatty acids and an increased ratio of glucagon/insulin. Evidence is given here that glucagon and *n*-octanoate increase the activity of PDH kinase in long-term culture of hepatocytes, and that the increase may approximate to that induced *in vivo* by starvation or diabetes.

EXPERIMENTAL

Materials

Details relating to male albino Wistar rats and sources of chemicals and biochemicals (unless given below) are as in Kerbey *et al.* (1977, 1984). Collagenase was from BCL, Lewes, East Sussex, U.K.; glucagon was from

Sigma (London) Chemical Co., Poole, Dorset, U.K.; Falcon tissue-culture bottles (surface area of plating 175 cm²) were from Scientific Supplies Co., London E.C.1, U.K.; tissue-culture medium 199 and foetal-calf serum were from Gibco, Paisley, Renfrewshire, Scotland, U.K. Streptomycin sulphate (to 100 mg/l) (Evans Medical, Beaconsfield, Bucks., U.K.) and benzylpenicillin (sodium) (to 60 mg/l = 10⁶ units/l) (Glaxo Laboratories, Greenford, Middx., U.K.) were added to medium 199. The major potential respiratory substrates in medium 199 were 5.5 mM-glucose and 0.6 mM-sodium acetate (for other aspects of composition, see Paul, 1975).

Hepatocytes and culture

Hepatocytes were obtained from livers of 250–350 g rats by perfusion at 38 °C with bicarbonate-buffered saline equilibrated with O₂/CO₂ (19:1) (Krebs & Henseleit, 1932) to remove blood, followed by recirculation of 100 ml of the same medium containing 24 mg of collagenase (20–30 min). The liver was then diced on a plastic sieve and the cells washed through with medium (see above). The cells were collected and washed by resuspension (twice) at 50 g for 2 min and then washed by resuspension (twice) in medium 199 containing 5% (v/v) foetal-calf serum. Viability was assessed by Trypan Blue exclusion (85–90% viable). Cell density was determined with a haemocytometer, and cells were diluted to 0.8 × 10⁶–1 × 10⁶/ml. Usually eight Falcon bottles were plated (each with 27 ml of cell suspension) and incubated for 4 h at 37 °C in air/CO₂ (19:1). The bottles were then inspected with a microscope to check attachment (usually > 90%), the medium was aspirated, attached cells were washed with medium 199 to remove foetal-calf serum, which was essential for attachment, and culture continued with 27 ml of medium 199 with additions (glucagon, sodium *n*-octanoate) as given in the text or Table 1. This constituted zero time in the incubation. Culture was then continued for a further fixed period of 21 h, with at least one further change of medium after 17 h. When short periods of exposure to glucagon and/or sodium *n*-octanoate were tested, the prescribed period (x) was from (21–x) h to 21 h by appropriate change of medium. After 21 h plates were examined (by

Abbreviations used: PDH, pyruvate dehydrogenase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide.

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microscope: loose cells had a rounded appearance; attached cells were polyhedral) and washed vigorously with 10 ml of sucrose medium (0.25 M-sucrose/5 mM-Tris/2 mM-EGTA, pH 7.4) and re-examined (by microscope). If the appearance of the attached cells was abnormal, the culture was rejected. Cells were detached with a rubber-coated spatula and 2×4 ml plus 1×2 ml of sucrose medium. The overall yield was 65–80%.

Mitochondria

Mitochondria were prepared from cells (usually two bottles/incubation condition) by disruption in a Teflon/glass Potter–Elvehjem homogenizer (10–12 strokes), followed by centrifugation at 900 *g* for 10 min to remove unbroken cells and debris. The pellet was rehomogenized and centrifuged again. The combined supernatants were centrifuged at 18000 *g* for 10 min, the mitochondrial pellet was washed once with sucrose medium and resuspended in approx. 250 μ l of the same medium (there was no fluffy layer). Mitochondria were prepared from rat liver by a similar method, except that the mitochondrial pellets were washed by resuspension and sedimentation (twice) and the fluffy layer was carefully removed.

For assay of PDH kinase, mitochondria were incubated at 30 °C for 25 min in KCl medium (for composition see Kerbey *et al.*, 1977) containing 10 μ M-CCCP to effect conversion of inactive PDH complex into active complex. Pilot experiments involving a complete time course up to 60 min showed that conversion was complete by 15–20 min and required CCCP. Mitochondria were centrifuged (30 s; Eppendorff 3200 centrifuge), the supernatant was aspirated and pellets were frozen in liquid N₂. For assay of PDH complex as made, mitochondria were added to KCl medium and pelleted immediately.

Extracts were prepared by freezing and thawing (three times) in 30 mM-potassium phosphate/5 mM-EGTA/5 mM-dithiothreitol/1% (v/v) ox serum/1 mM-tosyl-lysyl-chloromethane, pH 7.5. With cultured cells approximately two-thirds of the mitochondria were incubated in 2×0.5 ml of KCl medium and extracted with 230 μ l of extraction medium. With mitochondria from rat livers 1 mg of mitochondrial protein was incubated and extracted.

Assays

PDH complex was assayed spectrophotometrically by coupling to arylamine acetyltransferase as described by Kerbey *et al.* (1977). For assay of PDH kinase, 190 μ l of extract containing 2 μ mol of MgCl₂, 10 μ g of oligomycin and approx. 30–35 munits of PDH complex was warmed to 30 °C (3 min), ATP added to 0.5 mM (10 μ l), and three samples were taken for assay of PDH complex between 15 s and 90 s (the exact times depended on the expected rate of ATP-dependent inactivation, determined in pilot experiments). Typically 75% inactivation was achieved at the final time point. Zero-time activity was determined on a separate sample of extract. Extracts showed no loss of PDH activity over this period of incubation in the absence of ATP. PDH kinase activity was calculated as the apparent first-order rate constant for ATP-dependent inactivation (see Kerbey & Randle, 1982). The concentration of MgCl₂ used (10 mM) was shown to yield maximum rate constants. A unit of PDH complex forms 1 μ mol of product/min at 30 °C.

RESULTS AND DISCUSSION

Activities of PDH complex and PDH kinase in rat liver mitochondria

Rat liver mitochondria contain PDH complex and branched-chain 2-oxo acid dehydrogenase complex (branched-chain complex); both complexes are inactivated by phosphorylation. Purified rat liver branched-chain complex is active towards branched-chain oxo acids and pyruvate; PDH complex shows no measurable activity with ketoleucine (4-methyl-2-oxopentanoate). The K_m values (means $\pm$ S.E.M.) were: PDH complex (pyruvate) 27 ± 2.8 μ M; branched-chain complex (pyruvate) 734 ± 24 μ M (T. M. Beggs & P. J. Randle, unpublished work); branched-chain complex (ketoleucine) 15 ± 1.7 μ M (Parker & Randle, 1978). Relative V_{max} values for branched-chain complex were 1:5.0 (pyruvate: ketoleucine; T. M. Beggs & P. J. Randle, unpublished work). In extracts of mitochondria, PDH complex was assayed with 1 mM-pyruvate. Published values for branched-chain complex referred to in the present paper were assayed with 0.2 mM-ketoleucine because of substrate inhibition at higher concentrations (Parker & Randle, 1978); substrate inhibition is also seen with pyruvate above 12 mM (T. M. Beggs & P. J. Randle, unpublished work). At these substrate concentrations (1 mM-pyruvate: 0.2 mM-ketoleucine) the relative activities of branched-chain complex were 1:10.5.

The relative total activities of the two complexes in rat liver mitochondria as assayed with 1 mM-pyruvate (PDH complex) and 0.2 mM-ketoleucine (branched-chain complex) were approx. 1:5 (branched-chain complex:PDH complex) (for branched-chain complex activities see Patston *et al.*, 1986; for PDH complex activities see below). Therefore, in assays with 1 mM-pyruvate, as used in the present study, approx. 2% of total PDH complex activity in rat liver mitochondria may be attributed to branched-chain complex [i.e. $(1/10.5) \times (1/5)$]. In PDH kinase assays the potential contribution of branched-chain complex to assays of PDH complex has been ignored, because it can be computed that the effect of such a contribution is to underestimate the apparent first-order rate constant by no more than 3.6% (the highest error assumes no inactivation of branched-chain complex by phosphorylation).

The total activity of PDH complex in rat liver mitochondria (corrected for branched-chain complex) was 43.4 ± 1.2 (fed rats) and 44.1 ± 1.8 (rats starved for 48 h) munits/mg of protein (means $\pm$ S.E.M. for five observations). The percentage of the complex in the active form in freshly prepared rat liver mitochondria was 20.5 ± 2.0 (fed rats) and 6.9 ± 0.70 (rats starved for 48 h) (mean $\pm$ S.E.M. for five observations; corrections for branched-chain complex based on estimates by Patston *et al.*, 1984). The PDH kinase activities were 0.89 ± 0.04 min⁻¹ (fed rats) and 2.52 ± 0.06 min⁻¹ (rats starved for 48 h) (mean $\pm$ S.E.M. for five observations). The ratio of PDH kinase activities (starved/fed) was 2.83 ± 0.11 .

Activities of PDH kinase and PDH complex in mitochondria from rat hepatocytes

PDH kinase activities in extracts of mitochondria from livers of fed rats (see the preceding section) or freshly prepared hepatocytes or hepatocytes cultured for 21 h in medium 199 (Table 1, lines 1 and 2) did not differ

Table 1. Effect of glucagon (55 nM) and sodium *n*-octanoate (1 mM) on the activities of PDH kinase and PDH complex (active form) in mitochondria from rat hepatocytes

Mitochondria were from freshly prepared hepatocytes (line 1) or from hepatocytes cultured for 4 h in medium 199 containing 5% (v/v) foetal-calf serum and for a further 21 h in medium 199 (5.5 mM-glucose; 0.6 mM-sodium acetate) with further additions as shown (lines 3–9). The period of exposure to glucagon or *n*-octanoate or both (column 2) was at the end of incubation, i.e. 4 h of exposure was from 17 h to 21 h of incubation. PDH complex (active form) was assayed in extracts of freshly prepared mitochondria. Total PDH complex (sum of active and inactive forms) and PDH kinase were assayed in extracts of mitochondria incubated for 25 min at 30 °C in KCl medium containing 10 μ M-CCCP. Results are means $\pm$ S.E.M.; the number of observations (in parentheses) is the number of hepatocyte preparations (livers), and each individual observation was usually the mean of two assays. * $P < 0.01$, † $P < 0.05$ for effects of glucagon or *n*-octanoate or both, the control being hepatocytes incubated for 21 h in their absence in the same experiment (line 2). ‡ $P < 0.01$ for differences from glucagon alone or *n*-octanoate alone. For other differences $P > 0.05$.

Addition to culture medium	Period of culture with glucagon and/or <i>n</i> -octanoate (h)	Activity	
		PDH complex active form (% of total)	PDH kinase pseudo-first-order rate constant (min ⁻¹)
—	(freshly prepared hepatocytes)	16.3 $\pm$ 3.0 (2)	0.67 $\pm$ 0.07 (3)
Nil	0	20.4 $\pm$ 1.8 (11)	0.81 $\pm$ 0.03 (11)
Glucagon/ <i>n</i> -octanoate	0.25	35.7 $\pm$ 3.6* (3)	0.87 $\pm$ 0.04 (3)
Glucagon/ <i>n</i> -octanoate	1	32.6 $\pm$ 3.8† (5)	1.02 $\pm$ 0.03* (5)
Glucagon/ <i>n</i> -octanoate	4	23.7 $\pm$ 2.8 (6)	1.20 $\pm$ 0.04* (8)
Glucagon/ <i>n</i> -octanoate	21	13.7 $\pm$ 1.2* (8)	2.00 $\pm$ 0.07*‡ (8)
Glucagon	4	—	1.01 $\pm$ 0.05* (4)
Glucagon	21	—	1.21 $\pm$ 0.17† (2)
<i>n</i> -Octanoate	21	—	1.33 $\pm$ 0.10* (3)

significantly. When hepatocytes were cultured for 21 h in medium 199 supplemented with glucagon (55 nM) and sodium *n*-octanoate (1 mM), PDH kinase activity was increased 2.5-fold (Table 1, line 6). Significant increases in PDH kinase were also seen with glucagon or *n*-octanoate separately, but these were significantly less than when both were present (Table 1, lines 6, 8 and 9). However, the sum of the increases induced by glucagon or *n*-octanoate (0.92 min⁻¹) was not significantly less than that induced by the two together (1.19 min⁻¹). With glucagon plus *n*-octanoate, a significant but much smaller increase in PDH kinase activity was detected at 1 h and 4 h, but there was no increase at 0.25 h (Table 1, lines 3–5), i.e. the major increase occurred between 4 and 21 h. Because culture was mainly overnight it was not practicable to investigate time periods between 4 and 21 h. The increase in PDH kinase activity was not necessarily maximum at 21 h, but problems of viability of cells with more prolonged culture have yet to be resolved.

Preparation of hepatocytes or culture in medium 199 had no significant effect on the percentage of PDH complex in the active form in freshly isolated mitochondria (see the preceding section, and Table 1, lines 1 and 2). Culture of hepatocytes with glucagon plus *n*-octanoate increased the percentage of PDH complex in the active form at 0.25 and 1 h, but this fell subsequently to reach approximately two-thirds of the control value at 21 h of culture (Table 1, lines 2–6).

General discussion and conclusions

Long-term regulation of reversible phosphorylation of PDH complex (or more generally) does not appear to have been studied *in vitro*. The major purpose of the present study was to ascertain whether culture of rat hepatocytes may be used to investigate long-term

regulation of PDH kinase activity. In this study PDH kinase has been assayed by the rate of ATP-dependent inactivation, which is known to be correlated with the rate of phosphorylation of the major inactivating site (site 1) in pig and rat complexes (Sale & Randle, 1981, 1982). In rat heart mitochondria, phosphorylation of PDH complex may be measured directly by incorporation of ³²P from [γ -³²P]ATP into protein, because phosphorylation of other proteins is negligible (Sale & Randle, 1982). This technique is not applicable to liver mitochondria, because incorporation of ³²P into protein (approx. 6 nmol/unit of PDH complex inactivated) was 7–8-fold greater than incorporation into site 1/unit of PDH complex inactivated, and 2–3-fold greater than incorporation into all three phosphorylation sites (results not shown; G. S. Denyer & A. L. Kerbey, unpublished work). Because of this, the 2.5–3-fold increase in the rate of ATP-dependent inactivation of PDH complex in extracts of liver mitochondria induced by 48 h starvation is not associated with significantly increased rates of ³²P incorporation into protein. When PDH complex was purified from rat liver mitochondrial extracts, the ratio (starved/fed) for PDH kinase activity was found to be approx. 2.5 by both ATP-dependent inactivation and ³²P incorporation. The incorporation of ³²P/unit of PDH complex inactivated was as expected (G. S. Denyer & A. L. Kerbey, unpublished work). Purification of PDH complex was impracticable with the number of hepatocytes that could be cultured, but it seems reasonable to assume that ATP-dependent inactivation of PDH complex was due to phosphorylation of the complex.

The total activity of PDH complex in rat liver mitochondria was approx. 43 munits/mg of mitochondrial protein; this corresponds to approx. 4.3 units/g wet wt. of hepatocytes, or 16 units/g dry wt. of hepatocytes (based on 100 mg of mitochondrial protein/g wet wt. of

hepatocytes and a wet-wt./dry-wt. ratio of 3.7; Foden & Randle, 1978). These values are greater than those given for rat liver mitochondria of 16–26 munits/mg or for rat hepatocytes of 1.9–3.3 units/g wet wt. (Walajtys *et al.*, 1974; Leiter *et al.*, 1978; Walajtys-Rode & Williamson, 1980; Oviasu & Whitton, 1984). The value for mitochondrial total PDH complex in the present study is, however, virtually identical with the value of 44 munits/mg published by McCormack (1985).

The values for percentage of PDH complex in the active form in mitochondria isolated from freshly prepared hepatocytes of fed rats or livers of fed or starved rats in the present study are comparable with the values for whole livers or hepatocytes given by Oviasu & Whitton (1984). The short-term activation of PDH complex with glucagon plus *n*-octanoate may be due to glucagon, as described by Oviasu & Whitton (1984) for whole hepatocytes; the increase in activity observed was comparable. Measurement of percentage of active complex in mitochondria isolated from tissues is not optimal for measurements in the whole tissue *in vivo*, but limitations in the availability of cultured hepatocytes made it necessary for this technique to be utilized. Published evidence may suggest that the method affords valid qualitative comparisons (see, e.g., Hutson *et al.*, 1978), and the values obtained with this method in the present study agree closely with those obtained on whole hepatocytes by Oviasu & Whitton (1984). The possible contribution of branched-chain complex to estimates of PDH complex in rat liver appears to have been overlooked in earlier studies; data given here define the potential contribution.

It has been shown here that PDH kinase activity in extracts of liver mitochondria from normal fed rats was unaffected by isolation of hepatocytes or by culture of hepatocytes for 21 h in medium 199, and that PDH kinase activity was increased approx. 2.5-fold by 21 h of culture with glucagon plus *n*-octanoate. The increase in PDH kinase activity effected by glucagon plus *n*-octanoate in culture was comparable with that effected in liver *in vivo* by 48 h starvation of rats. Tissue culture thus appears to have the potential to define in detail the hormonal and other factors that may mediate the effects of starvation

and diabetes on PDH kinase activity in liver, heart, skeletal muscle, kidney and adipose tissue.

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