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Insulin reverses effects of starvation on the activity of pyruvate dehydrogenase kinase in cultured hepatocytes

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In tissue culture of hepatocytes, insulin (0.1–1 munits/ml for 4 h) reversed completely the effects of starvation of rats to decrease the activity of pyruvate dehydrogenase (PDH) complex and to increase the activities of PDH kinase and PDH kinase activator protein. It had no effect in hepatocytes from fed rats. Significant effects of insulin were detected with 0.01 munit/ml after 4 h, and in 1–2 h with 1 munit/ml.

INTRODUCTION

In animal tissues the mitochondrial pyruvate dehydrogenase (PDH) complex is regulated by reversible phosphorylation, being inactivated by phosphorylation catalysed by PDH kinase intrinsic to the complex, and reactivated after dephosphorylation by a mitochondrial PDH phosphatase [1,2]. In rats, starvation or induction of alloxan-diabetes leads over 24–48 h to enhanced phosphorylation of PDH complex, resulting in an up to 30-fold decrease in the percentage of the active (dephosphorylated) form in liver, muscles, kidney and adipose tissue. These effects are reversed over 24–48 h by refeeding (starvation) or insulin injections (diabetes) (for reviews, see [3,4]). The effects of starvation to enhance phosphorylation of PDH complex in rat heart and liver are associated with increased activity of PDH kinase as measured in extracts of mitochondria. The effect may be mediated by an increase in the specific activity of a protein activator of PDH kinase (KAP) which can be separated from PDH complex and PDH kinase intrinsic to the complex by gel filtration [5,6].

Hepatocytes from fed rats cultured in medium 199 for 21 h maintained unchanged activities of PDH complex (percentage in active form) and of PDH kinase [7]. Addition of glucagon and n-octanoate led over 21 h to a decrease in the percentage of active PDH complex and to an increase in the activity of PDH kinase comparable with that induced *in vivo* by starvation [7]. We show here that culture for 21 h in medium 199 had no effect on the activities of PDH complex (percentage in active form), PDH kinase or KAP in hepatocytes from livers of starved rats. Insulin added at physiological concentrations over the last 4 h of culture completely reversed the effects of starvation on these activities.

EXPERIMENTAL

Materials

Details relating to male albino Wistar rats and sources of chemicals, biochemicals, Falcon tissue-culture bottles, tissue-culture medium 199 [8] and antibiotics were as given in [6,7]. The major potential respiratory substrates in medium 199 are 5.5 mM-glucose and 0.6 mM-sodium acetate. Bovine insulin (approx. 24 units/mg) was from Sigma Chemical Co., Poole, Dorset, U.K.; stock solutions (20 units/ml) were prepared in 3.3 mM-HCl.

Hepatocytes and culture

The general details pertaining to isolation of hepatocytes and to tissue culture were as in [7]. Hepatocytes were plated at a cell density of approx. 10^6 cells/ml, and 27 ml of cell suspension was added to each bottle. Cell attachment was achieved by incubation for 4 h at 37 °C in air/CO_2 (19:1) in medium containing 5% (v/v) foetal-calf serum. The medium was then aspirated, the cells washed with medium 199 to remove serum, and culture continued with 27 ml of medium 199 for a fixed period of 21 h with one further change of medium after 17 h. Insulin, when present, was added for a period (x) from ($21-x$) h to 21 h by appropriate addition to the medium. In general hepatocytes were prepared from a single liver and cultured in any one experiment in twelve Falcon bottles. In studying the effect of insulin, hepatocytes from each liver were divided between cultures made in the presence and in the absence of the hormone. Viability was assessed as in [7], and cultures adjudged unsatisfactory were rejected. At the end of culture the attached cells were washed vigorously with 10 ml of sucrose medium (0.25 M-sucrose/5 mM-Tris/2 mM-EGTA, pH 7.4) and detached with a rubber-coated spatula into 2×4 ml plus 1×2 ml of sucrose medium.

Mitochondria

Hepatocytes were disrupted in sucrose medium in a Potter–Elvehjem homogenizer [7]. For assay of PDH complex and PDH kinase in mitochondrial extracts, mitochondria were isolated by differential centrifugation as in [7]. For assay of PDH complex (active form), freshly prepared mitochondria were added to KCl medium [7] and immediately pelleted (30 s; Eppendorff 3200 centrifuge; supernatant aspirated) and frozen in liquid N_2. For assay of total PDH complex (sum of active and inactive forms) and PDH kinase mitochondria were incubated for 25 min at 30 °C in KCl medium containing 10 μM-CCCP to effect conversion of inactive complex into active complex and then pelleted. 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-

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

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Table 1. Effect of prior starvation (48 h) and of insulin added *in vitro* on the activities of PDH complex and PDH kinase in extracts of mitochondria isolated from cultured rat hepatocytes

Mitochondria were from hepatocytes cultured for 21 h (after attachment) in medium 199, with change of medium after 17 h. Insulin where present was added at the time and concentration shown. 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 prepared from mitochondria incubated for 25 min at 30 °C in KCl medium containing 10 μM-CCCP. The number of hepatocyte preparations was four (lines 1–4) or three (lines 5–8). Total PDH complex averaged 41.4±0.35 units/g of mitochondrial protein (mean±S.E.M. for 47 hepatocyte cultures). There was no significant differences in total PDH complex between different cultures (not shown). *$P < 0.01$ for effect of starvation; †$P < 0.01$ for effect of insulin; ‡$P < 0.01$ for difference from 0.1 munit of insulin/ml; §$P < 0.02$ for difference from '(18th–21st)' h. Results are means±S.E.M.

Insulin added (munits/ml) (h) of culture	PDH complex (active form, % of total)		PDH kinase [expressed as pseudo-first-order rate constant (min^{-1})]	
	Fed rats	Starved rats	Fed rats	Starved rats
None				
None	23±0.64	11±0.63*	0.96±0.08	2.38±0.07*
0.01 (17th–21st)	25±0.42	17±1.32*†	0.86±0.03	1.55±0.03*†
0.1 (17th–21st)	25±0.31	21±1.20†	0.98±0.05	1.18±0.06†
1 (17th–21st)	24±0.82	23±0.41†	0.85±0.07	0.84±0.04†‡
None		10±0.27*		2.57±0.11*
1 (20th–21st)		12±0.03†		2.57±0.01
1 (19th–21st)		15±0.42†		1.86±0.06†
1 (18th–21st)		18±0.75†		1.28±0.04†
1 (17th–21st)		22±0.65†§		0.90±0.04†§

chloromethane ('TLCK'), pH 7.5. In these experiments, hepatocytes were prepared and cultured on day 1, mitochondria isolated on day 2 and assays done on day 2 or 3.

For separation of PDH complex and KAP by gel filtration, mitochondria were isolated after incubation with digitonin to remove lysosomes by the procedure in [6]. After incubation in KCl medium containing CCCP and pelleting, extracts were prepared in 50 mM-potassium phosphate/10 mM-EGTA/2 mM-dithiothreitol/1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride/2.5% (v/v) Triton X-100, pH 7. Triton X-100 was necessary to prevent unacceptable losses of PDH complex when extracts were clarified by centrifugation before gel filtration. Gel filtration (Sephacryl S-300) was carried out as in [6], except that the running buffer contained 2.5% (v/v) Triton X-100. In these experiments, mitochondria from hepatocytes equivalent to 2.5–3 livers were accumulated over 7–10 days (−80 °C), and pooled extracts were prepared. The yield of mitochondria [based on the recovery of PDH complex (active plus inactive forms)] was approx. 35%.

Assays and statistical analysis

PDH complex was assayed spectrophotometrically as in [6,7]. PDH kinase activity was assayed by the rate of ATP-dependent inactivation as in [6,7], and was calculated as the apparent first-order rate constant [5]. KAP activity was assayed in the pooled second protein peak (A_{280}) after Sephacryl S-300 chromatography [9] by its effects on the apparent first-order rate constant for ATP-dependent inactivation of purified pig heart PDH complex and on the rate of incorporation of ^{32}P from [γ-^{32}P]ATP into pig heart complex as in [6]. The significance of differences between means was calculated by Student's t test.

RESULTS AND DISCUSSION

Activities of PDH complex and PDH kinase in hepatocyte mitochondria and the effect of insulin in culture

In what follows, values for PDH complex activity (total and active form) and PDH kinase activity have not been corrected for the possible contribution of branched-chain-2-oxoacid dehydrogenase complex to assays of PDH complex. The approximate potential contributions, taken from [7] are: total PDH complex, 2%; active form of PDH complex, 4% (fed or starved); and PDH kinase, no more than a 3.6% underestimation of the apparent first-order rate constant.

The total activities of PDH complex (sum of active and inactive forms) in mitochondria from cultured rat hepatocytes averaged (units/g of mitochondrial protein) 41.4 (all cultures), 42.5 (fed rats) and 41 (starved rats), and are comparable with previously published estimates [7,10]. There were no obvious effects of insulin on total activity of PDH complex (results not shown). In hepatocytes cultured in the absence of insulin the percentages of PDH complex in the active form (23%, fed rats; 11 or 10%, starved rats) and the activities of PDH kinase (0.96 min^{-1}, fed rats; 2.38 or 2.57 min^{-1}, starved rats) (Table 1, lines 1 and 5) are comparable with values given in [6,7] for mitochondria prepared from rat livers. The percentage of PDH complex in the active form is based on active PDH complex measured in extracts of freshly prepared mitochondria; evidence that this method may give valid estimates has been given in [7].

When hepatocytes from fed rats were exposed to insulin (0.01–1 munit/ml) for the last 4 h of culture, the percentage of PDH complex in the active form and the activity of PDH kinase remained unchanged (Table 1,

Table 2. Effect of insulin added *in vitro* to hepatocytes from starved rats in cultures on activities of PDH kinase and KAP

Mitochondria were derived from hepatocytes prepared from the livers of six rats (starved for 48 h) and divided between cultures + insulin (where present insulin was added to 1 munit/ml from the 17th to the 21st h of culture). Rat liver PDH complex (fraction 1) and KAP (fraction 2) were separated by gel filtration from extracts containing 2.3 units of PDH complex. KAP was assayed by the increase which it effects in the PDH kinase activity of rat liver PDH complex (fraction 1; 0.3 unit/ml) or highly purified pig heart PDH complex (0.3 unit/ml; PDH kinase activity 0.118 ± 0.02 min^{-1}). Results are means ± S.E.M. for three or four assays. *$P < 0.01$ for effect of insulin; †$P < 0.01$ for effect of fraction 2.

Mitochondrial fraction	Fraction 2 (mg/ml of assay mixture)	Conditions of culture ...	PDH kinase activity [expressed as pseudo-first-order rate constant (min^{-1})]	
			No insulin	Insulin
Unfractionated extract	–		2.94 ± 0.18	0.97 ± 0.08*
Fraction 1	–		0.08 ± 0.01	0.005 ± 0.001*
Fractions (1 + 2)	0.22		0.46 ± 0.02†	0.09 ± 0.003*†
Fraction 2 with pig heart PDH complex	0.86		1.58 ± 0.16†	0.60 ± 0.04*†
	0.43		1.03 ± 0.04†	0.27 ± 0.04*†
	0.22		0.52 ± 0.03†	–

lines 1–4). In the corresponding experiment with hepatocytes from starved rats, insulin increased the percentage of active complex and decreased the activity of PDH kinase (Table 1, lines 1–4). The effect was evident at an insulin concentration of 0.01 munit/ml (the lowest concentration tested) (compare lines 1 and 2, Table 1), but the values achieved differed from those obtained with hepatocytes from fed rats under comparable conditions of culture (Table 1, line 2). Insulin at 1 munit/ml restored the values to those obtained with hepatocytes from fed rats (Table 1, line 4), but at 0.1 munit/ml the values for hepatocytes from starved rats were still different from those for hepatocytes from fed rats at $P < 0.05 > 0.02$ (P values not shown in Table 1, line 3). The time course of the action of insulin (1 munit/ml) on hepatocytes from starved rats is shown in Table 1, lines 5–9. Restoration to values not significantly different from those of fed rats took 4 h (compare lines 4 and 9 in Table 1), but significant effects of insulin both on the percentage of active PDH complex and on activity of PDH kinase were evident within 2 h (compare lines 5 and 7, Table 1).

Activity of KAP in mitochondria from culture hepatocytes and effect of insulin in culture

It has seemed important to show more directly that the action of insulin to decrease the activity of PDH kinase in hepatocytes from starved rats may be mediated by a decrease in the activity of KAP. This has been accomplished by separating KAP from PDH complex by gel filtration on Sephacryl S-300 and assay of KAP activity with purified pig heart PDH complex. The results are shown in Table 2.

Separation of rat hepatocyte PDH complex (fraction 1) from KAP (fraction 2) led to the expected and substantial decrease in PDH kinase activity (compare lines 1 and 2, Table 2), but the decrease in kinase activity effected by insulin was still apparent. Recombination of fractions 1 and 2 (line 3) showed stimulation of rat liver PDH kinase by KAP and showed further that the effect of insulin was retained after recombination (line 3 in Table 2). The activity of PDH kinase in purified pig heart PDH complex was increased by KAP in a concentration-dependent manner (compare lines 4–6, Table 2). The stimulation at the highest concentration used was 14-fold (hepatocytes not exposed to insulin) and 1.8-fold (hepatocytes exposed to insulin) (line 5 of Table 2 and line 5 of the legend to Table 2). Thus with pig heart PDH complex also, the activity of KAP was decreased by insulin action in hepatocytes from starved rats. The interpretation of these experiments assumes equivalent recoveries of KAP after gel filtration (the contents of PDH complex in the mitochondrial extracts loaded were equivalent).

The incorporation of ^{32}P from [γ-^{32}P]ATP into purified pig heart PDH complex was enhanced by KAP, and the effect of KAP from hepatocytes of starved rats was decreased significantly by exposure to insulin (results not shown).

General discussion and conclusions

In short-term experiments *in vitro* with tisues from fed rats, insulin increased the proportion of PDH complex in the active form in adipocytes (up to 2-fold) [11,12], in hepatocytes (up to 1.3-fold) in [13,14] but not in [15]; and not in perfused liver [16] or in heart or diaphragm muscles [17,18]. In adipocytes and hepatocytes this effect of insulin (where seen) was maximum in 3–10 min. It is apparently mediated in adipocytes by an increase in the activity of PDH phosphatase [19,20].

In the present study in which hepatocytes were maintained in culture for 21 h before addition of insulin, the hormone had no effect on the percentage of active PDH complex in hepatocytes from fed rats after 4 h. In hepatocytes from starved rats, which retained their lower percentage of active PDH complex in culture, insulin increased active PDH complex 2-fold to the level observed in hepatocytes from fed rats. The effect took 4 h to complete, but was detectable within 1 h. This effect of insulin was associated with a decrease in the activities of PDH kinase and of KAP. It would appear that this effect of insulin may be distinguished from the more rapid effects of the hormone which others have observed in adipocytes and hepatocytes from fed rats and which, in adipocytes, is apparently mediated through PDH phosphatase.

The effect of insulin which we have described here was evident, after 4 h of exposure, at an insulin concentration of 0.01 munit/ml. Complete reversal of the effects of starvation required 4 h of exposure of hepatocytes to insulin at a concentration of 0.1–1 munit/ml. This concentration is comparable with the mean portal-vein concentration of insulin in fed rats *in vivo* reported as 1.3 nM in [21], i.e. approx. 0.19 munit/ml. The time required for reversal of these effects of starvation on hepatocytes *in vivo* is not known. In rat heart muscle, reversal *in vivo* of the effects of starvation on the activities of PDH complex (active form) and of KAP required 20 h of refeeding [6]. It may be that a longer period is required for reversal *in vivo*, because of effects of other circulating hormones and/or nutrients not present in the culture medium *in vitro*.

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