Longer-term regulation of pyruvate dehydrogenase kinase in cultured rat hepatocytes

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The activities of pyruvate dehydrogenase (PDH) kinase and of PDH kinase activator protein (KAP) were increased 2–2.4-fold during 25 h of culture of hepatocytes from fed rats with glucagon plus n-octanoate. PDH kinase activity in hepatocytes from starved rats (initially $2.2 \times$ fed control) fell during 25 h of culture in medium 199 (to $1.5 \times$ fed control), but was maintained by glucagon plus octanoate. Dibutyryl or 8-bromo cyclic AMP increased PDH kinase activity 2–2.2-fold in hepatocytes from fed rats, but phenylephrine and isoproterenol (isoprenaline) were without effect. Insulin blocked the action of glucagon to increase PDH kinase activity and decreased the effect of octanoate and octanoate plus glucagon. It is suggested that the effects of starvation to increase activities of PDH kinase and of KAP in liver are mediated by alterations in circulating concentrations of glucagon, fatty acids and insulin and in hepatic cyclic AMP.

INTRODUCTION

The mitochondrial pyruvate dehydrogenase (PDH) complex of animal tissues is inactivated by phosphorylation catalysed by PDH kinase, which is intrinsic to the PDH complex [1]. The activity of PDH kinase in extracts of mitochondria from rat liver, heart and skeletal muscle is increased 2–3-fold over 24–48 h by starvation or induction of alloxan-diabetes [2–4]. This effect of starvation and diabetes is associated with a stable 2–3-fold increase in the activity of PDH kinase that is mediated by a comparable increase in the activity of a protein activator of PDH kinase (KAP). KAP may be separated from the PDH complex by differential centrifugation or gel filtration [4–7]. The effects of starvation or diabetes on the activities of PDH complex or PDH kinase are reversed over 24–48 h by refeeding or insulin injection respectively.

The effects of starvation *in vivo* to increase activity of PDH kinase may be reproduced in primary hepatocytes from fed rats in tissue culture with glucagon plus n-octanoate. This effect required 21 h of exposure, and each agent was partially effective alone [8]. The effect of starvation to increase activity of PDH kinase was maintained over 25 h of culture, but could be reversed within 4 h by insulin, which was shown also to decrease activity of KAP [9]. In the present study, effects of glucagon and octanoate have been compared in hepatocytes from fed and starved rats. In hepatocytes from fed rats, interactions of insulin with glucagon and octanoate, the effects of dibutyryl cyclic AMP, 8-bromo cyclic AMP and of phenylephrine and isoproterenol (isoprenaline) on the activity of PDH kinase, and the effects of glucagon plus octanoate on the activity of KAP have been investigated.

EXPERIMENTAL

Materials

Details of rats, sources of chemicals, biochemicals, Falcon tissue-culture bottles, medium 199, antibiotics and insulin are given in [4,7,9]. Phenylephrine and isoproterenol were from Sigma Chemical Co., Poole, Dorset, U.K.

Hepatocytes and culture

Details of isolation of hepatocytes and tissue cultures were as in [8,9]. After each use, the liver-perfusion apparatus was washed exhaustively with water followed by ethanol, and heated to 70 °C overnight. This procedure completely prevented infection of cultures, which otherwise occurred occasionally. The glass components were cleaned in chromic acid every 1–2 months. The yield of hepatocytes in the present study was 50 % greater than in [8,9], allowing at least 12 Falcon bottles to be plated with 27 ml of cell suspension (10^6 cells/ml) for each liver. The period of culture was fixed at 25 h [4 h in medium 199 containing 5 % (v/v) foetal-calf serum, followed by a total of 21 h in medium 199, with a change of medium 199 after 21 h]. The only exception was a 4 h culture (starved rats) in Table 1 (below). The major potential respiratory substrates in medium 199 are 5.5 mM-glucose, 0.6 mM-sodium acetate and amino acids.

Assay of PDH kinase in mitochondria extracts

Hepatocytes were harvested, after vigorous washing, with a rubber-coated spatula and disrupted. Mitochondria were isolated in sucrose medium and incubated for 30 min at 30 °C in KCl medium containing 10 μ M-CCCP to convert inactive PDH complex into active

Abbreviations used: PDH, pyruvate dehydrogenase; KAP, pyruvate dehydrogenase kinase activator protein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

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complex. Further details were as given in [8,9], except that mitochondria extracts were prepared in the extraction buffer given in [4]. PDH kinase was assayed by the rate of ATP-dependent inactivation of PDH complex as in [4,8], and activity was calculated as the apparent first-order rate constant [6].

Assay of KAP

Mitochondria derived from hepatocytes prepared from three to six livers were extracted and KAP separated from PDH complex by gel filtration on Sephacryl S-300 (bed volume 150 ml) as in [9]. PDH kinase was assayed in peak 1 (PDH complex). KAP was assayed through its effect on the PDH kinase activity of highly purified pig heart PDH complex as in [4]. Equivalence of recovery of KAP fractions from individual mitochondrial extracts was assumed from identity between the amounts of PDH complex loaded and recovered from the Sephacryl S-300 columns (for details, see the legend to Table 4 below).

Statistical analysis

Analysis of variance (33 hepatocyte cultures, fed rats, medium 199; 97 assays of PDH kinase) showed that variance between hepatocyte preparations was greater than variance between assays ($0.05 > P > 0.02$). This is at the margin of statistical significance. In Table 1, S.E.M. and P values (t test) are based on the number of cultures. In Tables 2–4, S.E.M. values are based on the total number of assays. Unless stated to the contrary, levels of significance (P) for effects of additions to culture medium in Tables 2–4 were based on the paired t test (i.e. differences due to variations in culture conditions were calculated for each hepatocyte preparation, and the t test was used to ascertain whether the mean difference for all paired hepatocyte cultures is significantly greater than zero).

RESULTS AND DISCUSSION

Mitochondrial PDH kinase activity in hepatocytes from fed and 48 h-starved rats

Mitochondrial PDH kinase activity in fed rats was not changed by preparation of hepatocytes and culture in

medium 199 for 25 h (Table 1, column 2, lines 1 and 4) as in [8,9]. In starved rats, mitochondrial PDH kinase activity was not changed by preparation of hepatocytes (Table 1, column 3, lines 1, 2) as in [8,9], but culture in medium 199 led to a decrease in activity (Table 1, column 3, lines 2–4). The decrease was approx. 30% of the starved–fed difference at 4 h and 60% at 25 h. The results differ from those of a previous study [9] in which PDH kinase activity was fully maintained in culture in the absence of insulin. The difference is assumed to be related to the improvements in technique which have resulted in a consistently greater degree of viability of hepatocytes in culture, and a much higher yield of hepatocytes overall, in the present study. The starved/fed ratio for PDH kinase (liver) was 2.35 (Table 1, line 1), in conformity with previous studies [4,9].

Glucagon (50 nM) plus n-octanoate (1 mM) maintained PDH kinase activity during culture of hepatocytes from starved rats (Table 1, column 3, lines 1 and 5) and increased PDH kinase activity in hepatocytes from fed rats to a near-equivalent level (Table 1, columns 2 and 3, line 5). This could be consistent with the proposition (cf. [8,9]) that glucagon and fatty acids may mediate effects of starvation on liver PDH kinase activity *in vivo*. Glucagon (50 nM) failed to maintain PDH kinase activity during culture of hepatocytes from starved rats (Table 1, column 3, lines 1, 4 and 7). The hormone increased PDH kinase activity of hepatocytes from fed rats to a level comparable with that of hepatocytes from starved rats (Table 1, columns 2 and 3, line 7). This might suggest that octanoate was the major factor maintaining the activity of PDH kinase in hepatocytes from starved rats during incubations with glucagon plus octanoate (cf. Table 1, column 3, lines 5 and 7). In hepatocytes from fed rats, octanoate was more effective than glucagon in increasing PDH kinase activity (Table 1, column 2, lines 6 and 7). As shown in Table 2, the increase in PDH kinase activity induced in hepatocytes from fed rats by glucagon plus octanoate was associated with an increase in activity of KAP (line 3, columns 2 and 3).

All subsequent studies were performed with hepatocytes from fed rats. With this preparation PDH kinase activity is maintained at the '*in vivo*' level after preparation of hepatocytes and culture in medium 199 (the

Table 1. Activity of PDH kinase in hepatocytes from fed or 48 h-starved rats

Hepatocytes were cultured in medium 199 containing 5% (v/v) foetal-calf serum for 4 h (0–4 h) and then in medium 199 for a further 21 h (4–25 h). At the end of culture, hepatocytes were harvested, mitochondria isolated, extracts prepared and PDH kinase activity assayed and calculated as the apparent first-order rate constant. Significance: * $P < 0.01$ for effect of starvation; † $P < 0.01$ for effect of culture; ‡ $P < 0.01$ for effect of glucagon and/or n-octanoate; § $P < 0.01$, || $P < 0.05$ for difference from glucagon + n-octanoate. (), Total number of assays; [], number of preparations of hepatocytes or liver mitochondria.

Details of culture; additions to medium 199 (for period of culture shown)	PDH kinase activity (min^{-1} ; mean $\pm$ S.E.M.) for hepatocytes from:	
	Fed rats	Starved rats
Liver mitochondria (no culture)	1.10 $\pm$ 0.13 (6)	2.44 $\pm$ 0.21 [6]*
Fresh hepatocytes (no culture)	—	2.19 $\pm$ 0.19 [5]
No additions		
4 h of culture	—	1.78 $\pm$ 0.12 [5]
25 h of culture	0.91 $\pm$ 0.03 [22]§	1.42 $\pm$ 0.13 [9]*†
50 nM-Glucagon + 1 mM-n-octanoate (0–25 h)	2.18 $\pm$ 0.14 [11]‡	2.62 $\pm$ 0.16 [6]‡
1 mM-n-octanoate (0–25 h)	1.87 $\pm$ 0.05 [11]‡§	2.64 [1]
50 nM-glucagon (0–25 h)	1.47 $\pm$ 0.05 [14]‡	1.43 $\pm$ 0.06 [3]§

Table 2. Effect of glucagon plus n-octanoate on the activities of PDH kinase and KAP in rat hepatocytes

Hepatocytes from fed rats were cultured in medium 199 containing 5% (v/v) foetal-calf serum for 4 h and then for a further 21 h in medium 199. Glucagon (50 nM), plus n-octanoate (1 mM) when added, were present throughout. At the end of culture, hepatocytes were harvested, mitochondria isolated and extracts prepared and clarified by centrifugation, and PDH kinase activity assayed and calculated as the apparent first-order rate constant. The extracts were subjected to gel filtration on Sephacyl S-300, and fraction 1 (containing PDH complex) assayed for PDH kinase. Fraction 2 (containing KAP) was located and its activity assayed with purified pig heart PDH complex. Total PDH complex activities of centrifuged extracts subjected to gel filtration were 1.31 units (control hepatocytes) and 1.34 units (hepatocytes cultured with glucagon plus n-octanoate). Recoveries of PDH complex in fraction 1 were approx. 80%. The PDH kinase activity of purified pig heart PDH complex was $0.12 \pm 0.008 \text{ min}^{-1}$ (mean $\pm$ S.E.M. for 18 assays). Significance: * $P < 0.01$ for difference from control; † $P < 0.01$ for difference from unity; ‡ $P < 0.01$ for effect of fraction 2. (), Number of assays. For controls there were two experiments and six hepatocyte preparations; for glucagon plus octanoate, one experiment and 12 hepatocyte preparations.

Fraction	PDH kinase activity (min^{-1}) (mean $\pm$ S.E.M.)		Ratio (B)/(A)
	Control (A)	Glucagon + octanoate (B)	
Mitochondrial extract	0.54 ± 0.023 (10)	1.55 ± 0.092 (3)*	2.87 ± 0.15 †
Peak 1 (PDH complex)	0.08 ± 0.015 (12)	0.10 ± 0.015 (5)	1.28 ± 0.24
Peak 2 (with pig heart PDH complex)	0.184 ± 0.01 (17)‡	0.40 ± 0.054 (8)*‡	2.20 ± 0.20 †

Table 3. Effect of glucagon, n-octanoate and insulin on the activity of PDH kinase in rat hepatocytes

Hepatocytes from fed rats were cultured in medium 199 containing 5% (v/v) foetal-calf serum for 4 h (0–4 h) and then for a further 21 h (4–25 h) in medium 199. Glucagon and/or n-octanoate were present from 0 to 25 h; insulin when present was added over the period of culture shown in column 3. At the end of culture, hepatocytes were harvested, mitochondria isolated, extracts prepared and PDH kinase activity assayed and calculated as the apparent first-order rate constant. Values in columns 4 and 5 are multiples of values with control hepatocytes (i.e. cultured in medium 199 with no additions). Significance: * $P < 0.01$, § $P < 0.05$ for effect of insulin; † $P < 0.01$ for difference from unity; ‡ $P < 0.01$ for effect of n-octanoate; for other differences $P > 0.05$. (), Total number of assays; [], number of hepatocyte preparations.

Additions to medium		Period of addition of insulin (h)	PDH kinase activity (multiple of control activity)	
Glucagon (nM)	n-Octanoate (mM)		No insulin	Insulin (1 munit/ml)
25	0	21–25	1.69 ± 0.09 (3) [1]†	–
50	0		1.72 ± 0.04 (47)[15]†	1.38 ± 0.07 (14)[4]*†
		0–25	–	0.87 ± 0.06 (9)[4]*
100	0	–	1.93 ± 0.14 (9)[3]†	–
50	1	21–25	2.42 ± 0.12 (20)[6]†‡	2.40 ± 0.13 (10)[3]†‡
		0–25	–	1.87 ± 0.08 (11)[4]†‡*
0	1	21–25	2.11 ± 0.08 (31)[11]†	1.87 ± 0.09 (19)[6]†
		0–25	–	1.65 ± 0.12 (17)[5]†§

present study; [8,9]), and insulin had no effect when present alone [9].

Interactions of glucagon and/or n-octanoate with insulin

These results are given in Table 3. In this Table (and in Table 4), PDH kinase activities are multiples of the control (i.e. hepatocytes cultured in medium 199). The PDH kinase activity for all hepatocytes (fed rats) cultured in medium 199 in the present study was $0.76 \pm 0.26 \text{ min}^{-1}$ (mean $\pm$ S.E.M. for 47 hepatocyte preparations and 147 assays). This value is not significantly different from those given in [8,9].

The effects of glucagon at 25, 50 and 100 nM on PDH kinase activity were comparable (Table 3, column 4, lines 1, 2 and 4). The effect of glucagon (50 nM) was inhibited completely by insulin (1 munit/ml) when both hormones were present throughout culture. The inhibitory effect of

insulin was incomplete (approx. 50%) when it was added from 21 to 25 h of culture (column 4, line 2; column 5, lines 2 and 3). With octanoate (1 mM), inhibition by insulin was incomplete (approx. 40%) when present throughout culture, and no inhibition was seen when insulin was added from 21 to 25 h (columns 4 and 5, lines 7 and 8). With glucagon plus octanoate, inhibition by insulin was approx. 40% when the hormone was present throughout culture, but it had no effect when added from 21 to 25 h (columns 4 and 5, lines 5 and 6).

Effects of dibutyryl cyclic AMP, 8-bromo cyclic AMP and insulin, and of phenylephrine and isoproterenol

These results are given in Table 4. Dibutyryl cyclic AMP and 8-bromo cyclic AMP increased PDH kinase activity approx. 2-fold (column 3, lines 1–3). Insulin failed to decrease PDH kinase activity in the presence of

Table 4. Effect of dibutyryl cyclic AMP, 8-bromo cyclic AMP and insulin, and of phenylephrine and isoproterenol, on the activity of PDH kinase in rat hepatocytes

Hepatocytes from fed rats were cultured in medium 199 containing 5% (v/v) foetal-calf serum for 4 h (0–4 h) and then for a further 21 h (4–25 h) in medium 199. Dibutyryl cyclic AMP or 8-bromo cyclic AMP were present from 0 to 25 h; insulin when present was added over the period of culture shown in column 3. At the end of culture, hepatocytes were harvested, mitochondria isolated, extracts prepared and PDH kinase activity assayed and calculated as the apparent first-order rate constant. Values in columns 4 and 5 are multiples of values with control hepatocytes (i.e. cultured in medium 199 with no additions). Significance * $P < 0.01$ for difference from unity; for other differences, $P > 0.05$. (), Total number of assays; [], number of hepatocyte preparations. † P , values based on paired t test and number of assays.

Additions to medium	Concn. (μM)	Period of addition of insulin (h)	PDH kinase activity (multiple of control)	
			No insulin	Insulin (1 munit/ml)
Dibutyryl cyclic AMP	50	21–25	1.99 ± 0.09 (15)[5]*	1.98 ± 0.12 (6)[2]†
8-Bromo cyclic AMP	50	0–25	2.20 ± 0.09 (8)[3]*	1.83 ± 0.12 (9)[3]†
Phenylephrine	0.1	–	0.93 ± 0.04 (3)[1]	–
	1.0	–	0.88 ± 0.06 (6)[2]	–
	10.0	–	0.90 ± 0.06 (17)[6]	–
Isoproterenol	10.0	–	0.91 ± 0.07 (5)[2]	–

dibutyryl cyclic AMP (columns 3 and 4, lines 1 and 2). Phenylephrine had no effect on PDH kinase activity either at the concentrations shown in Table 4 (column 3, lines 4–6) or at 0.5, 5, 25 or 50 μM (results not shown). Phenylephrine (1 or 50 μM) was also without effect in the presence of 50 nM-glucagon (results not shown in Table 4). Vasopressin (not shown in Table 3) and 10 μM -isoproterenol (column 3, line 7) were also ineffective.

General discussion and conclusions

In the present study, glucagon (50 nM), dibutyryl cyclic AMP and 8-bromo cyclic AMP increased PDH kinase activity in hepatocytes from fed rats to a comparable extent. This suggests that the action of glucagon is mediated by cyclic AMP. In the present study, isoproterenol (a β -adrenergic agonist) had no effect on PDH kinase activity in hepatocytes of fed rats. However, the β -adrenergic effect of adrenaline on cyclic AMP concentration in liver is small and relatively short-lived compared with that of glucagon (2–3-fold compared with 50-fold in perfused liver [15], and 2-fold compared with 30-fold in hepatocytes [11]).

Insulin (1 munit/ml), when present throughout culture, completely inhibited the effect of glucagon to increase PDH kinase activity, but it failed to inhibit the effect of dibutyryl cyclic AMP. Insulin only partially inhibited (by approx. 40%) the effect of n-octanoate or of glucagon plus octanoate. The concentration of glucagon used in the present study is some 100-fold greater than the physiological concentration of 0.2–0.5 nM cited in [10]. This high concentration was used because glucagon is readily inactivated by proteolysis and because high concentrations of the hormone are required for sustained elevation of cyclic AMP as measured in shorter-term experiments [11]. Assays of cyclic AMP confirmed that sustained elevation of cyclic AMP throughout culture by glucagon was achieved in the present study (results not shown). The concentration of insulin used may approximate to that in the portal vein of fed rats. Insulin can oppose actions of glucagon in liver by lowering cyclic AMP concentration (through activation of cyclic AMP

phosphodiesterase and/or inhibition of adenylate cyclase [10]), and it can also inhibit actions of cyclic AMP or dibutyryl cyclic AMP [12]. Because insulin has no effect on PDH kinase activity in the absence of glucagon [9] or in the presence of dibutyryl cyclic AMP (the present study), this might suggest that insulin antagonized glucagon by lowering the cyclic AMP concentration. However, in shorter-term experiments in [10,13], insulin effects on cyclic AMP concentration or on metabolic responses mediated by cyclic AMP (e.g. enhanced gluconeogenesis) were either small or absent at high glucagon concentrations.

Collectively these observations regarding effects of glucagon, cyclic AMP analogues, fatty acid (octanoate) and insulin provide a basis for the effect of starvation to increase activities of PDH kinase and KAP in liver *in vivo*. It has been established in the present study that glucagon plus octanoate increase the activity of KAP in cultured hepatocytes. In the rat, starvation increases plasma concentrations of non-esterified fatty acids and glucagon, decreases plasma concentration of insulin and increase the hepatic concentration of cyclic AMP [14,15]. The observation (the present study) that glucagon + fatty acid (octanoate) maintain, but do not increase, the activity of PDH kinase during 25 h of culture of hepatocytes from starved rats further supports their proposed role as mediators of the effect of starvation to increase PDH kinase activity.

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