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Pyruvate inhibition of pyruvate dehydrogenase kinase

Effects of progressive starvation and hyperthyroidism in vivo, and of dibutyryl cyclic AMP and fatty acids in cultured cardiac myocytes

David A. Priestman, Karen A. Orfali, Mary C. Sugden*

Department of Biochemistry, Medical Sciences, St Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, Mile End Road, London E1 4NS, UK

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Abstract Both prolonged starvation and hyperthyroidism evoke stable increases in cardiac pyruvate dehydrogenase kinase (PDHK) activity. Pyruvate inhibits PDHK in rat heart mitochondria with activation of PDHC. The sensitivity of PDHK to inhibition by pyruvate declines after prolonged starvation. In the present study, pyruvate concentrations giving 50% active complex (PDHa) in mitochondria from fed, control and fed, hyperthyroid rats were 0.3 and 0.8 mM, respectively, compared with 1.0 and 2.8 mM, respectively in mitochondria from 24-h-starved and 48-h-starved rats. The results demonstrate that altered pyruvate sensitivity is not of necessity linked with altered PDHK activity. PDHK activities in mitochondria prepared from cardiac myocytes from fed rats were increased after culture for 24 h with dibutyryl cyclic AMP (50 μM) plus *n*-octanoate (1 mM), with a concomitant decline in sensitivity of PDHK to pyruvate inhibition, suggesting that changes in sensitivity of PDHK to pyruvate inhibition in vivo may be secondary to increased fatty acid supply and cyclic AMP concentrations.

Key words: Pyruvate dehydrogenase kinase; Pyruvate inhibition; Cardiac myocyte; Fatty acid; Cyclic AMP; Rat heart

1. Introduction

The rate of glucose oxidation is controlled by flux through the mitochondrial multienzyme pyruvate dehydrogenase complex (PDHC). In starvation, the activity of mammalian PDHC is suppressed in order to conserve glucose which is essential to the brain and other specialised cells. This critical management role in cellular fuel utilisation is primarily achieved through regulatory control of a dephosphorylation/phosphorylation cycle ([1]; reviews [2,3]). Phosphorylation (inactivation) of PDHC is catalysed by pyruvate dehydrogenase kinase (PDHK).

NADH and acetyl-CoA are produced as key intermediates in the mitochondrial oxidation of all fuels, as well as being direct products of the PDC reaction. Complementing direct feed-back regulation, increasing ratios of [acetyl-CoA]/[CoA] and of [NADH]/[NAD^+] activate PDH kinase [4]. PDHK activity can be rapidly suppressed by pyruvate [5,6]. Accordingly, physiological conditions that promote glycolytic activity, such as overconsumption of carbohydrate, would be expected to increase pyruvate levels and thereby to activate PDHC. Inhibition of PDHK results from pyruvate binding to the PDHK-ADP intermediate, pyruvate and ADP acting synergistically [7]. The presence of the dead-end, catalytically inactive PDHK-ADP-pyruvate complex decreases the proportion of active PDHK, with the result that the apparent V_m of PDHK is reduced.

In mitochondria oxidising 2-oxoglutarate+malate, the effect of added pyruvate to increase the proportion of PDHa is greatly reduced when the mitochondria are prepared from 48-h-starved or diabetic animals [4,8–10]. In addition, the percentage of PDHa in rat heart mitochondria oxidising 2-oxoglutarate+malate is much lower in mitochondria from starved rats, than with those from fed rats [4]. The differences persist in the presence of PDHK inhibitors (pyruvate or dichloroacetate) at concentrations sufficient to effect almost complete conversion of PDHb to PDHa from normal rats, and are not explicable in terms of altered mitochondrial concentrations of known effectors of PDHK [4]. This latter effect of prolonged starvation may be attributed to long-term stable regulation of PDHK through increases in its specific activity (reviewed in [11]). Culture of cardiac myocytes prepared from fed rats for 24 h with a fatty acid (*n*-octanoate) or with dibutyryl cyclic AMP (Bt_2cAMP) increases PDHK to approximately the same extent as 24 h of starvation in vivo [12–14]. Culture of cardiac myocytes from starved rats in the absence of fatty acid or cAMP analogue largely reverses the effect of starvation [14]; reversal is prevented by *n*-octanoate plus Bt_2cAMP [12]. It was therefore proposed that the effect of starvation to increase cardiac PDHK might be mediated by increases in cAMP and fatty acids [11]. To address possible mechanisms through which long-term changes in pyruvate sensitivity might be achieved, we examined whether the addition of *n*-octanoate in combination with Bt_2cAMP to cultured cardiac myocytes leads (in conjunction with enhanced PDHK activity) to an altered sensitivity of PDHK to acute inhibition by pyruvate.

In addition to prolonged starvation, the experimental induction of hyperthyroidism through the administration of tri-iodothyronine (T3) for 3 days leads to a stable increase in cardiac PDHK activity [13]. This effect of hyperthyroidism is associated with increased lipolysis and changes in cardiac ratios of free and acylated carnitine indicative of increased fatty acid oxidation [15]. The effects of starvation and hyperthyroidism to increase cardiac PDHK are, however, additive [13]. Furthermore, the effect of hyperthyroidism in vivo can be reproduced by the addition of T3 to cardiac myocytes in culture, indicating a direct effect of T3 on the heart [13]. These observations suggest that different mechanisms may be involved [13]. We therefore examined whether the stable enhancement of cardiac PDHK activity observed in vivo after

*Corresponding author.

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T3 administration (3 days) in the fed state was, like that of prolonged starvation, associated with a reduced sensitivity of PDHK activity to acute suppression by pyruvate.

2. Materials and methods

Female 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 at the end of the dark phase. Rats were permitted free access to standard rodent diet. Additional groups of rats, maintained on standard diet, were either starved for 24 h or 48 h immediately before use, or made hyperthyroid by the daily subcutaneous injection of T3 (100 mg/100 g body wt per day for 3 days, sampling on day 3) [13,15]. Daily food intake was not significantly affected by the experimental induction of hyperthyroidism.

Heart mitochondria were prepared according to [4]. Assays for active PDHC and citrate synthase in freeze-clamped tissue extracts and in isolated mitochondria were as described in [16]. Total PDHC was assayed as active complex in incubations for 10 min in the absence of respiratory substrate. Total PDHC activity (measured in heart mitochondria) was not affected by starvation or by T3 administration (control, 86.1 ± 5.1 mU/U of citrate synthase; starved, 93.5 ± 9.4 mU/U of citrate synthase; T3-treated, 98.7 ± 2.2 mU/U of citrate synthase). Citrate synthase activities were also unaffected (results not shown). PDHK activities were measured in extracts of mitochondria as described in [10] and were computed as the apparent first-order rate constant of ATP-dependent PDHa inactivation. To test the effects of pyruvate, incubations were performed in KCl media in the presence of 5 mM 2-oxoglutarate/0.5 mM L-malate, together with the concentrations of pyruvate indicated. Incubations were terminated by centrifugation after 5 min, and assayed for active PDHC complex (PDHa) [4,6].

Calcium-tolerant ventricular cardiac myocytes were isolated by collagenase digestion of adult rat hearts and cultured as described in [12]. Individual culture flasks were plated at a density of 10^5 cells/ml (27 ml of cell suspension per flask) with the additions specified. Cardiac myocytes from 2–4 flasks were pooled for the preparation of each batch of mitochondria. Cardiac myocyte suspensions were centrifuged at 145 × *g* for 2 min and the cell pellets were each suspended in 5 ml sucrose medium. Mitochondria were prepared from the cells using a Polytron tissue homogeniser (PT 10 probe, position 3, 5–10s) followed by centrifugation at 900 × *g* for 10 min. The pellets were resuspended and centrifuged. The combined supernatants were centrifuged at 18 000 × *g* for 10 min to obtain mitochondrial pellets.

Statistical significance of differences between groups was assessed by Student's unpaired *t*-test. Results are means ± S.E.M. for the numbers of rats (mitochondrial preparations) indicated.

3. Results

3.1. Active and inactive forms of PDH in heart mitochondria incubated with respiratory substrate

Prolonged (24 or 48 h) starvation [10,12] or T3 treatment (3 days) [13] both increase cardiac PDHK activity, as measured in mitochondrial extracts. In the present study, T3 treatment elicited a 2.2 ± 0.2 ($n = 11$) fold increase in PDHK activity, whereas 48 h starvation was associated with a 2.0 ± 0.2 ($n = 10$) fold increase in PDHK activity. The mean concentration of active PDH complex in freshly prepared mitochondria from control fed euthyroid rats incubated for 5 min at 30°C in the presence of respiratory substrate (2-oxoglutarate/L-malate) in the absence of pyruvate was 26 ± 4% (4) of total complex. Starvation for 24 or 48 h both decreased the proportion of

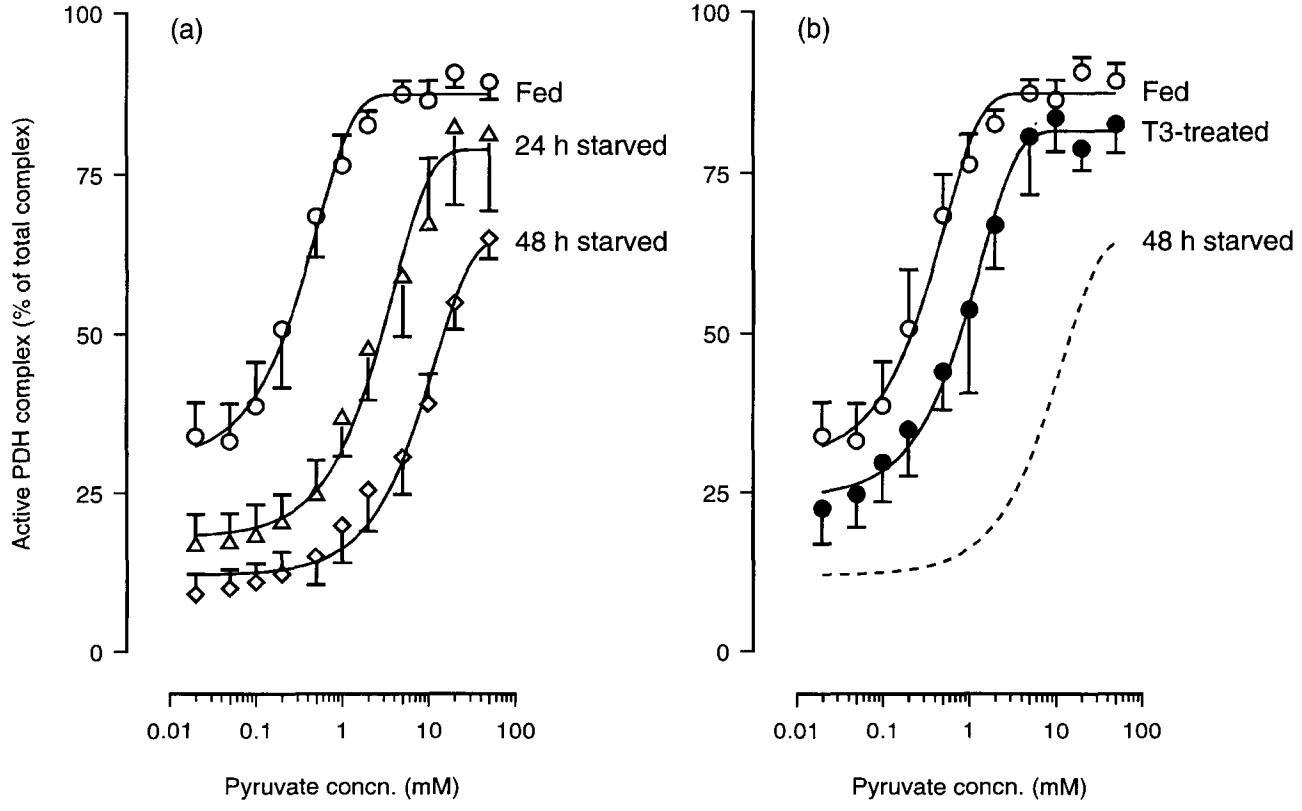

Fig. 1. Effect of pyruvate concentrations on steady-state PDH-complex activity in cardiac mitochondria Mitochondria were prepared from fed, 24-h-starved, 48-h-starved rats, or fed, hyperthyroid rats as indicated. Mitochondria (0.5–1 mg of protein) were incubated for 5 min at 30°C in 0.5 ml of KCl medium containing 5 mM 2-oxoglutarate/0.5 mM L-malate with the concentrations of pyruvate shown. Each point is the mean of eight observations (fed), 10 observations (24 h starved), 10 observations (48 h starved) or six observations (T3-treated, fed) from four, five, five and three mitochondrial preparations respectively.

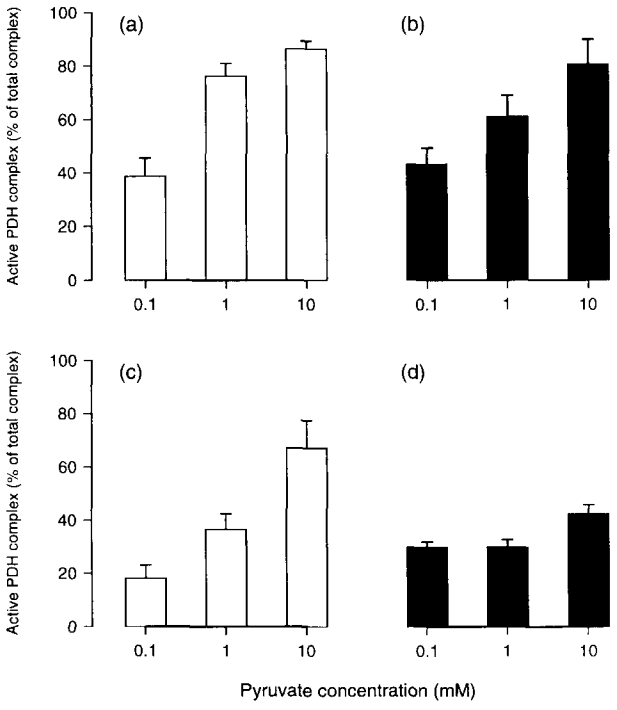

Fig. 2. Effects of pyruvate concentrations on steady-state PDH-complex activity in cardiac mitochondria prepared from hearts of fed (a) or 24-h-starved (c) rats and in mitochondria prepared from cardiac myocytes prepared from fed rats after culture in the absence (b) or presence (d) of Bt_2cAMP (50 μM) and *n*-octanoate (1 mM). Experimental details are given in Section 2 and the legend to Fig. 1. Bars show mean ± S.E.M. of eight observations (fed, panel a) and 10 observations (24 h starved, panel c) from four and five mitochondrial preparations from whole hearts, and 10 observations (no additions to culture, panel b) and six observations (culture plus Bt_2cAMP and *n*-octanoate, panel d) from five and three cardiomyocyte cultures (each utilizing cells prepared from two rats). Statistically significant differences between mitochondria from fed and 24-h-starved rats were observed at 0.1 and 1 mM sodium pyruvate ($P < 0.05$). Statistically significant differences between mitochondria prepared from cardiac myocytes cultured for 24 h in the absence or presence of Bt_2cAMP and *n*-octanoate were observed at 1 and 10 mM sodium pyruvate ($P < 0.01$).

active complex in mitochondria incubated for 5 min with 2-oxoglutarate/L-malate [values (% of total activity) were 15 ± 5% (5) for 24-h-starved rats and 9 ± 3% (5) for 48-h-starved rats (means ± S.E.M. for the number of mitochondrial preparations in parentheses, two observations per preparation, $P < 0.05$ for 48-h-starved versus control). The concentration of active complex in freshly prepared mitochondria from T3-treated fed rats incubated for 5 min at 30°C with 2-oxoglutarate/L-malate in the absence of pyruvate was 27 ± 4% of total complex ($P > 0.5$ for T3-treated versus control).

3.2. *Active and inactive forms of PDH in heart mitochondria incubated with respiratory substrate and pyruvate*

The effects of sodium pyruvate (20 μM–50 mM) on the concentrations of active PDH complex are shown in Fig. 1. As observed in previous studies, sodium pyruvate inhibited PDHK in rat heart mitochondria with activation of PDHC. The effect of prolonged (24 or 48 h) starvation to decrease the sensitivity of PDHC to activation by pyruvate is clearly demonstrated in Fig. 1a. In mitochondria from 48-h-starved rats, the concentration of PDHa was lower than in the fed control at all concentrations of pyruvate used and therefore the maximum activation attainable by inclusion of 50 mM pyruvate was diminished by 48 h starvation. Although starvation for 24 h was similarly associated with reduced suppression of PDHK by pyruvate addition, the maximum activation attainable by inclusion of 50 mM pyruvate was not statistically affected by this period of starvation. Sodium pyruvate concentrations giving 50% active complex in mitochondria from fed control, 24-h-starved or 48-h-starved rats were 0.3, 1.0 and 2.8 mM, respectively. The sodium pyruvate concentrations giving 50% active complex in mitochondria from T3-treated fed rats was 0.8 mM (Fig. 1b). This value does not differ significantly from that of control fed rats. The maximum activation attainable by inclusion of pyruvate was also unaffected by T3 treatment in vivo (Fig. 1b).

3.3. *PDHK activities in freshly prepared and cultured cardiac myocytes from fed control rats*

It was not possible to construct detailed dose-response curves for the effects of pyruvate for mitochondria from cardiac myocytes because of the small yield of mitochondria; however, preliminary experiments indicated the response of PDHa to pyruvate addition in mitochondria prepared from cardiac myocytes resembled that found in mitochondria prepared from whole hearts but a brief period of culture (2 h) after myocyte preparation was required in order to obtain coupled mitochondria (results not shown). It was possible to obtain percentages of PDHa at 0.1, 1.0 and 10 mM sodium pyruvate routinely by preparing and pooling mitochondria from two separate myocyte preparations after culture. Inspection of the percentages of PDHa at these selected pyruvate concentrations permits discrimination between mitochondrial preparations obtained from fed and starved rats (Fig. 2a,c). Thus, the percentages of PDHa at 0.1, 1 and 10 mM sodium pyruvate in mitochondria incubated with 2-oxoglutarate/L-malate were 39 ± 7% (4), 76 ± 5% (4) and 86 ± 3% (4) with mitochondria from fed rats (Fig. 2a), compared with 18 ± 5% (5), 37 ± 6% (5) and 67 ± 10% (5) with mitochondria from 24-h-starved rats (Fig. 2c) ($P < 0.05$ for differences between mitochondria from fed and 24-h-starved rats at 0.1 and 1 mM sodium pyruvate) and 11 ± 3% (5), 20 ± 6% (5) and 39 ± 5% (5) with mitochondria from 48-h-starved rats (see Fig. 1a, $P < 0.01$ for differences between mitochondria from 48-h-starved rats at all three sodium pyruvate concentrations).

Mitochondria were prepared from cardiac myocytes from fed rats after culture for 24 h in medium 199 supplemented with 4% foetal calf serum, antibiotics and antimycotics. In the absence of further additions the percentages of PDHa at 0.1, 1 and 10 mM sodium pyruvate in mitochondria incubated with 2-oxoglutarate/L-malate were 43 ± 6, 61 ± 8 and 80 ± 9%, respectively (mean ± S.E.M. for 5 myocyte preparations) (Fig. 2b). The addition of Bt_2cAMP (50 mM) plus *n*-octanoate (1 mM) during the 24 h period of culture led to a stable 1.5-fold increase in PDHK activity, together with a decline in the sensitivity of PDHK to pyruvate inhibition, such that the percentages of active complex at 0.1, 1 and 10 mM pyruvate were 30 ± 2, 30 ± 3 and 43 ± 4%, respectively (Fig. 2d). Statistically significant differences between mitochondria prepared from cardiac myocytes cultured for 24 h in the absence or presence of Bt_2cAMP and *n*-octanoate were observed at 1 and 10 mM sodium pyruvate ($P < 0.01$). Thus, the effect of 24 h starvation both to increase cardiac PDHK activity and to

decrease the sensitivity of PDHK to pyruvate inhibition could be reproduced in cultured cardiac myocytes by the addition of Bt_2cAMP plus *n*-octanoate.

4. Discussion

When rat heart mitochondria are incubated in the absence of respiratory substrates, the ATP concentration is low and $\gg$90–100% of extracted PDHC is in the active form [4,6]. The addition of respiratory substrates (5 mM 2-oxoglutarate/L-malate) increases the ATP concentration, and this allows substantial phosphorylation of the dehydrogenase component of PDHC, with decrease in the percentage of the active form [4,6]. The rise in ATP is smaller in mitochondria from hearts of diabetic rats than in the non-diabetic controls, whereas the PDHa activity falls to a lower value [4,6]. Pyruvate prevents this inactivation, and can reverse it when it has been induced [4,6]. In the present study, the ability of pyruvate to effect the conversion of PDHC into its active form was markedly reduced in mitochondria from hearts of 24-h- or 48-h-starved rats. This difference between mitochondria from fed and 48-h-starved rats was evident at all concentrations of pyruvate (to 50 mM). Thus, the characteristics of PDHK within mitochondria are modified in a stable manner by starvation such that the effect of pyruvate in increasing PDHa activity is severely impaired. The mechanism underlying this effect of prolonged starvation has not been elucidated.

Studies to date have identified two isoenzymic forms of PDHK (PDHK1 and PDHK2) in rat heart sharing up to 70% amino acid identity [17,18]. The physiological significance for the existence of multiple isoenzymes of PDHK in the heart has not been established but studies with recombinant isoenzymes of PDHK have indicated that they may differ in terms both of their basal activities and in their regulation by NADH/NAD^+ and acetyl-CoA/CoA [18]. It is therefore tempting to speculate that the two isoenzymes may also differ in sensitivity to pyruvate inhibition. Starvation in the rat increases the specific activity of PDHK α-chain without any notable increase in its tissue concentration (as assessed by purification to homogeneity and by ELISA of α-chain in mitochondrial extracts) [19,20]. Nevertheless, it remains possible that the relative proportions of the two isoforms changes during prolonged starvation. If so, it would be hypothesised that the isoform preferentially expressed during starvation would have increased V_m and decreased pyruvate sensitivity.

In hepatocytes in culture, Bt_2cAMP and palmitate increases the activity of PDHK 2-fold without any increase in the concentration of PDHK α-chain [20]. This has led to the conclusion that the effect of cyclic AMP and palmitate to increase PDHK activity must be achieved through structural modification [11]. The present results with cultured cardiac myocytes demonstrate that changes in the sensitivity of PDHK to pyruvate inhibition can be achieved in conjunction with increased PDHK activity in response to 24 h of culture with Bt_2cAMP and fatty acid. It is therefore strongly suggested that changes in pyruvate sensitivity are also due to modification of the structure of PDHK.

After 24 h of starvation the sensitivity of PDHK to inhibition by pyruvate at low concentrations is decreased compared with that found in the fed state, but the effect of 24 h starvation can be overcome by increasing the pyruvate concentration to 20–50 mM. At 10 mM pyruvate, PDHa activity in cardiac mitochondria from 24-h-starved rats oxidising oxoglutarate and malate is only $\approx$10% less than that observed when the mitochondria are prepared from fed rats. In contrast, when cardiac myocytes are cultured for 24 h with Bt_2cAMP and *n*-octanoate, PDHa activity in mitochondria oxidising oxoglutarate and malate at 10 mM pyruvate is only $\approx$50% of that found when the mitochondria are prepared from myocytes cultured in the absence of additions. Thus, the pattern of response of PDHK activity to pyruvate obtained after 24 h culture with fatty acid and cAMP analogue more closely resembles that observed after 48 h starvation in vivo than that which is observed after 24 h starvation in vivo. The progressive response to starvation is suggestive of a change in enzyme expression, possibly of an enzyme catalysing covalent modification of PDHK, or turnover of a modulator affecting pyruvate sensitivity. Early studies have demonstrated no direct effect of cAMP on PDHK activity when assayed on pig heart PDHC by radioassay [6].

Cardiac PDHK activity in mitochondria from 48-h-starved rats does not differ significantly from that found after T3 treatment [13]. Nevertheless, PDHK in heart mitochondria from hyperthyroid animals retain sensitivity to suppression by pyruvate (present study). The present results therefore unambiguously demonstrate that changes in pyruvate sensitivity are not necessarily linked to changes in the V_m of PDHK. The lack of any obligatory link between enhanced PDHK in the absence of pyruvate and diminished sensitivity of the PDHK reaction to pyruvate inhibition in hyperthyroidism suggests that acute conditions leading to increased generation of pyruvate (such as increased heart work) will be able to oppose the longer-term increase in PDHK activity. However, the results also clearly demonstrate that an elevated pyruvate concentration would not be an adequate metabolic condition to increase the level of active PDHC after prolonged starvation. The induction of pyruvate insensitivity may be viewed within the physiological context of the necessity for Cori cycling for the maintenance of blood glucose concentrations. The demonstration that dichloroacetate induces hypoglycaemia, preceded by a fall in blood lactate concentrations, in starved rats [21] demonstrates that inappropriate PDHC activation through inhibition of PDHK can have profound effects on glucoregulation in vivo through decreasing the supply of gluconeogenic substrates. It is presumed that after prolonged starvation, cardiac energy requirements can be fully met through fatty acid oxidation. In contrast, it appears that in hyperthyroidism any increase in pyruvate concentration will be countered by activation of the PDHC and subsequent removal of the pyruvate by oxidation. Since dietary carbohydrate is provided there is no necessity for glucose conservation. Ratios of free to acylated carnitine and of short-chain to long-chain acylcarnitine differ between starved and hyperthyroid rats [15] and the differences between the groups are consistent with the hypothesis that lipid oxidation alone may be insufficient to sustain the cardiac ATP requirement in hyperthyroidism, particularly as rates of cardiac glucose utilisation (transport and phosphorylation) in the hyperthyroid state are relatively high [15]. In view of the strong suppression of cardiac mitochondrial long-chain fatty acid oxidation by malonyl-CoA at the level of carnitine palmitoyltransferase I [22,23] together with the present results obtained using cardiac myocytes cultured with *n*-octanoate (a medium chain length fatty acid), it is tempting to speculate that changes in pyruvate sensitivity oc-

curring in vivo after starvation result from fatty acylation consequent upon sustained high rates of mitochondrial fatty acid oxidation.

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