Studies of the long-term regulation of hepatic pyruvate dehydrogenase kinase

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The administration of a low-carbohydrate/high-saturated-fat (LC/HF) diet for 28 days or starvation for 48 h both increased pyruvate dehydrogenase kinase (PDHK) activity in extracts of rat hepatic mitochondria, by approx. 2.1-fold and 3.5-fold respectively. ELISAs of extracts of hepatic mitochondria, conducted over a range of pyruvate dehydrogenase (PDH) activities, revealed that mitochondrial immunoreactive PDHKII (the major PDHK isoform in rat liver) was significantly increased by approx. 1.4-fold after 28 days of LC/HF feeding and by approx. 2-fold after 48 h of starvation. The effect of LC/HF feeding to increase hepatic PDHK activity was retained through hepatocyte preparation, but was decreased on 21 h culture with insulin (100 μ-i.u./ml). A sustained (24 h) 2–4-fold elevation in plasma insulin concentration *in vivo* (achieved by insulin infusion via an osmotic pump) suppressed the effect of LC/HF feeding so that hepatic PDHK activities did not differ significantly from those of (insulin-infused) control rats. The increase in hepatic PDHK activity evoked by 28 days of LC/HF feeding was prevented and reversed (within 24 h) by the replacement of 7 % of the dietary lipid with long-chain $\omega-3$ fatty acids. Analysis of hepatic membrane lipid revealed a 1.9-fold increase in the ratio of total polyunsaturated $\omega-3$ fatty acids to total mono-unsaturated fatty acids. The results indicate that the increased hepatic PDHK activities observed in livers of LC/HF-fed or 48 h-starved rats are associated with long-term actions to increase hepatic PDHKII concentrations. The long-term regulation of hepatic PDHK by LC/HF feeding might be achieved through an impaired action of insulin to suppress PDHK activity. In addition, the fatty acid composition of the diet, rather than the fat content, is a key influence.

INTRODUCTION

Pyruvate dehydrogenase kinase (PDHK) phosphorylates the pyruvate dehydrogenase (PDH) component of the PDH complex (PDHC), leading to its inactivation (reviewed in [1–3]). Hepatic PDHK activity is subject to long-term regulation during prolonged starvation [4–7]. The stable enhancement of its activity has been variously attributed to increased specific activity of PDHK [8] or to increased enzyme concentration [9]. The PDHK activity enhancement elicited by starvation can be reproduced over a similar timescale (21–24 h) by the addition of glucagon and a fatty acid (n-octanoate) in cultured hepatocytes [10–12]. As the increase in hepatocyte PDHK activity in culture is comparable with that of starvation *in vivo* [10], it has been suggested that increased fatty acid oxidation rates and cAMP concentrations mediate the response to starvation [3]. The effect of starvation for 48 h is reversed within 4–8 h of refeeding [6], over which period hepatic cAMP concentrations decline [13] and fatty acid oxidation is suppressed [14]. The initial response of PDHK to refeeding after starvation is mimicked by 2 h of euglycaemic hyperinsulinaemia [7], suggesting that it might be related to restoration of plasma insulin concentrations. PDHK activity in hepatocytes from rats starved for 48 h (initially approx. 2-fold higher than control) declines on culture in the presence of insulin [15].

A low-carbohydrate/high-saturated-fat (LC/HF) diet containing approximately half the normal amount of carbohydrate and approx. 4-fold the normal lipid content also leads to a stable enhancement of hepatic PDHK activity [7]. It is not known whether the enhanced hepatic PDHK activity observed in response to LC/HF feeding reflects increased specific activity or concentration of PDHK. The effect of an LC/HF diet to enhance hepatic PDHK activity is achieved only after a relatively prolonged period (between 10 and 28 days) and is absent over the timescale during which starvation-induced increases in PDHK activity can be achieved [16]. Long-term changes in hepatic PDHK enzyme concentration are therefore not excluded. Plasma insulin concentrations are maintained at relatively high values [7,17] and, unlike starvation, LC/HF feeding is not associated with increased steady-state hepatic concentrations of cAMP [7]. Nevertheless, an acute elevation in plasma insulin concentrations in post-absorptive rats reverses the effect of prior LC/HF feeding to increase hepatic PDHK activity [7].

In the present experiments we studied the long-term regulation of hepatic PDHK activity by LC/HF feeding. First, we used ELISAs of PDHKII (p45, the major PDHK isoform in liver [18,19]) to determine whether LC/HF feeding increases the specific activity or concentration of hepatic PDHK. Similar studies were undertaken with rats starved for 48 h. Secondly, we examined whether the effect of LC/HF feeding could be reversed by elevating insulin while maintaining the dietary lipid supply. Lastly, we determined whether the effect of LC/HF feeding was secondary to the increased lipid supply or to the decreased supply of carbohydrate by replacing a small percentage of dietary fatty acids with long-chain $\omega-3$ fatty acids, maintaining a constant carbohydrate-to-lipid ratio. This procedure enhances insulin sensitivity in liver and other cell systems [20–23].

EXPERIMENTAL

Animals and diets

Female Wistar rats (200–250 g) were maintained on standard high-carbohydrate/low-fat diet (47 % fat, 33 % carbohydrate, 20 % protein), starved for 48 h, or provided with one of two

Abbreviations used: LC/HF diet, low-carbohydrate/high-saturated-fat diet; PDH, pyruvate dehydrogenase; PDHa, active (dephosphorylated) form of PDH; PDHC, pyruvate dehydrogenase complex; PDHK, PDH kinase.

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isocaloric LC/HF diets of identical caloric distributions. The first LC/HF diet, containing lard as the major lipid source (90% of total lipid calories), was that shown previously to enhance tissue PDHK activities [7,17]. In the second LC/HF diet, 7% of the saturated and $\omega-6$ fatty acids were replaced with long-chain $\omega-3$ fatty acids from marine oil (Marine TG 18/30; Scotia Pharmaceuticals, Guildford, Surrey, U.K.) [24]. This diet is referred to as $\omega-3$ fatty acid-supplemented LC/HF diet. Daily energy intake was not significantly affected by the type of diet consumed. To assess the effects of long-term exposure to an elevated insulin concentration, groups of rats were fitted with Alzet osmotic pumps. Insulin was infused subcutaneously at a fixed rate (2 i.u./day). Rats were allowed free access to the relevant diet throughout and were sampled in the fed state at the end of 24 h of infusion. Pump implantation and insulin infusion were without effect on food intake over the 24 h period (results not shown).

ELISA

Antibodies against purified recombinant PDHKII (kindly provided by Zeneca Pharmaceuticals) were raised in New Zealand White rabbits. The priming dose was 10 μg of protein in Freund's complete adjuvant given subcutaneously at four dorsal sites. After 6 weeks, boosting injections (10 μg) in Freund's incomplete adjuvant were given at intervals of 4 weeks. Blood was sampled from an ear vein at 10 days after boosts. Serum was screened for antibodies by Western blotting, with preimmune serum as control. The antiserum was specific for PDHKII with negligible cross-reaction with PDHKI. ELISAs were performed with clarified extracts of liver mitochondria as described previously [8].

Hepatocytes and culture

Methods used for preparation and culture of hepatocytes are described in [10,12]. Culture was for 4 h in medium 199 containing 5% (v/v) foetal calf serum, followed by 21 h in medium 199 with a change of medium 199 after 17 h. Additions of n-octanoate, dibutyryl cAMP and insulin were made to the culture medium for the final 21 h of culture with the exception of some studies in which it is specified that insulin was included only for the final 4 h of culture.

Enzyme assays

Active PDH (PDHa) activity was assayed spectrophotometrically by coupling to arylamine acetyltransferase [25]. PDHK activities were determined in mitochondrial extracts by the rate of ATP-dependent inactivation of PDHa as described in [26]. PDHK activity was computed as the apparent first-order rate constants for ATP-dependent PDHa inactivation [26]. PDHK activities were usually assayed at pH 7.5 in extracts of liver or hepatocyte mitochondria [7,12]. In experiments investigating the effects of insulin infusion, PDHK activities were measured at pH 7.0. This results in higher apparent first-order rate constants for ATP-dependent inactivation of PDHC in extracts of liver mitochondria (results not shown). As the assay of PDHK is pH-dependent, paired experiments were undertaken where direct comparisons were made.

Insulin, metabolite and membrane fatty acid analysis

Plasma insulin was assayed with a radioimmunoassay kit from Phadeseph Pharmacia (Uppsala, Sweden). Blood glucose concentrations were determined with a glucose analyser (YSI, Yellow Springs, Ohio, U.S.A.). Hepatic glycogen and cAMP concentrations were assayed in freeze-clamped liver samples [7,24]. Total membrane fractions were prepared from freeze-clamped livers and membrane phospholipid composition was determined as in [24]. Fatty acids were identified by comparing their retention times with those of authentic standards.

Statistical analysis

Experimental data are expressed as means ± S.E.M. Statistical significance of differences between groups was assessed by Student's unpaired t test. Curve-fitting was performed with FIG P software.

RESULTS

Immunoreactive PDHK in liver mitochondria

Starvation (48 h) increased hepatic PDHK activity by 3.5-fold [from 0.090 ± 0.010 ($n = 13$) min^{-1} to 0.315 ± 0.034 min^{-1} ($n = 6$); $P < 0.001$] (see also [5,7,10,15]). Provision of LC/HF diet for 28 days also increased hepatic PDHK activity [by 2.1-fold, to 0.192 ± 0.022 min^{-1} ($n = 6$); $P < 0.001$]. As reported previously [7], total PDHC activities were unchanged by starvation or LC/HF feeding. Results for ELISAs of PDHKII in clarified extracts of mitochondria from livers of control, 48 h-starved and

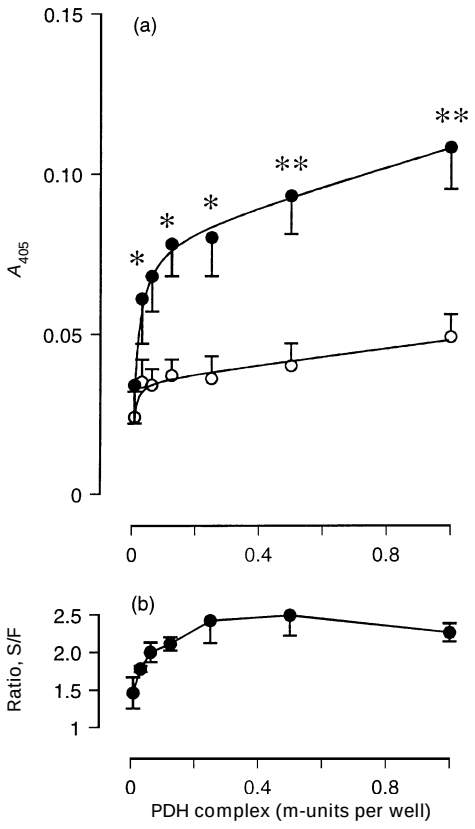

Figure 1 ELISAs of PDHKII in extracts of rat liver mitochondria from fed and starved rats

Liver mitochondria were extracted by freezing and thawing (three times) in mitochondrial extraction buffer. The extracts were clarified by centrifugation and ELISAs were performed as described previously [8]. (**a**) Results from four mitochondrial preparations from control (fed) rats (○) and rats starved for 48 h (●); means ± S.E.M. for four ELISAs (each four wells per assay). (**b**) Ratios of starved to fed (S/F; means ± S.E.M.) for individual points in the ELISA, providing an estimate of the fractional increase induced by starvation. Statistically significant effects of starvation, $^{*}P < 0.05$; $^{**}P < 0.01$.

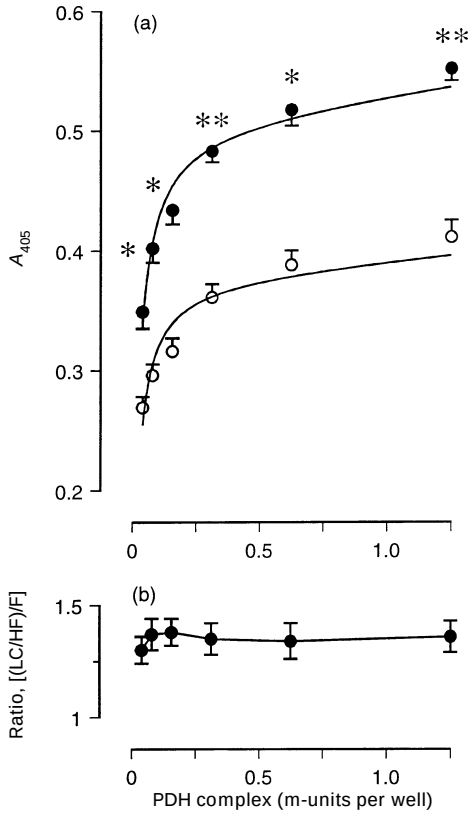

Figure 2 ELISAs of PDHKII in extracts of rat liver mitochondria from control and LC/HF-fed rats

Liver mitochondria were extracted by freezing and thawing (three times) in mitochondrial extraction buffer. The extracts were clarified by centrifugation and ELISAs were performed as described previously [8]. (**a**) Results from four mitochondrial preparations from control rats (○) and LC/HF-fed rats (●); means ± S.E.M. for four ELISAs (each four wells per assay). (**b**) Ratios of LC/HF-fed to control fed rats [(LC/HF)/F; means ± S.E.M.] for individual points in the ELISAs. Statistically significant effects of LC/HF feeding, $^{*}P < 0.05$; $^{**}P < 0.01$.

LC/HF-fed rats are shown in Figures 1 and 2 respectively. In these experiments we compared the different groups (fed compared with starved; LC/HF compared with control) directly by side-by-side comparison, in the same well block and over the same range of PDHC activities. The amount of immunoreactive PDHKII was reproducibly and significantly increased after 48 h of starvation over the range from 0.0625 to 1 m-unit of PDHC per well (Figure 1a) and was reproducibly and significantly increased by the provision of the LC/HF diet for 28 days over the range 0.078–1.25 m-unit of PDHC per well (Figure 2a). Mean ratios of activities of extracts from starved and fed, and from LC/HF and fed, animals at individual points in the ELISAs (Figures 1b and 2b respectively) were 1.98 ± 0.04 ($n = 4$) and 1.36 ± 0.01 ($n = 4$).

Mitochondrial PDHK activity after hepatocyte culture in the presence or absence of insulin

The effect of 28 days of LC/HF feeding to increase liver PDHK activity was stable to hepatocyte and mitochondria preparation (Table 1). PDHK activity was not significantly changed by culture in medium 199 for 25 h when hepatocytes were prepared from control (fed) rats (Table 1), but declined (by approx. 30 %) if the hepatocytes were prepared from rats starved for 48 h [PDHK activities of 0.484 ± 0.052 ($n = 6$) min^{-1} and 0.336 ± 0.029 min^{-1} ($n = 6$) respectively before and after culture in the latter group; $P < 0.05$]. Culture of hepatocytes prepared from LC/HF-fed rats resulted in an approx. 10 % decline in activity after 25 h of culture (Table 1). Consequently, PDHK activities in mitochondria from hepatocytes from LC/HF-fed rats after culture remained significantly higher than those observed for hepatocytes from rats maintained on standard diet after culture (Table 1).

Studies with hepatocyte cultures have shown that the effects of prolonged starvation to increase hepatic PDHK activity can be reversed by adding insulin to the culture medium [15]. In the present experiments the addition of insulin (100 μ-i.u./ml) during the second (21 h) culture period did not significantly influence PDHK activities in hepatocytes from fed control rats, but evoked a significant 25 % decline in PDHK activity, over and above that evoked by culture alone, with hepatocytes from LC/HF-fed rats (Table 1). PDHK activities in hepatocytes from LC/HF-fed rats cultured with 100 μ-i.u./ml insulin did not differ significantly from those of control hepatocytes cultured with or without insulin. A modest effect of insulin *in vitro* to reverse the effect of LC/HF feeding *in vivo* was detectable as a 14 % (not significant) decline in PDHK activity within 4 h (Table 1).

Response of hepatic PDHK activity to insulin infusion *in vivo*

Acute (2.5 h) exposure to elevated plasma insulin concentrations reverses the effect of prior LC/HF feeding to increase hepatic PDHK activity [7]. We examined whether reversal was also possible when the dietary lipid supply was maintained. Rats maintained on the LC/HF diet for 28 days were infused with insulin for a further 24 h. Rats continued to have unrestricted

Table 1 Effects of culture and insulin in culture on hepatocyte PDHK activity in cells from control and LC/HF-fed rats

Full experimental details are given in the Experimental section. The numbers of hepatocyte preparations from individual rats are given in parentheses. Statistically significant effects of the administration of LC/HF diet are indicated by †† $P < 0.01$. Statistically significant effects of the addition of insulin (100 μ-i.u./ml) to the culture medium are indicated by ** $P < 0.01$. Abbreviation: n.d., not determined.

	PDHK activity (min^{-1})			
		Cultured hepatocytes		
Dietary group	Freshly prepared hepatocytes	No addition	Insulin (17–21 h)	Insulin (0–21 h)
Standard diet (control)	0.138 ± 0.011 (11)	0.124 ± 0.011 (11)	n.d.	0.113 ± 0.009 (15)
LC/HF diet	0.188 ± 0.010†† (10)	0.169 ± 0.011†† (10)	0.144 ± 0.011 (9)	0.125 ± 0.009** (6)

Table 2 Effects of $\omega-3$ fatty acid supplementation on the metabolic response to LC/HF feeding

Full experimental details are given in the Experimental section. The LC/HF diets were both provided for 28 days. Numbers of rats are given in parentheses. Statistically significant effects of $\omega-3$ fatty acid supplementation are indicated by * $P < 0.05$.

Dietary group	PDHK activity (min^{-1})	Hepatic glycogen (mg/g)	Plasma glucose (mM)	Plasma insulin (μ-i.u./ml)
LC/HF	0.192 ± 0.022 (6)	79.3 ± 6.5 (9)	9.05 ± 0.33 (7)	27 ± 5 (16)
$\omega-3$ fatty acid-supplemented LC/HF	0.113 ± 0.012* (6)	58.2 ± 3.4* (9)	8.26 ± 0.90 (8)	21 ± 4 (16)

access to the LC/HF diet over this period. At a fixed insulin infusion rate, plasma insulin concentrations at the end of the infusions were 62 ± 8 μ-i.u./ml ($n = 7$) and 121 ± 4 μ-i.u./ml ($n = 4$) in control and LC/HF-fed rats respectively ($P < 0.001$). In this series of studies, PDHK activities were measured at pH 7.0, rather than at pH 7.5, yielding higher apparent rate constants (see the Experimental section). Hepatic PDHK activities in LC/HF-fed rats were significantly suppressed by insulin infusion [from 1.188 ± 0.157 min^{-1} ($n = 9$) to 0.501 ± 0.063 min^{-1} ($n = 11$); $P < 0.001$]. Insulin infusion for 24 h did not significantly influence hepatic PDHK activities in rats maintained on standard diet [control, 0.589 ± 0.088 min^{-1} ($n = 8$); insulin-infused, 0.436 ± 0.062 min^{-1} ($n = 11$)].

Hepatic PDHK activities, metabolic parameters and membrane lipid profiles in LC/HF-fed rats after dietary supplementation with long-chain $\omega-3$ fatty acids

In the rat, provision of long-chain $\omega-3$ fatty acids in a high-saturated-fat diet prevents the development of insulin resistance [27]. The reversal of the effect of LC/HF feeding by insulin infusion suggested that the enhanced hepatic PDHK activity observed in LC/HF-fed rats might result from an impaired action of insulin. The replacement of 7% of total dietary fatty acids by long-chain $\omega-3$ fatty acids from marine oil abolished the increase in hepatic PDHK activity evoked by 28 days of LC/HF feeding, but did not improve the hepatic carbohydrate status (as assessed by glycogen storage) or affect plasma glucose or insulin concentrations (Table 2). A significant effect of long-chain $\omega-3$ fatty acid supplementation was achieved within 24 h, after which time hepatic PDHK activities had declined to 0.117 ± 0.017 min^{-1} ($n = 6$).

Several significant changes in the fatty acid profile of liver membrane lipids occurred in response to supplementation of the LC/HF diet with long-chain $\omega-3$ fatty acids (Table 3). Although the content of saturated fatty acids was not decreased, there was a 1.9-fold increase in the ratio of total polyunsaturated $\omega-3$ fatty acids to total mono-unsaturated fatty acids in hepatic membrane lipid (from 0.89 to 1.70). This was predominantly due to a marked decline in hexadecenoate ($C_{16:1}$, $\omega-7$) to almost negligible levels, together with a 2-fold increase in docosahexaenoate ($C_{22:6}$, $\omega-3$) from 5.6 ± 1.7% to 11.6 ± 1.5% of total fatty acid ($P < 0.05$).

Table 3 Profile of fatty acids in the membrane fraction of livers of rats provided with either LC/HF diet or $\omega-3$-supplemented LC/HF diet for 28 days

Full experimental details are given in the Experimental section. Statistically significant effects of long-chain $\omega-3$ fatty acid supplementation are indicated by * $P < 0.05$. The numbers in parentheses indicate the positions of double bonds.

		Proportion of total fatty acids (%)	
Fatty acids	Dietary group ...	LC/HF	$\omega-3$ fatty acid-supplemented LC/HF
Saturated (total)		40.1 ± 3.7	42.2 ± 5.7
$C_{16:0}$		19.7 ± 1.9	17.9 ± 5.7
$C_{18:0}$		20.4 ± 3.8	24.4 ± 4.9
Mono-unsaturated (total)		16.1 ± 2.7	12.0 ± 1.8
$C_{16:1}$ (9)		3.9 ± 1.3	0.3 ± 0.1*
$C_{18:1}$ (9)		15.1 ± 2.3	11.9 ± 1.9
Polyunsaturated $\omega-6$ (total)		28.9 ± 2.9	24.2 ± 2.1
$C_{18:2}$ (9,12)		13.1 ± 0.5	12.1 ± 1.6
$C_{20:4}$ (5,8,11,14)		15.8 ± 3.4	12.1 ± 1.3
Polyunsaturated $\omega-3$ (total)		14.4 ± 3.3	20.4 ± 4.1
$C_{20:5}$ (5,8,11,14,17)		11.5 ± 2.6	7.2 ± 2.9
$C_{22:5}$ (7,10,13,16,19)		0.7 ± 0.5	2.1 ± 0.4
$C_{22:6}$ (4,7,10,13,16,19)		5.6 ± 1.7	11.6 ± 1.5*

Effects of hepatocyte culture in the presence of n-octanoate and/or dibutyryl cAMP

Irrespective of whether hepatocytes were prepared from control or LC/HF-fed rats, the addition of n-octanoate (1 mM) alone to the culture medium led to only modest increases in PDHK activity (Table 4). In paired experiments, mean fold increases were 1.37 ± 0.09 ($n = 9$) for hepatocytes from control rats and 1.45 ± 0.09 ($n = 10$) for hepatocytes from LC/HF-fed rats. The observed increase in hepatic PDHK activity after the addition of dibutyryl cAMP (50 μM) was 33% less with hepatocytes from LC/HF-fed rats than with hepatocytes from control rats. As a consequence, the mean fold increases in hepatic PDHK activities were 1.32 ± 0.10 ($n = 5$) and 1.64 ± 0.09 ($n = 11$) in hepatocytes prepared from LC/HF-fed and control rats respectively. The observed increase in hepatic PDHK activity after the addition of n-octanoate and dibutyryl cAMP in combination was 36% lower with hepatocytes prepared from LC/HF-fed rats compared with the response with hepatocytes prepared from control rats. Thus the effect of n-octanoate plus dibutyryl cAMP with cells from control rats (1.97 ± 0.28-fold increase; $n = 8$) was significantly ($P < 0.05$) greater than that observed in response to n-octanoate alone, but only slightly (not significantly) greater than that with dibutyryl cAMP alone. In contrast, with cells from LC/HF-fed rats, the effect of n-octanoate plus dibutyryl cAMP (1.56 ± 0.13-fold increase; $n = 5$) was not significantly greater than that of either n-octanoate or dibutyryl cAMP alone. On the basis of these studies, we examined whether hepatic cAMP concentrations were altered by LC/HF feeding. Steady-state cAMP concentrations in freeze-clamped livers of control and LC/HF-fed rats were 425 ± 22 pmol/g ($n = 12$) and 366 ± 14 pmol/g ($n = 8$) respectively ($P < 0.05$). The steady-state cAMP concentration was unaffected by the inclusion of $\omega-3$ fatty acids in the high-fat diet (380 ± 19 pmol/g; $n = 8$).

Table 4 Effects of n-octanoate and dibutyryl cAMP in culture on PDHK activity in hepatocytes prepared from control or LC/HF-fed rats

Additions of n-octanoate (1 mM) and/or dibutyryl cAMP (50 μM) were made to the culture medium for the final 21 h of culture. Full experimental details are given in the Experimental section. Numbers of observations are given in parentheses. Statistically significant effects of additions are indicated by † $P < 0.05$: †† $P < 0.01$; ††† $P < 0.001$. Statistically significant effects of the administration of LC/HF diet are indicated by * $P < 0.05$; *** $P < 0.001$.

	PDHK activity (min^{-1})	
Addition to culture	Control diet	LC/HF diet
None	0.112 ± 0.009 (15)	0.169 ± 0.011*** (10)
n-Octanoate	0.157 ± 0.015† (9)	0.219 ± 0.015†* (8)
Dibutyryl cAMP	0.184 ± 0.015††† (11)	0.217 ± 0.021 (5)
n-Octanoate + dibutyryl cAMP	0.201 ± 0.012††† (8)	0.226 ± 0.009†† (5)

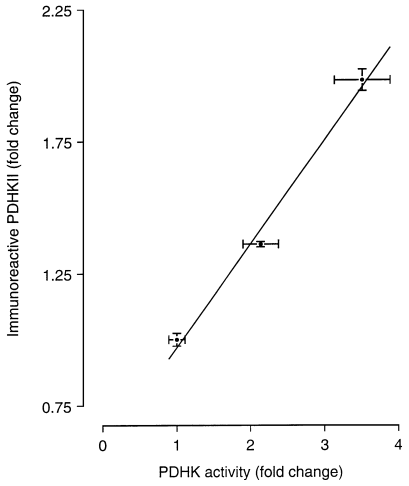

Figure 3 Correlation between mean hepatic PDHK activities and mean PDHKII concentrations

Fold changes in PDHK activity and fold changes in immunoreactive PDHKII observed in the three experimental groups (fed, starved and LC/HF) were measured in internally controlled experiments. The results are presented as fold changes observed in paired experiments for PDHK activity (measured in mitochondrial extracts from control and experimental animals prepared and assayed in parallel) and fold changes in immunoreactive PDHKII observed directly through side-by-side comparisons in the same well block. The relationship revealed a strong positive linear correlation ($r = 0.995$) between mean fold increases in hepatic PDHK activities and mean fold increases in PDHKII concentrations.

DISCUSSION

PDHK comprises one component of the regulatory phosphorylation cycle of mammalian PDHC. PDHK consists of two dissimilar α and β subunits, the α subunit being the catalytic subunit. PDHKα is found tightly bound to PDHC and free in the mitochondrial matrix [3,9,28,29]. The more loosely bound PDHK is identical with the PDHK activator protein KAP [3,9,28,29]. Previous ELISA studies with rabbit anti-(rat PDHKα) serum (raised against PDHKα purified from extracts of liver mitochondria prepared from rats starved for 48 h) demonstrated that PDHKα immunoreactivity was significantly increased by 1.3-fold with mitochondrial extracts from rats starved for 48 h compared with those of fed rats [8]. However, it was concluded that the increase in PDHKα immunoreactivity observed in response to starvation could account for at most 15% of the observed increase in PDHK activity, and thus that starvation increased the specific activity of PDHK in rat liver mitochondria [3,8]. In the present study, we employed ELISAs with rabbit anti-(recombinant PDHKII) serum. We demonstrated that both 48 h of starvation and prolonged (28 days) LC/HF feeding led to significant stable increases in the hepatic concentration of PDHKII, the only PDHK isoform normally present in rat liver [18,19]. The responses were consistently observed over a range of PDHC activities and occurred without any change in total PDHC activities, indicating that PDHKII expression is specifically targeted.

Analysis of the data for the three experimental groups (fed, starved and LC/HF) revealed a strong positive linear correlation ($r = 0.995$) between mean fold changes in hepatic PDHK activities and mean fold changes in hepatic immunoreactive PDHKII (Figure 3). Extrapolating from this relationship, it is estimated that increases in PDHKII concentrations can account for approx. 44% of the increases in PDHK activities, assuming that the specific activity of PDHKII is unchanged. It cannot be concluded from the present study whether isoforms of PDHK exist that exhibit different specific activities, whether PDHK can undergo post-translational modification with a resultant change in its specific activity, whether interactions between the two PDHK subunits influence PDHK activity or whether interactions between PDHK and other components of PDHC modify PDHK activity.

The enhancement of hepatic PDHK activity observed in response to the LC/HF diet was relatively stable to hepatocyte preparation and declined by only approx. 10% after 21 h of culture. LC/HF feeding seems to render hepatic PDHK activity relatively insensitive to further up-regulation by cAMP and fatty acids. In addition, the effect of prior LC/HF feeding was rendered non-significant when insulin was present in the culture medium or insulin was infused *in vivo*. Furthermore the replacement of 7% of the dietary fatty acids in the LC/HF diet by long-chain $\omega-3$ fatty acids, which prevents the development of the insulin resistance evoked by high-fat feeding [27], both suppressed the increase in hepatic PDHK activity evoked by the provision of the unsupplemented LC/HF diet and reversed the effect of 28 days of (unsupplemented) LC/HF feeding to increase hepatic PDHK. As the two LC/HF diets contained identical amounts of lipid and carbohydrate, the response of hepatic PDHK to high-fat feeding does not result from a lack of dietary carbohydrate. The inclusion of long-chain $\omega-3$ fatty acids in the LC/HF diet increased the ratio of polyunsaturated long-chain $\omega-3$ fatty acid to mono-unsaturated fatty acid in hepatic membrane lipid. An increase in this ratio is associated with increased membrane fluidity and enhanced sensitivity to insulin [20,30]. Taken together, the results suggest that enhanced hepatic PDHK activity in response to LC/HF feeding occurs because insulin action is deficient.

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