Role of protein synthesis and of fatty acid metabolism in the longer-term regulation of pyruvate dehydrogenase kinase

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Antibodies were raised in rabbits to free rat liver pyruvate dehydrogenase (PDH) kinase α -chain and shown to react with PDH kinase α -chain in rat heart and liver PDH complexes, in purified pig heart PDH complex and in bovine kidney dihydrolipoamide acetyltransferase–protein X–PDH kinase subcomplex. E.l.i.s.a for PDHE1 (pyruvate dehydrogenase) and PDH kinase have been developed and applied to assays of these proteins in extracts of rat liver and rat heart mitochondria; the measured immunoreactivities for PDHE1 (heart > liver) and for PDH kinase α -chain (liver > heart) paralleled known differences in PDH complex and PDH kinase activities respectively. The results

of e.l.i.s.a of PDH kinase α -chain in extracts of rat liver mitochondria showed that the effects of starvation to increase PDH kinase activity *in vivo*, and the effects of dibutyryl cyclic AMP or palmitate to increase PDH kinase activity in hepatocytes cultured *in vitro*, are due largely (> 90%) to an increase in the specific activity of PDH kinase. The effect, in cultured hepatocytes, of dibutyryl cyclic AMP to increase PDH kinase activity was blocked by cycloheximide; the effect of palmitate was blocked by an inhibitor of carnitine palmitoyltransferase I (Etomoxir), but not by cycloheximide.

INTRODUCTION

Mitochondrial pyruvate dehydrogenase (PDH) complexes from mammalian tissues are inactivated by phosphorylation of the E1 α component catalysed by a PDH kinase intrinsic to the complex [1]. Rat heart, liver and skeletal-muscle mitochondria contain a protein fraction (kinase-activator protein; KAP), separated from rat PDH complex by gel filtration or ultracentrifugation, which enhances the intrinsic PDH kinase activity of purified rat or pig PDH complexes [2–4]. Starvation or alloxan-diabetes increases the activities of PDH kinase and KAP in rat heart and liver mitochondria, 2–3-fold over 24–48 h [2–6]. In 24 h of culture of rat hepatocytes, cardiac myocytes and soleus muscle, n-octanoate, dibutyryl cyclic AMP (dbcAMP) and glucagon (in hepatocytes only) increased activity of PDH kinase 2–3-fold [7–9], i.e. the effects of starvation or diabetes *in vivo* may be mediated by increased circulating concentrations of non-esterified fatty acids and tissue cyclic AMP [9].

Five lines of evidence have suggested that KAP is a free PDH kinase which can be separated by gel filtration from the kinase intrinsic to PDH complex [10,11]. Thus KAP induces [³²P]phosphorylation and inactivation of pig or ox heart PDHE1 free of PDH kinase [10,11], and of PDH complex from *Saccharomyces cerevisiae* which lacks PDH kinase [10]. KAP also catalyses [³²P]phosphorylation of a synthetic peptide substrate for PDH kinase [11]. KAP is inactivated by fluorosulphonyl-benzoyl-adenosine [10], which is known to form adducts with the ATP-binding sites of protein kinases, and by thiol-reactive reagents [10], which are known to inactivate the intrinsic PDH kinase of bovine kidney PDH complex [12].

The intrinsic PDH kinase of PDH complex is composed of two subunits, α and β , and the kinase activity is in the α -subunit [12]. A protein fraction has been purified to apparent homogeneity from liver mitochondrial KAP of fed and starved rats as a single subunit which appeared to correspond to the free α -subunit of

the intrinsic kinase (PDHK α) [13]. KAP may also contain the heterodimeric ($\alpha\beta$) form of PDH kinase ([11]; D. A. Priestman, S. C. Mistry, A. Halsall and P. J. Randle, unpublished work), which is separated from PDHK α during the purification [13]. Furthermore, it was shown that starvation effects an approx. 4-fold increase in specific activity, with no apparent change in the concentration of PDHK α protein [13]. This increase in specific activity could be due to induction/repression of isoforms of differing specific activity or to covalent modification of PDH kinase.

In the present study, we: describe an e.l.i.s.a. for PDH kinase which assays both PDHK α and the α -subunit of the PDH kinase intrinsic to PDH complex; show in cultured hepatocytes that dbcAMP and palmitate increase the specific activity of PDH kinase; show that cycloheximide blocks the effect of dbcAMP on PDH kinase, but not that of palmitate; and show that the β -oxidation inhibitor Etomoxir inhibits the effect of palmitate.

EXPERIMENTAL

Materials

Details relating to male Wistar rats (source, feeding), and sources of chemicals, biochemicals, Centriflo cones, reagents for gel filtration and ion-exchange resins for f.p.l.c. are as in [3,4,10,13], except that reagents for Western blotting were from Sigma Chemical Co.; molecular-mass markers were from Bio-Rad Laboratories; [U-¹⁴C]leucine was from Amersham International; sodium Etomoxir (Byk Gulden Chemische Fabrik G.m.b.H., Konstanz, Germany) was given by Professor E. D. Saggerson, University College London, and Dr H. S. A. Sherratt, University of Newcastle upon Tyne; and Protosol was from NEN Research Products. PDH complex was purified from pig hearts [1] and freed of KAP and PDH phosphatase by centrifuging at 150000 g [5]. PDHE1 and dihydrolipoamide acetyltransferase (PDHE2) were separated from pig heart PDH complex by the method of

Abbreviations used: PDH, PDHE1, pyruvate dehydrogenase; PDHE2, dihydrolipoamide acetyltransferase; X, protein X component of the PDH complex; PDHK α , free rat PDH kinase α -chain; dbcAMP, dibutyryl cyclic AMP; KAP, kinase-activator protein.

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Pettit et al. [14]. The resulting preparation of PDHE1 was largely freed of PDH kinase (> 98%) by two successive ion-exchange chromatographic runs on DEAE-cellulose as in [10]. PDHK α was purified from extracts of liver mitochondria of 48 h-starved rats as in [13]. Purified bovine kidney PDHE2-X (protein X component)-kinase subcomplex was kindly given by Dr. T. E. Roche, Department of Biochemistry and Physics, Kansas State University, Manhattan, KS, U.S.A.

Polyclonal antibodies to purified PDHE1 and to purified PDHK α were raised in New Zealand White rabbits. The priming doses were 100 μ g of protein given subcutaneously at multiple sites in Freund's complete adjuvant. Boosting injections at 4-weekly intervals were 50 μ g of protein subcutaneously in Freund's incomplete adjuvant. Blood (ear vein) was taken 10 days later, and serum was screened for antibodies by Western blots from SDS/PAGE by using goat (anti-rabbit IgG) IgG-alkaline phosphatase with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (from Sigma). SDS/PAGE and Western blots included PDHK α (2 μ g of protein) [13], KAP (20 μ g of protein; Sephacryl S300 eluate [13]), extracts of rat liver and heart mitochondria (20 μ g of protein), and purified pig heart PDH complex (5 μ g of protein), together with molecular-mass markers (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa; prestained molecular-masses 106, 49.5, 32.5, 27.5 and 18.5 kDa). Parallel controls (Western blots) were included with non-immune sera. Plates (96-well) for e.l.i.s.a. were from Nunclon through Life Technologies Ltd., Paisley, Scotland, U.K.

Sources of materials for hepatocyte preparation and tissue culture were as given in [7], except that collagenase types A and H were from Boehringer Corp. (London).

Assays

PDH complexes

PDH complexes were assayed spectrophotometrically by monitoring NAD reduction at 340 nm or (when lactate dehydrogenase was present) by coupling to arylamine acetyltransferase as in [15]. PDHE1 was assayed as PDH complex after recombination with an excess of PDHE2 and dihydrolipoamide dehydrogenase (PDHE3) as in [2].

KAP and PDHK α

KAP and PDHK α were assayed as PDH kinase by their effects to increase the rate of ATP-dependent inactivation of purified pig heart PDH complex, under conditions given in [4], activity being computed as the apparent first-order rate constant [2,4]. Routinely, samples were taken for assay of PDH complex or PDHE1 activity at four times chosen to yield approx. 75% inactivation by the last time point; control incubations in the absence of ATP were included. As routinely prepared, the intrinsic PDH kinase activity of pig heart PDH complex is low (apparent first-order rate constant < 0.2 min⁻¹). PDHK α preparations were standardized by PDH kinase assays employing ATP-dependent inactivation of pig heart PDH complex; one unit is defined as the amount required to give $t_{0.5} = 30$ s, i.e. an apparent first-order rate constant of 1.4.

E.l.i.s.a.

The procedures for attachment of proteins to Nunclon well plates, washing, reaction with antibodies, and detection and assay of antigen-antibody complexes were as described in [16]. Outline details are given in the legend to Figure 3.

Miscellaneous

Protein was assayed with Coomassie Blue [17]. ATPase activities of PDH complex and of KAP and PDHK α were assayed as in [15], and were < 0.4% hydrolysis of ATP/min under the conditions of the PDH kinase assay. D-3-Hydroxybutyrate was assayed enzymically [18].

Hepatocytes and mitochondria

Preparation and culture

Hepatocytes were prepared from livers of fed or 48 h-starved rats of 200–280 g essentially by the method of Berry and Friend [19] as modified by Seglen [20], and cultured in Medium 199 at a cell density of 10⁶ cells/ml as described in [7]. Viability of cells (Trypan Blue exclusion) was 80–85%. Routinely, cells were cultured in medium 199/5% (v/v) foetal-calf serum for 4 h for attachment and for a further 21 h in the absence of serum and with additions as shown in Tables, Figures or the text. At the end of culture, medium was poured off, and the cells were washed once with 10 ml of sucrose medium (0.25 M sucrose/5 mM Tris/2 mM EGTA, pH 7.4), and harvested (4–8 flasks) into 15–20 ml of sucrose medium by scraping.

Mitochondria

Mitochondria were isolated from rat liver, heart (Polytron method) and hepatocytes, and extracts were prepared for assay of PDH kinase as described in [3,4,7].

RESULTS AND DISCUSSION

PDH kinase assays in cultured hepatocytes: effects of cycloheximide and Etomoxir

The general design of these experiments was to divide the hepatocytes between 16 Falcon tissue-culture flasks in four groups of four (i.e. control, control plus cycloheximide, dbcAMP, dbcAMP plus cycloheximide). The results are presented in Table 1 as means $\pm$ S.E.M. of ratios computed in each experiment together with the number of experiments (i.e. hepatocyte preparations). (It has been shown previously by analysis of variance [7] that the between-hepatocyte variance is greater than the between-assays variance, at 0.05 > *P* > 0.02.)

Control experiments in which [U-¹⁴C]leucine incorporation into protein was measured in hepatocytes cultured for 20 h showed that 30 μ M cycloheximide inhibited incorporation by 89% (column 2, line 1). Etomoxir is a known inhibitor of carnitine acyltransferase I [21,22]; control experiments in which Etomoxir completely suppressed the 3.3-fold increase over 20 h in 3-hydroxybutyrate output induced by palmitate confirmed its effectiveness in these hepatocytes (column 5, lines 8, 9). Control experiments showed also that cycloheximide or Etomoxir was without significant effect on total PDH complex activity (column 3, lines 1, 9).

The effects on PDH kinase activity are shown in column 4. At 30 μ M, cycloheximide alone had no significant effect on PDH kinase over 20 h, but it inhibited by 92% the 3-fold increase in activity induced by dbcAMP (lines 2, 3). The 1.9-fold increase in PDH kinase induced by 0.6 mM palmitate/0.8% BSA was inhibited by 21% by cycloheximide, but the inhibition was not statistically significant (lines 6, 7). It was important to ascertain whether BSA might interfere with the action of cycloheximide. As shown in columns 2 and 4 (lines 3, 5), inhibition by cycloheximide was unaffected by BSA. As shown in column 4 (lines 8, 9), 1 mM Etomoxir totally inhibited the effect of palmitate on PDH kinase activity.

Table 1 Effects of cycloheximide and Etomoxir on the PDH kinase responses to dbcAMP and palmitate in rat hepatocytes in culture

Hepatocytes were cultured in medium 199 for 25 h and with the additions as shown for 4–25 h of culture. For [^{14}C]leucine incorporation, cells were harvested, proteins precipitated and washed and radioactivity was assayed after dissolution with Protosol. For assay of total PDH-complex activity, mitochondria were isolated from cells from four flasks and incubated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 30 min at 30 °C to effect conversion of inactive into active complex, and PDH complex was assayed after extraction. PDH kinase was also assayed in such extracts. 3-Hydroxybutyrate was assayed in deproteinized culture medium. Results (means $\pm$ S.E.M.) are shown as multiples of control values (no. of hepatocyte preparations in parenthesis; three assays/preparation). Control values were: [^{14}C]leucine incorporation, 114 ± 8.8 nmol/mg of protein (4); total PDH-complex activity 130.7 ± 14.6 m-units (6) (combined mitochondria from four flasks). PDH kinase activities ranged in separate experiments from 0.47 ± 0.07 (4) to $0.91 \pm 0.02 \text{ min}^{-1}$ (4); 3-hydroxybutyrate outputs ranged from 25 ± 4.2 to 59.5 ± 2.16 nmol/ 10^6 cells plated. * $P < 0.01$, § $0.05 > P > 0.02$ for difference from unity; † $P < 0.01$ for effect of cycloheximide or of Etomoxir. For other differences $P > 0.05$. Abbreviation: CHX, cycloheximide.

Addition to culture medium	Multiple of control for:			
	[^{14}C]Leucine incorporation	Total PDH complex activity	PDH kinase activity	3-Hydroxybutyrate output
30 μM CHX	$0.11 \pm 0.01^*$ (12)	0.95 ± 0.12 (6)	1.23 ± 0.12 (10)	—
50 μM dbcAMP	0.90 ± 0.06 (4)	—	$3.01 \pm 0.39^*$ (4)	—
50 μM dbcAMP + 30 μM CHX	$0.13 \pm 0.03^*$ (4)	—	$1.15 \pm 0.05^\S$ † (4)	—
50 μM dbcAMP/0.8% BSA	—	—	$2.91 \pm 0.22^*$ (3)	—
50 μM dbcAMP/0.8% BSA/30 μM CHX	$0.06 \pm 0.01^*$ (3)	—	$1.15 \pm 0.03^\S$ (3)	—
0.6 mM palmitate/0.8% BSA	—	—	$1.90 \pm 0.07^*$ (5)	—
0.6 mM palmitate/0.8% BSA/30 μM CHX	—	—	$1.71 \pm 0.07^*$ (5)	—
0.6 mM palmitate/0.8% BSA	—	—	$2.36 \pm 0.24^*$ (4)	$3.34 \pm 0.33^*$ (4)
0.6 mM palmitate/0.8% BSA/1 mM Etomoxir	—	0.93 ± 0.029 (4)	$0.93 \pm 0.06^\dagger$ (4)	$0.86 \pm 0.12^\dagger$ (4)

Figure 1 E.I.s.a. of purified rat liver PDHK α with rabbit anti-(rat PDHK α) serum (▲) or IgG purified from the antiserum with Protein A-Sepharose (○)

For details of the e.i.s.a. procedure see the legend to Figure 3. IgG was purified by applying 1 ml of antiserum in 100 mM Tris/HCl, pH 8, to a 1 ml column of Protein A-Sepharose CL4B, then washing with 10 ml of the same buffer followed by 10 ml of 10 mM-Tris/HCl, pH 8, and IgG was eluted with 0.1 M glycine/HCl, pH 3, and located by protein assay. Results are means $\pm$ S.E.M. (the error bars are sometimes smaller than the symbols).

E.I.s.a. for PDHK α ; development and evaluation

Antisera to rat liver PDHK α were evaluated by Western blots of SDS/PAGE which showed reactivity with PDHK α , purified bovine kidney PDHE2-X-kinase subcomplex, purified pig heart PDH complex, and extracts of rat heart and liver mitochondria. The molecular mass on SDS/PAGE of PDHK α was 45 kDa, and on Western blots extracts of rat liver and heart mitochondria gave a band in the same position, and a fainter band of lower molecular mass. The molecular mass values on SDS/PAGE of bovine kidney PDHE2-X-kinase subcomplex were 79.8, 56.4, 45.5 and 43.7 kDa (means from two gels), representing E2, protein X, and the α - and β -subunits of PDH kinase respectively. The molecular mass of the α -subunit of PDH kinase was

Figure 2 Comparative e.I.s.a. of purified rat liver PDHK α (▲) and purified bovine kidney PDHE2-X-kinase subcomplex (○) with rabbit anti-(rat PDHK α) serum

For details of the e.i.s.a. procedure see the legend to Figure 3. PDHK α was purified as described in the Experimental section; purified bovine kidney PDHE2-X-kinase subcomplex was a gift from Dr. T. E. Roche (see the Experimental section). Results are means $\pm$ S.E.M. (the error bars are smaller than the symbols).

comparable with that obtained on SDS/PAGE by Dr. T. E. Roche (personal communication), but lower than the value of 48 kDa in [14]. In all SDS/PAGE, the PDHK α band is obviously below that of the α -subunit of bovine kidney PDH kinase when run in an adjacent track; the computed molecular mass was 44.7 kDa for PDHK α , compared with 45.5 kDa for bovine kidney PDH K α . Western blots of pig heart PDH complex with antisera to PDHK α also showed a very faint band in the position of protein X. Photographs of Western blots are not shown, because they did not reproduce satisfactorily. We interpret these findings to show that antibodies to PDHK α cross-react with the α -subunit of PDH kinase in bovine kidney PDHE2-X-kinase subcomplex and pig heart PDH complex, and that PDHK α purified from KAP corresponds to the α -subunit of PDH kinase intrinsic to rat PDH complex or is closely related structurally.

Figure 3 E.I.I.s.a. of (a) PDHE1 and (b) PDHK α in extracts of rat heart (○) and rat liver (▲) mitochondria

Heart and liver mitochondria from fed rats were extracted by freezing and thawing (three times) in mitochondrial extraction buffer [4,9] at 14.5 mg of protein/ml (liver) and 8.9 mg/ml (heart). The extracts were used without further clarifying by centrifugation. Activities were for PDH kinase (min^{-1}) 0.43 ± 0.01 (liver) and 0.18 ± 0.03 (heart) and for PDH complex (m-units/mg of protein) 55 (liver) and 140 (heart). Portions ($100 \mu\text{l}$) of extracts were added in quadruplicate to individual wells in a 96-well plate and incubated overnight at 4°C . The extracts were removed, wells washed, and residual protein-binding sites blocked by incubation for 1 h with BSA. After washing, rabbit anti-(rat PDHK α) or rabbit anti-(pig PDHE1) serum was added as appropriate to dilutions of 1:1000 and 1:500 respectively and incubated at room temperature for 60 min. After washing, goat anti-(rabbit IgG) IgG-alkaline phosphatase was added and incubated at room temperature for 60 min. After washing, *p*-nitrophenyl phosphate was added and colour allowed to develop for 20 min at room temperature in the dark. The colour was then read with a Bio-Rad ELISA Reader at 405 nm (for further details see [16]). Corrections were applied for controls incorporating non-immune rabbit serum. Results are means $\pm$ S.E.M. (the error bars are smaller than the symbols).

In preliminary experiments with $0.25 \mu\text{g}$ of highly purified PDHK α /well, it was found that maximum binding of antibodies was achieved with antiserum dilutions of 1:6400 to 1:800 (results not shown). In e.i.i.s.a. 1:1000 dilution was used routinely.

Figure 1 shows that e.i.i.s.a. of PDHK α , with rabbit antiserum and with IgG purified from the antiserum, give identical curves at equivalent titres. Routinely antisera have been used in our e.i.i.s.a. Figure 2 shows comparative e.i.i.s.a. of rat liver PDHK α and of purified bovine kidney PDHE2-X-PDH kinase subcomplex with rabbit anti-(rat PDHK α) serum. Saturation was achieved with both substrates, but the plateau levels differed by some 2.8-fold. This might suggest the presence of antibodies reacting with epitopes in rat liver PDHK α which are absent from the bovine kidney PDHK α subunit, or that the reactivity of the bovine PDHK α is altered through binding to PDHE2 or to the PDH kinase β -subunit in the subcomplex. In both of these experiments with PDHK α (Figures 1 and 2), plateaux were

Figure 4 E.I.I.s.a. of PDHK α in extracts of liver mitochondria from fed (○) or 48 h-starved (▲) rats

For details of the e.i.i.s.a. procedure and preparation of mitochondrial extracts, see the legend to Figure 3, except that mitochondrial extracts were clarified by centrifugation. (a) shows mean values for two e.i.i.s.a.s, each with four wells per protein concentration. (b) shows combined values ($\pm$ S.E.M.) for four e.i.i.s.a.s (each four wells per assay). The error bars are sometimes smaller than the symbols. Inset shows ratios of starved/fed (S/F; means $\pm$ S.E.M.) for individual points in the e.i.i.s.a., giving an estimate of the fractional increase induced by starvation.

achieved with $0.5\text{--}1 \mu\text{g}$ of protein/well. Much higher protein concentrations were required to achieve a plateau with extracts of mitochondria (approx. $10 \mu\text{g}$ /well; see Figures 3–6). It is not therefore possible to use PDHK α for quantitative e.i.i.s.a. with mitochondrial extracts. We have therefore compared the different groups (fed versus starved; hepatocytes with or without dbcAMP; hepatocytes with or without palmitate) directly through side-by-side comparison, in the same well block and over the same range of protein concentrations.

Earlier studies have shown that total PDH-complex activity (active + inactive forms)/mg of protein in extracts of rat heart mitochondria is approx. 3 times that in liver, and that PDH kinase activity of rat liver PDH complex is approx. 3 times that of heart [3,4,9]. It was important to ascertain whether comparable differences might be detected by e.i.i.s.a.; these results are shown in Figure 3 with rabbit antisera to PDHE1 (panel a) and to PDHK α (panel b). The results in Figure 3(a) yield estimates for the relative concentration of PDHE1 by e.i.i.s.a. in mitochondrial extracts of 2.40 ± 0.16 (heart/liver) for the ratio of total PDH-complex activities in mitochondrial extracts (heart/liver) of 2.55. Figure 3(b) shows that the concentration of PDHK α /unit of PDH complex as measured by e.i.i.s.a. in extracts of rat liver mitochondria is 3.44 ± 0.58 times that of rat heart mitochondria; the ratio of measured PDH kinase activities (liver/heart) was

Figure 5 E.I.I.s.a. (a) of PDHK α in KAP (●) and in PDH complex (Δ) separated from liver mitochondria of starved rats and (b) of PDHK α intrinsic to the PDH-complex fraction separated from liver mitochondrial extracts from fed (○) and 48 h-starved (▲) rats

For details of the e.i.i.s.a. procedure see the legend to Figure 3. In (a) KAP and PDH complex were separated by gel filtration of liver mitochondrial extracts from 48 h-starved rats on Sephacryl S300 as in [4]. PDH kinase activities were: PDH complex (0.43 unit/mg; 300 m-units/ml in assay), 4.44 min^{-1} ; KAP, 46 units/mg (0.32% pure; cf. [13]). In (b) PDH complex was separated from KAP by sedimentation (250 000 g for 90 min) and redissolved in extraction buffer. PDH kinase activities of PDH complex were 1.67 min^{-1} (fed) and 6.8 min^{-1} (starved). Inset shows ratios of starved/fed (S/F; means $\pm$ S.E.M.) for individual points in the e.i.i.s.a., giving an estimate of the fractional increase induced by starvation.

2.42 ± 0.31 . Thus tissue differences in mitochondrial activities of PDH complex and PDH kinase were accompanied by comparable differences in concentrations of PDHE1 and PDHK α subunits as measured by e.i.i.s.a. This provides support for the validity of direct comparisons by e.i.i.s.a.

E.I.I.s.a of PDHK α in extract of rat liver mitochondria and PDH complex therefrom; effects of starvation *in vivo*, and of dbcAMP and of palmitate in cultured hepatocytes

Figure 4 shows the results of e.i.i.s.a with rabbit anti-(rat PDHK α) serum of extracts of mitochondria from livers of fed and 48 h-starved rats. In Figure 4(a) the assay employed a concentration range of mitochondrial protein from 5–320 μg of protein per well, and the results are means of assays on two pairs of extracts; in Figure 4(b) the range was 0.5–32 μg per well, and the results are means of assays on four pairs of extracts. In Figure 4(a) the amount of immunoreactive PDHK α in the extract from fed rats was approx. 10% greater than for starved rats, though there was no significant difference between the individual values. In Figure 4(b) the reactivity of the extract from

Figure 6 E.I.I.s.a. of PDHK α (a and c) and of PDHE1 (b and d) in extracts of mitochondria from rat hepatocytes cultured for 25 h with or without dbcAMP (a and b) or with or without palmitate (c and d)

For details of the e.i.i.s.a. procedure and preparation of mitochondrial extracts, see the legend to Figure 3. For details of preparation of hepatocytes and tissue culture see the legend to Table 1. Symbols are: (a) and (b), Δ, control (0.8% BSA); ●, 50 μM dbcAMP + 0.8% BSA; (c) and (d), ▲, control (0.8% BSA); □, 0.6 mM palmitate + 0.8% BSA. Results are means $\pm$ S.E.M. (the error bars are sometimes smaller than the symbols) for four or five hepatocyte preparations with four wells at each protein concentration per e.i.i.s.a.

the starved is up to 1.3-fold greater than that of the fed ($P < 0.01$; see the inset). In both sets of assays the plateau was reached at approx. 32 μg of mitochondrial protein/well. It is concluded that the concentrations of PDH kinase α -chain in extracts of rat liver mitochondria from fed and starved rats are closely similar by this assay and that the approx. 30% increase in the starved rats is too small to account for the 2.5–3-fold increase in PDH kinase activity. This indicates that starvation increased the specific activity of PDH kinase in rat liver mitochondria, in conformity with previous evidence based on relative specific activities of highly purified PDHK α [13].

Mitochondrial extracts contain both PDHK α and PDH kinase intrinsic to PDH complex. Figure 5(a) shows that the e.i.i.s.a. detects both PDHK α and the kinase α -chain intrinsic to the complex in Sephacryl S300 eluates of liver mitochondrial extract from starved rats. Figure 5(b) shows the results of e.i.i.s.a. of intrinsic PDHK α in the PDH complex, after separation from KAP in extracts of mitochondria from livers of fed and 48 h-starved rats. In the e.i.i.s.a. the ratio starved/fed was no greater than 1.44 at any point and averaged 1.28 (see the inset). The ratio of PDH kinase activities was 4.1 (see the legend to Figure 5). It is concluded that starvation increases the specific activity of PDH kinase intrinsic to the PDH complex and that the possible contribution of an increase in PDH kinase concentration is unlikely to account for more than approx. 7–11% of the increase in activity, i.e. starvation increases the specific activity of the PDH kinase intrinsic to the PDH complex.

Figure 6 shows the results of e.i.i.s.a. with rabbit antisera to rat PDHK α (panels a and c) and to PDHE1 (panels b and d) in

extracts of mitochondria from hepatocytes cultured with or without dbcAMP (panels a and b) or with or without palmitate (panels c and d). As shown in panels (a) and (b), 50 μ M dbcAMP was without effect on the concentrations of PDHE1 or of PDHK α over 25 h of culture as measured with this assay. Likewise, as shown in panels (c) and (d), 0.6 mM palmitate on 0.8% BSA was without effect on the concentrations of PDHE1 or of PDHK α . The results with antiserum to PDHE1 are in agreement with the observation that culture with dbcAMP [7] or palmitate (Table 1) has no significant effect on total PDH complex activity. The results with antiserum to rat PDHK α would indicate likewise that dbcAMP and palmitate have no effect on the concentration of PDHK α , i.e. that dbcAMP and palmitate, like starvation *in vivo*, increase the specific activity of PDH kinase.

Conclusions

Previous studies have shown that starvation and alloxan-diabetes in the rat increase the activity of PDH kinase 2.5–3-fold by a longer-term (24–48 h) and stable mechanism in rat heart, liver and skeletal muscle. This increase involves both the PDH kinase intrinsic to PDH complex and PDHK α . Studies with cultured hepatocytes, cardiac myocytes and soleus muscles have indicated that the effects of starvation and diabetes may be mediated by longer-term effects of cyclic AMP and non-esterified fatty acids. The results of the present study have confirmed by e.l.i.s.a that starvation increases the specific activity of PDHK α in liver and have shown further that starvation also increases the specific activity of PDHK α intrinsic to the PDH complex. The results with e.l.i.s.a also indicate that starvation may result in an approx. 20% increase in the concentration of PDHK α in liver. The present study has shown further that culture of hepatocytes with dbcAMP or with palmitate also increases the specific activity of PDHK α without detectable change in its total concentration. The study has also shown for the first time that protein synthesis is required for the effect of dbcAMP to increase PDH kinase specific activity in cultured hepatocytes, but that protein synthesis is not required for the effect of palmitate. Lastly, the studies with Etomoxir (an inhibitor of carnitine palmitoyltransferase I [21,22]) suggest that the effect of fatty acids are mediated either by intramitochondrial fatty acyl-CoA or by a product of β -oxidation of fatty acids. The results generally indicate that starvation, dbcAMP and palmitate effect a change in the primary structure of PDHK α ; this could presumably involve two isoforms with different specific activities and/or post-synthetic covalent modification of PDHK α .

Note added in proof (received 5 April 1994)

Since this paper was accepted, Popov et al. [23] have deduced the primary structure of a rat heart PDH kinase α -chain from a cloned cDNA and shown that the recombinant protein has PDH

kinase activity, and that antiserum raised to the recombinant protein cross-reacts with the α -chain of PDH kinase in highly purified rat heart PDH complex. The N-terminal sequence of the rat liver PDHK α which we have purified [13] is not contained within the deduced sequence of Popov et al. [23]. Professor R. A. Harris has kindly provided us with a sample of his recombinant kinase, and in assays with purified pig heart PDH complex we find that its specific activity is comparable with that of the PDHK α which we have purified from liver mitochondria of fed rats. Preliminary studies by Western blotting show that our rabbit anti-(rat PDHK α) serum shows little, if any, cross-reaction with the recombinant PDH kinase α -chain. Because N-terminal sequence analyses of five preparations of highly purified rat liver PDHK α showed no detectable contaminating protein [13], and because the specific activity of our highly purified rat liver PDHK α is comparable with that of the recombinant PDH kinase α -chain, we conclude that there are two PDH kinase isoenzymes. It seems likely that only one of these has been assayed by e.l.i.s.a. in the present study.

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REFERENCES

- 1 Linn, T. C., Pettit, F. H. and Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241
- 2 Kerbey, A. L. and Randle, P. J. (1982) *Biochem. J.* **206**, 103–111
- 3 Kerbey, A. L., Richardson, L. J. and Randle, P. J. (1984) *FEBS Lett.* **176**, 115–119
- 4 Denyer, G. S., Kerbey, A. L. and Randle, P. J. (1986) *Biochem. J.* **239**, 347–354
- 5 Hutson, N. J. and Randle, P. J. (1978) *FEBS Lett.* **92**, 73–76
- 6 Fuller, S. J. and Randle, P. J. (1984) *Biochem. J.* **219**, 635–646
- 7 Marchington, D. R., Kerbey, A. L., Giardina, M. G., Jones, E. A. and Randle, P. J. (1989) *Biochem. J.* **257**, 487–491
- 8 Marchington, D. R., Kerbey, A. L. and Randle, P. J. (1990) *Biochem. J.* **267**, 245–247
- 9 Stace, P. B., Fatania, H. R., Jackson, A., Kerbey, A. L. and Randle, P. J. (1992) *Biochim. Biophys. Acta* **1135**, 201–206
- 10 Mistry, S. C., Priestman, D. A., Kerbey, A. L. and Randle, P. J. (1991) *Biochem. J.* **275**, 775–779
- 11 Jones, B. S. and Yeaman, S. J. (1991) *Biochem. J.* **275**, 780–783
- 12 Stepp, L. R., Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1983) *J. Biol. Chem.* **258**, 9545–9548
- 13 Priestman, D. A., Mistry, S. C., Kerbey, A. L. and Randle, P. J. (1992) *FEBS Lett.* **308**, 83–86
- 14 Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1982) *Methods Enzymol.* **90**, 195–200
- 15 Kerbey, A. L. and Randle, P. J. (1985) *Biochem. J.* **231**, 523–529
- 16 Heseltine, L., Turner, I. B., Fussey, S. P. M., Kelly, P. J., James, O. F. W., Yeaman, S. J. and Bassendine, M. F. (1990) *Gastroenterology* **99**, 1786–1792
- 17 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 18 Williamson, D. H., Mellanby, J. and Krebs, H. A. (1962) *Biochem. J.* **82**, 90–96
- 19 Berry, M. M. and Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- 20 Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- 21 Declercq, P. E., Falck, J. R., Kuwajima, M., Tyminski, H., Foster, D. W. and McGarry, J. D. (1987) *J. Biol. Chem.* **262**, 9812–9821
- 22 Esser, V., Kuwajima, M., Britton, C. H., Krishnan, K., Foster, D. W. and McGarry, J. D. (1993) *J. Biol. Chem.* **268**, 5810–5816
- 23 Popov, K. M., Kedishvili, N. Y., Zhao, Y., Shimomura, Y., Crabb, D. W. and Harris, R. A. (1993) *J. Biol. Chem.* **268**, 26602–26606