Volume 336, number 3, 501–505 FEBS 13469 December 1993

Long-term regulation of pyruvate dehydrogenase kinase by high-fat feeding

Experiments in vivo and in cultured cardiomyocytes

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Received 24 October 1993

The provision of the high-fat diet (47% of calories as fat) for 28 days evoked a significant decline in cardiac PDH_a activity, together with marked increases in the activity of PDH kinase measured in isolated mitochondria and freshly-prepared cardiomyocytes from adult rats. Plasma insulin concentrations in fat-fed rats were not significantly different from control, but plasma NEFA concentrations were elevated. PDH kinase activity in cardiomyocytes from fat-fed rats fell substantially in culture (25 h). This decline was prevented by the inclusion of *n*-octanoate and DBcAMP in combination, but not individually, in the culture medium. The results are discussed in relation to the role for fatty acids and insulin in the long-term modulation of cardiac PDH kinase activity by high-fat feeding.

Pyruvate dehydrogenase complex; Pyruvate dehydrogenase kinase; Starvation; High-fat diet; Glucose/fatty acid cycle; Insulin

1. INTRODUCTION

The pyruvate dehydrogenase (PDH) complex is a mitochondrial multienzyme complex catalysing the oxidative decarboxylation of pyruvate to acetyl-CoA. In mammals, including man, the activity of the PDH complex reaction is regulated by reversible phosphorylation and end-product inhibition [1,2]. The PDH complex is inactivated by phosphorylation of the E1 alpha component by PDH kinase and reactivated by its dephosphorylation, catalysed by PDH phosphatase.

In heart, a decline in active PDH complex (PDH_a) activity can be achieved by acute activation of PDH kinase by metabolite effectors [3]. The activity of cardiac PDH kinase is enhanced by increasing mitochondrial concentration ratios of acetyl-CoA/CoA, NADH/NAD^+ and ATP/ADP. The effects of fatty acids and ketone bodies to promote phosphorylation and inactivation of the PDH complex in the perfused heart are mediated by the increase in the mitochondrial acetyl-CoA/CoA concentration ratio and enhanced by the increase in mitochondrial NADH/NAD^+ concentration ratio which the oxidation of these lipid fuels is known to induce [3,4]. Similarly, the mechanism whereby cardiac PDH_a activity is decreased during starvation involves direct, short-term effects of increased oxidation of lipid-derived fuels [3–5] which lead to activation of PDH kinase by an increased mitochondrial acetyl-CoA/CoA concentration ratio [4].

In progressive starvation, the short-term effects of oxidation of fatty acids and ketone bodies to decrease the percent PDH_a in heart are supplemented by a more long-term mechanism, involving a stable increase in the specific activity of PDH kinase which is observed after 24 to 48 h [6]. Primary cultures of rat cardiomyocytes have been used to investigate potential factors that may mediate the increase in PDH kinase activity [7]. Results from these studies are consistent with the concept that the effect of prolonged starvation to increase cardiac PDH kinase specific activity may be mediated by fatty acids and cyclic AMP [7].

The present study examined the consequences of the administration of a high-fat diet on cardiac active and total PDH complex and PDH kinase activities in vivo. Previous studies [8,9] had demonstrated that high-fat feeding for 19–23 days evokes a significant decline in cardiac PDH_a activity in vivo, without a change in PDH total activity, but the mechanism(s) underlying this response were not defined. The studies in vivo were extended by an investigation of the longer-term regulation of PDH kinase in primary cultures of adult cardiomyocytes from fat-fed rats in relation to the effects of fatty acids and cyclic AMP.

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Abbreviations: DBcAMP, dibutyryl cyclic AMP; NEFA, non-esterified fatty acids; PDH, pyruvate dehydrogenase; PDH_a, the active form of PDH.

2. MATERIALS AND METHODS

Biochemicals were from Boehringer Mannheim Ltd., Lewes, East Sussex, UK or Sigma Chemical Co. Ltd., Poole, Dorset, UK. Medium 199 and foetal calf serum (FCS) was purchased from Life Technologies Ltd., Uxbridge, Middlesex, UK. Primaria culture dishes were from Marathon Laboratory Supplies Co., London, UK. Antibiotics and antimycotics were from Sigma Chemical Co. Ltd., Poole, Dorset, UK. Kits for measurements of plasma insulin concentrations were from LadyBee Ltd., Horam, East Sussex, UK. Kits for measurements of plasma non-esterified fatty acid (NEFA) concentrations were from Alpha Laboratories, Eastleigh, Hampshire, UK. Rodent diets were from Special Diets Services, Witham, Essex, UK.

Female Wistar rats were maintained on a 12-h light/12-h dark cycle (light from 10.00 h). Fed rats were sampled in the absorptive state (within 0.5 h of the end of the dark (feeding) phase). Rats were permitted free access to either a standard rodent diet or a semi-synthetic high-fat diet. The dietary lipid source in the high-fat diet was lard, supplemented with corn oil (4% of total calories) to prevent essential fatty acid deficiency. Caloric distribution was 20% protein, 72% carbohydrate and 8% fat in the standard diet and 20% protein, 33% carbohydrate and 47% fat in the high-fat diet. One group of rats, maintained on standard diet, was starved for 48 h (from the end of the dark phase) immediately before use.

PDH complex, active form (PDH_a) activities were measured in freeze-clamped heart extracts by coupling to arylamine acetyltransferase as described in [4], with the addition of protease inhibitors (benzamidine (1 mM), leupeptin (10 μM), TLCK (0.3 mM)) but not Triton X-100 to the extraction medium (see [10]). PDH_a activities are expressed relative to citrate synthase, measured as in [4], to correct for possible differences in the efficiency of mitochondrial extraction between groups. Total PDH activities were measured in heart mitochondria [11]. A unit of PDH or citrate synthase activity is defined as that which converts 1 μmol of substrate into product/min at 30°C.

Calcium-tolerant ventricular cardiomyocytes were isolated by collagenase digestion of adult rat hearts [12]. Following isolation, they were washed 3 to 4 times at 37°C with bicarbonate-buffered saline [13] containing 10 mM glucose, 2% BSA and 50–500 μM Ca^{2+}, and allowed to settle under gravity. Following aspiration of the supernatant, the cardiomyocytes were resuspended in medium 199 containing 4% (v/v) foetal calf serum (which had been extensively dialysed before use), antibiotics and antimycotics (see [7]) and the additions indicated. Cardiomyocytes were plated in petri dishes at a density of 10^5 cells · ml^{-1} and cultured in an atmosphere of 95% O_2/5% CO_2 for 25 h at 37°C without change of medium. At the end of culture CCCP was added to the medium to 15 μM and incubation was continued for 30 min to effect PDH activation (see [7]). The cells were then dispersed and aspirated into plastic centrifuge tubes. The cardiomyocytes were spun down (2 min, 145 × g) and then resuspended in extraction buffer [14] for measurement of PDH kinase activity as described in [7], except that Triton X-100 was not included in the extraction buffer. For assay of PDH kinase activity in cardiac mitochondria, mitochondrial pellets (after 30 min incubation of mitochondria at 30°C without respiratory substrate in the presence of 15 μM FCCP) were frozen, thawed and dispersed into 50 mM KH_2PO_4, 50 mM K_2HPO_4, 10 mM EGTA, 2 mM dithiothreitol, 1 mM benzamidine, 10 μM leupeptin, 0.3 mM TLCK, pH 7.5 and then extracted by freeze-thawing 3 times. PDH kinase activities were estimated in mitochondrial and cardiomyocyte extracts by the rate of ATP-dependent inactivation of PDH complex as described in [14]. PDH kinase activity was computed as the apparent first-order rate constant [6]. The incubation mixture contained 0.5 mM ATP, 1 mM $MgCl_2$, 36 μg/ml oligomycin B and 150–350 m-units PDH complex/ml. Samples for assay of active PDH were taken at 3–4 time intervals over up to 8 min (depending on PDH kinase activity).

Plasma NEFA and TG concentrations were measured using commercial kits.

Statistical significance of differences between groups was assessed by Student's unpaired t-test. Results are means ± S.E.M. with the numbers of obs. in parentheses.

3. RESULTS

3.1. *Insulin and NEFA concentrations*

Plasma insulin concentrations in rats provided with the high-fat diet ad libitum for either 10 or 28 days were not significantly different from those observed in rats fed ad libitum on standard (high carbohydrate, low fat) diet (Table I). Starvation (48 h) decreased plasma insu-

Table I

Cardiac PDH_a and PDH kinase activities and plasma insulin and non-esterified fatty acid (NEFA) concentrations in rats provided with standard or high-fat diet

	PDH_a activity (mU/U of citrate synthase)	PDH kinase (pseudo-first-order rate constant (min^{-1}))	Insulin conc. (ng/ml)	NEFA conc. (mM)
Standard diet				
Fed	32.00 ± 4.99 (5)	0.130 ± 0.008 (13)	2.18 ± 0.25 (18)	0.11 ± 0.03 (20)
Starved (48 h)	0.37 ± 0.08§ (3)	0.264 ± 0.029§ (10)	0.47 ± 0.13§ (3)	0.56 ± 0.02§ (4)
High-fat diet				
10 day	24.60 ± 2.13 (4)	0.144 ± 0.009 (5)	2.10 ± 0.52 (9)	0.15 ± 0.04 (13)
28 day	11.20 ± 4.28* (5)	0.206 ± 0.026* (9)	1.52 ± 0.22 (7)	0.39 ± 0.06*** (10)

Full experimental details are given in section 2. PDH_a activities were measured in extracts of freeze-clamped hearts. Total PDH activities (mitochondria) were: standard diet, 65.5 ± 4.6 mU/U of citrate synthase; high-fat diet, 73.2 ± 19.2 mU/U of citrate synthase. Citrate synthase activities, measured in extracts of freeze-clamped tissue or mitochondria were unaffected by high-fat feeding (results not shown). PDH kinase activities were measured in extracts of cardiomyocytes. Rate constants were calculated by least-squares linear regression analysis of (ln % zero time activity) against time. Results are means ± S.E.M. for the number of observations in parentheses. Statistically-significant effects of high-fat feeding are shown by: *$P < 0.05$; ***$P < 0.001$. Statistically-significant effects of starvation are shown by: §$P < 0.001$.

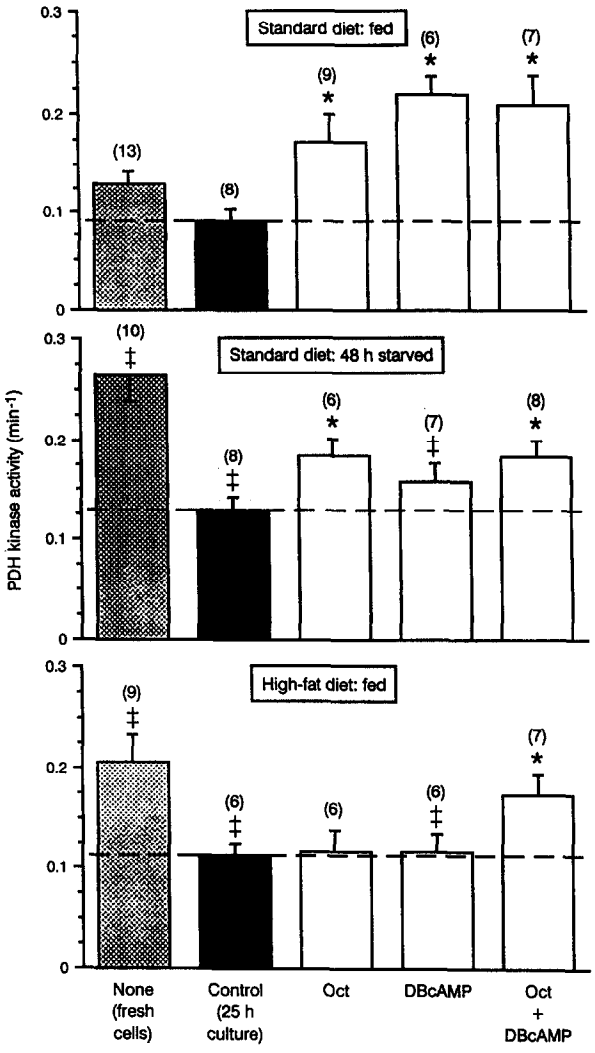

Fig. 1. Effects of culture, and of *n*-octanoate, DBcAMP and isoprenaline in culture, on the PDH kinase activity of cardiomyocytes from rats fed standard diet, 48-h starved rats or 28 day fat-fed rats. Full experimental details are given in section 2. Bars show mean ± S.E.M. for the number of observations given in parentheses. Oct, *n*-octanoate (1 mM); DBcAMP, dibutyryl cyclic AMP (50 μM). *$P < 0.05$ for the effects of addition to culture medium, ‡ $P < 0.05$ for the effects of starvation for 48 h or of high-fat feeding for 28 days.

lin concentrations in rats previously allowed access to standard diet (Table I). Plasma NEFA concentrations measured in the fed state were increased significantly by the provision of the high-fat diet for 28 days, but not for 10 days (Table I). Starvation (48 h) of rats maintained on standard diet also increased plasma NEFA concentrations (Table I).

3.2. *Effects of fat-feeding on PDH_a and PDH kinase in freeze-clamped hearts and isolated mitochondria*

The provision of the high-fat diet for 28 days led to a significant (65%) decline in cardiac PDH_a activity, measured in extracts of freeze-clamped tissue (Table I). In contrast, only modest (25%, $P > 0.05$) suppression of cardiac PDH_a activity was observed after 10 days of fat-feeding. As demonstrated by others [8], total PDH activity (sum of active and inactive forms) was not changed by the provision of a high-fat diet for up to 28 days. PDH measured in extracts of freeze-clamped hearts from rats provided with standard diet was approx. 49% active, whereas the percentage of active PDH complex was 34% after 10 days of fat-feeding and 15% after 28 days of fat-feeding. Cardiac PDH_a activity was almost completely suppressed by 48 h of starvation (Table I). The concentrations of total PDH complex, active PDH complex and the proportion of active PDH complex observed for hearts of fed rats provided with standard diet and sampled at the end of the dark phase were comparable with those observed previously [4,5].

The provision of the high-fat diet for 28 days was associated with a substantial (2.0-fold) increase in the activity of PDH kinase measured in isolated mitochondria (control, 0.094 ± 0.013 (4) min^{-1}; fat-fed, 0.189 ± 0.034 (3) min^{-1}; $P < 0.05$). The activity of PDH kinase measured in isolated mitochondria after 28 days of fat-feeding was not significantly different from that found in isolated mitochondria prepared from hearts of rats maintained on standard diet and subsequently starved for 48 h.

3.3. *PDH kinase activities in isolated cardiomyocytes*

The effect of high-fat feeding for 28 days to increase PDH kinase in isolated cardiac mitochondria persisted in freshly-prepared cardiomyocytes (Table I). As measured in extracts of fresh cells, absolute PDH kinase activity was comparable in magnitude to that observed in extracts of mitochondria prepared from hearts of fat-fed rats (see above). Starvation (48 h) was associated with a 2-fold increase in PDH kinase in freshly-isolated cardiomyocytes (Table I). This fold increase in PDH kinase in response to starvation, measured in freshly-isolated cardiomyocytes, is similar to that observed previously by others [7].

3.4. *Effects of culture on PDH kinase activity in cardiomyocytes*

Culture (25 h) of cardiomyocytes from fed rats provided with standard (high carbohydrate, low fat) diet ad libitum led to a 31% decline in PDH kinase activity compared with freshly-prepared cells (Fig. 1). As observed by others [7], a more marked (51%) decline in PDH kinase activity was observed on culture (25 h) of cardiomyocytes from 48 h starved rats (Fig. 1). As occurs when cardiomyocytes from 48 h-starved rats are cultured, PDH kinase activity in cardiomyocytes from rats provided with the high-fat diet for 28 days fell substantially (by approx. 45%) on culture (Fig. 1).

3.5. *Effects of* n*-octanoate and dibutyryl cyclic AMP*

The activity of PDH kinase in cultured cardiomyocytes from rats provided with unrestricted access to

standard diet was increased by *n*-octanoate (1 mM), dibutyryl cyclic AMP (DBcAMP) (50 μM) and by *n*-octanoate (1 mM) and DBcAMP (50 μM) in combination (Fig. 1). The effects of *n*-octanoate and DBcAMP to increase PDH kinase were not additive (Fig. 1). The value of PDH kinase activity observed after culture for 25 h with *n*-octanoate was similar to that measured in cardiomyocytes from 48-h starved rats cultured under identical conditions. The PDH kinase activities observed after culture for 25 h with DBcAMP plus *n*-octanoate exceeded those observed in freshly-prepared cells. The fold increases in PDH kinase activities evoked by *n*-octanoate and DBcAMP are similar to those observed previously in primary cultures of cardiomyocytes of chow-fed rats [7].

PDH kinase activities in cardiomyocytes from starved rats cultured with either *n*-octanoate or DBcAMP remained significantly ($P < 0.05$) lower than those observed in freshly-prepared cells from starved rats (Fig. 1). DBcAMP, when added alone, failed to increase PDH kinase activity significantly above control values, obtained by culture without this addition (Fig. 1). The addition of *n*-octanoate (1 mM) was, however, associated with a significant (approx. 43%) increase in PDH kinase activity; this effect was not enhanced by the further addition of DBcAMP (50 μM) (Fig. 1). Culture of cardiomyocytes from starved rats with *n*-octanoate and DBcAMP in combination increased PDH kinase activity significantly above control values; however, the activity of PDH kinase observed was still significantly different from that of freshly-prepared cells from starved rats (Fig. 1).

The decline in activity of PDH kinase observed in cardiomyocytes from fat-fed rats after 25 h of culture was not prevented by the inclusion of either *n*-octanoate or DBcAMP individually in the culture medium (Fig. 1). However, the addition of these agents in combination lead to a significant ($P < 0.05$) enhancement of PDH kinase activity and compensation for the effects of 25 h of culture to suppress PDH kinase activity (Fig. 1). However, as with cardiomyocytes from starved rats, the activity of PDH kinase observed after culture of cardiomyocytes from fat-fed rats with *n*-octanoate + DBcAMP did not exceed that found in freshly-isolated cardiomyocytes from fat-fed rats (Fig. 1).

4. DISCUSSION

A high-fat diet has been demonstrated to decrease the proportion of active PDH in the rat heart in vivo [8] and in vitro [9] without affecting the total activity of PDH [8]. In these previous studies, the dietary content of fat was increased to 40% at a constant protein intake for 19–23 days [8]. In the present study, we used a semi-synthetic diet in which lipid contributed 47% of calories and, again, protein content was maintained. We observed that high-fat feeding for a period of 28 days was associated with significant suppression of cardiac PDH_a activity (to approx. 30% of control). However, a less extended period of fat-feeding was not associated with a significant decline in cardiac PDH_a activity. It was noted that the decline in cardiac PDH_a activity observed after 28 days of fat-feeding was accompanied by a significant elevation in plasma NEFA concentrations. These findings suggest that PDH inactivation in the fed state after long-term fat feeding is related to an increased NEFA supply. As cardiac PDH total activity was unchanged by high-fat feeding, decreased cardiac PDH_a activities can be attributed to inactivation of the PDH complex by reversible phosphorylation by PDH kinase.

The administration of a high-fat diet for 28 days led to a substantial stable increase in the activity of PDH kinase measured in isolated mitochondria or cardiomyocytes; this response was absent after 10 days of fat-feeding. The failure to increase PDH kinase after 10 days of fat-feeding when NEFA concentrations are low, compared with the significant increase in PDH kinase observed after 28 days of fat-feeding (when NEFA concentrations are increased), strongly implicates a primary role for fatty acids in the stable activation of cardiac PDH kinase. This is supported by the results obtained with cultured cardiomyocytes, namely the demonstration of a direct effect of *n*-octanoate to increase PDH kinase activity in cultured cardiomyocytes from rats fed standard diet but not from rats provided with the high-fat diet, and the observation that culture of cardiomyocytes from fat-fed rats with DBcAMP + *n*-octanoate can only maintain (rather than enhance) the activity of PDH kinase.

The fold increase in PDH kinase activity observed in response to 28 days of fat-feeding was similar to that evoked by 48 h-starvation. However, importantly, the high-fat diet we provided contained a significant carbohydrate content (33% of caloric intake) and thus plasma insulin concentrations in the fat-fed rats were similar to those observed in rats fed ad libitum on standard diet (and significantly higher than those observed in rats starved for 48 h). The results thus indicate that a high circulating insulin concentration in vivo is unable to oppose the enhancement of cardiac PDH kinase activity induced by long-term fat-feeding. In addition, the results imply that the increase in PDH kinase activity observed in response to starvation is not of necessity directly dependent on the observed decline in insulin, but instead reflects the increase in fatty acid supply occasioned by increased adipose-tissue lipolysis secondary to the decline in insulin.

Although the increase in the activity of PDH kinase observed in response to 28 days of fat-feeding was similar to that evoked by 48-h starvation, the suppression of cardiac PDH_a activity associated with 28 days of fat-feeding was considerably less than that evoked by

48-h starvation. These findings are significant since they indicate that, despite an increase in PDH kinase activity which can be clearly demonstrated both in isolated mitochondria and in cardiomyocytes, a relatively high cardiac PDH_a activity can be retained in the fat-fed rats in the fed state. It is possible that a relatively high insulin concentration can compensate for long-term increases in PDH kinase activity and/or counteract the acute effects of fatty acid oxidation to activate PDH kinase in the fed state, despite a failure to oppose the increase in cardiac PDH kinase activity evoked by fat-feeding.

Acknowledgements: This work was generously supported by the British Heart Foundation. K.A.O. holds a B.H.F. research studentship. M.J.H. is a Drummond Research Fellow.

REFERENCES

[1] Randle, P.J. (1986) Biochem. Soc. Trans. 14, 799–806.
[2] Patel, M.S. and Roche, T.E. (1990) FASEB J. 4, 3224–3233.
[3] Kerbey, A.L., Randle, P.J., Cooper, R.H., Whitehouse, S., Pask, H.T. and Denton, R.M. (1976) Biochem. J. 154, 327–348.
[4] Caterson, I.D., Fuller, S.J. and Randle, P.J. (1982) Biochem. J. 208, 53–60.
[5] Holness, M.J., Liu, Y.-L. and Sugden, M.C. (1989) Biochem. J. 264, 771–776.
[6] Kerbey, A.L. and Randle, P.J. (1982) Biochem. J. 206, 103–111.
[7] Marchington, D.R., Kerbey, A.L. and Randle, P.J. (1990) Biochem. J. 267, 245–247.
[8] Stansbie, D., Denton, R.M., Bridges, B.J., Pask, H.T. and Randle, P.J. (1976) Biochem. J. 154, 225–236.
[9] Vary, T.C. and Randle, P.J. (1984) J. Mol. Cell. Cardiol. 16, 723–733.
[10] Stace, P.B., Marchington, D.R., Kerbey, A.L. and Randle, P.J. (1990) FEBS Lett. 273, 91–94.
[11] Park, O.J., Cesar, D., Faix, D., Wu, K., Shackleton, C.H.L. and Hellerstein, M.K. (1992) Biochem. J. 282, 753–757.
[12] Fuller, S.J., Gaitanaki, C.J. and Sugden, P.H. (1990) Biochem. J. 266, 727–736.
[13] Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66.
[14] Denyer, G.S., Kerbey, A.L. and Randle, P.J. (1986) Biochem. J. 239, 347–354.