Investigation of potential mechanisms regulating protein expression of hepatic pyruvate dehydrogenase kinase isoforms 2 and 4 by fatty acids and thyroid hormone

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Liver contains two pyruvate dehydrogenase kinases (PDKs), namely PDK2 and PDK4, which regulate glucose oxidation through inhibitory phosphorylation of the pyruvate dehydrogenase complex (PDC). Starvation increases hepatic PDK2 and PDK4 protein expression, the latter occurring, in part, via a mechanism involving peroxisome proliferator-activated receptor-α (PPARα). High-fat feeding and hyperthyroidism, which increase circulating lipid supply, enhance hepatic PDK2 protein expression, but these increases are insufficient to account for observed increases in hepatic PDK activity. Enhanced expression of PDK4, but not PDK2, occurs in part via a mechanism involving PPAR-α. Heterodimerization partners for retinoid X receptors (RXRs) include PPARα and thyroid-hormone receptors (TRs). We therefore investigated the responses of hepatic PDK protein expression to high-fat feeding and hyperthyroidism in relation to hepatic lipid delivery and disposal. High-fat feeding increased hepatic PDK2, but not PDK4, protein expression whereas hyperthyroidism increased both hepatic PDK2 and PDK4 protein expression. Both manipulations decreased the sensitivity of hepatic carnitine palmitoyltransferase I (CPT I) to suppression by malonyl-CoA, but only hyperthyrodism elevated plasma fatty acid and ketone-body concentrations and CPT I maximal activity. Administration of the selective PPAR-α activator WY14,643 significantly increased PDK4 protein to a similar extent in both control and high-fat-fed rats, but WY14,643 treatment and hyperthyroidism did not have additive effects on hepatic PDK4 protein expression. PPARα activation did not influence hepatic PDK2 protein expression in euthyroid rats, suggesting that up-regulation of PDK2 by hyperthyroidism does not involve PPARα, but attenuated the effect of hyperthyroidism to increase hepatic PDK2 expression. The results indicate that hepatic PDK4 up-regulation can be achieved by heterodimerization of either PPARα or TR with the RXR receptor and that effects of PPARα activation on hepatic PDK2 and PDK4 expression favour a switch towards preferential expression of PDK4.

Key words: carnitine palmitoyltransferase, hyperthyroidism, liver, peroxisome proliferator-activated receptor-α (PPARα).

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) occupies a strategic role in hepatic intermediary metabolism and is a major target for substrate and hormonal regulation (reviewed in [1]). Activation of hepatic PDC under conditions of an abundant glucose supply promotes glucose oxidation, and allows malonyl-CoA formation and fatty acid (FA) synthesis from pyruvate. Conversely, inactivation of PDC limits glucose oxidation and facilitates diversion of available pyruvate towards gluconeogenesis. PDC is rendered inactive by phosphorylation of the α-subunit of its pyruvate dehydrogenase (PDH) component by PDH kinase (PDK) (reviewed in [1,2]). Inhibitory phosphorylation of PDC is catalysed by the PDKs [3,4]. The PDKs are activated by acetyl-CoA and NADH, the products of FA oxidation (reviewed in [5]). However, hepatic carnitine palmitoyltransferase I (CPT I) activity is suppressed by malonyl-CoA in the fed state, and therefore incoming FAs are largely re-esterified rather than oxidized (reviewed in [6]). Thus, while the PDKs constitute the molecular targets through which the capacity for glucose oxidation can be diminished by elevated mitochondrial acetyl-CoA and NADH concentrations, this mode of regulation is restricted to feedback control by the end products of glucose oxidation unless mitochondrial FA oxidation is increased. This can be achieved by virtue of lowered malonyl-CoA, decreased sensitivity of CPT I to inhibition by malonyl-CoA or enhanced CPT I maximal activity.

Increases in hepatic PDK activity that are stable to mitochondrial preparation and incubation [7,8], reproduced in hepatocytes by culture with a FA together with agents elevating cAMP [9–11], and slow in onset [10] are seen after prolonged (48 h) starvation. Such increases are not immediately reversed by refeeding [12], implicating a long-term mechanism. Four PDK isoenzymes (PDK1–PDK4) have been identified in mammalian tissues [3]. Rat liver contains two of these, namely PDK2 and PDK4 [7,13,14]. Incubation of Morris hepatoma 7800 C1 cells with FA (palmitate or oleate) increases PDK4 mRNA levels [15].

Abbreviations used: CPT, carnitine palmitoyltransferase; DR, direct repeat; ECL® (Amersham), enhanced chemiluminescence; FA, fatty acid; NEFA, non-esterified fatty acid; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDHa, active pyruvate dehydrogenase; PDK, PDH kinase; PPARα, peroxisome proliferator-activated receptor-α; RXR, retinoid X receptor; S14, spot-14; T_3, tri-iodothyronine; TAG, triacylglycerol; TBS, Tris-buffered saline; TDG, 2-tetradecylglycidate; TR, thyroid-hormone receptor.

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Increased hepatic PDK activity after prolonged starvation occurs in conjunction with increased protein expression of both PDK2 and PDK4 [7,13,14,16], increased CPT I activity and decreased CPT I sensitivity to malonyl-CoA inhibition [17,18]. Despite concomitant enhancement of protein expression of both hepatic PDK isoforms after starvation, available evidence suggests that the signals that regulate hepatic PDK4 expression are distinct from those that regulate hepatic PDK2 expression. In particular, PDK4, but not PDK2, protein expression may be linked to PPARα-linked FA oxidation. Culture of Morris hepatoma 7800 C1 cells with the peroxisome proliferator-activated receptor-α (PPARα) agonist WY14,643 increases PDK4, but not PDK2, mRNA and protein expression [15]. In addition, dietary supplementation with WY14,643 for 3 days increases hepatic PDK4 mRNA and protein expression *in vivo* [15,16]. Further evidence supporting a role for signalling via PPARα in the control of PDK4 expression in liver in response to prolonged starvation is the observation that enhanced hepatic PDK4 protein expression after starvation is attenuated in PPARα-deficient mice [14]. Interestingly, although rates of hepatic FA oxidation are lower in livers of starved PPAR-α-null mice compared with wild-type mice, this is not due to inappropriate expression of the hepatic CPT I gene, which is similar in both genotypes [19]. Culture of Morris hepatoma 7800 C1 cells with insulin suppresses PDK2 mRNA and protein expression, but is less effective in blocking WY14,643-induced up-regulation of PDK4 expression [15]. Hence a decline in insulin may be important for facilitating increased hepatic PDK2 expression but, as suggested from the ability of PPARα-activation to increase hepatic PDK4 expression even in the fed state, is not obligatory to allow enhanced hepatic PDK4 expression.

PPARs heterodimerize with the retinoid X receptor (RXR) [20]. Each of the partners of the PPAR–RXR heterodimer is believed to occupy one of the hexameric half-sites of the PP response element ('PPRE'). As well as of PPAR, RXR forms the dimerization partner of other nuclear receptors, including the thyroid-hormone receptor (TR) [21]. Hyperthyroidism increases adipose-tissue lipolysis (and therefore hepatic FA delivery from adipose tissue) [22], enhances hepatic CPT I activity and decreases the sensitivity of CPT I to malonyl-CoA-dependent inhibition [23]. Hyperthyroidism also increases hepatic PDK activity in association with increased hepatic protein expression of PDK2 [24]. However, enhanced hepatic PDK2 protein expression in the absence of a change in PDK2 specific activity is insufficient to account for the increase in PDK activity elicited by hyperthyroidism [7]. Similarly, increased provision of lipid as a component of a high-fat diet increases the hepatic protein expression of PDK2 [7], but the effect of an increased supply of dietary fat on hepatic PDK4 expression is not known. Relatively high insulin levels are maintained in both hyperthyroidism [25] and after high-fat feeding [11]. Insulin deficiency would therefore not be predicted to participate in the response of hepatic PDK isoform expression to these manipulations, unless it co-exists with hepatic insulin resistance.

The presence of PDK4 in liver [26] highlights the need to establish the possible regulation of hepatic PDK4 protein expression by thyroid or dietary lipid status, and to determine to what extent changes in PDK2 and/or PDK4 protein expression reflect altered hepatic lipid delivery, accumulation or mitochondrial oxidation via CPT I. In the present study, we investigated the effects of high-fat feeding and experimental hyperthyroidism on hepatic PDK4 protein expression. Our results are examined in relation to effects on hepatic lipid delivery and disposal, hepatic CPT I activity and sensitivity of CPT I to inhibition by malonyl-CoA. In addition, since both TRs and PPARs heterodimerize with RXRs, we investigated whether the potential regulation of PDK4 by thyroid hormones is modulated by PPARα activation.

MATERIALS AND METHODS

Materials

General laboratory reagents were from Roche Diagnostics (Lewes, East Sussex, U.K.) or from Sigma (Poole, Dorset, U.K.), with the following exceptions. Arylamine acetyltransferase was purified from pigeon liver acetone-dried powder purchased from Europa Bioproducts (Ely, Cambridgeshire, U.K.). Enhanced chemiluminescence (ECL®) reagents, hyperfilm and secondary antibodies purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Anti-PDK2 antibodies were generated in the authors' laboratory in rabbits against recombinant PDK2 protein [7]. Anti-PDK4 antibodies, generated in rabbits against recombinant PDK4 protein [3], were generously provided by Professor Bob Harris (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.). Protein A for Western-blot analysis was obtained from ICN Pharmaceuticals (Basingstoke, Hants., U.K.). WY14,643 and tri-iodothyronine (T_3) were purchased from Sigma (Poole, Dorset, U.K.). 2-Tetradecylglycidate (TDG) (McN-3802) was generously provided by McNeil Pharmaceuticals (Spring House, PA, U.S.A.). Bradford reagents for protein estimation were purchased from Bio-Rad Ltd. (Hemel Hempstead, Herts., U.K.). Kits for determination of non-esterified fatty acid (NEFA) and triacylglycerol (TAG) concentrations were purchased from Alpha Laboratories Limited (Eastleigh, Hants., U.K.).

Animals

All studies were conducted in adherence to the regulations of the United Kingdom Animal Scientific Procedures Act (1986). Adult female albino Wistar rats (200–250 g) were purchased from Charles River (Margate, Kent, U.K.). Rats were maintained at a temperature of 22 ± 2 °C and subjected to a 12 h light/12 h dark cycle. Control rats were allowed access *ad libitum* to water and standard high-carbohydrate/low-fat rodent laboratory diet [52 % (w/w) carbohydrate, 15 % (w/w) protein, 3 % (w/w) lipid and 30 % (w/w) non-digestible residue; 10.92 kJ (2.61 kcal) metabolizable energy/g] purchased from Special Diet Services (Witham, Essex, U.K.). High-fat-fed rats were maintained on a semi-synthetic low-carbohydrate high-saturated-fat diet (33 % carbohydrate, 20 % protein and 47 % lipid on an energy basis) (see [27] for details of composition) for 28 days. Both rat groups were sampled in the absorptive state at the end of the dark phase. Calorific intake was approx. 65 % higher in the high-fat-fed group compared with the control group. Similar increases in calorific intake in response to high-fat feeding have been observed in previous studies by other workers [28]. The increase in energy intake was not associated with an increase in body weight during the 4-week period of high-fat feeding, but there were no obvious differences in physical activity between the two dietary groups. In some studies, food was removed from cages at 48 h before killing the rats for determination of the effects of starvation. To inhibit CPT I, TDG was administered intragastrically as a suspension (2.5 mg/100 g body wt.) in 0.5 % (w/v) carboxymethylcellulose at 2 h before sampling [29]. Administration of 0.5 % (w/v) carboxymethylcellulose did not affect the parameters under investigation. In some experiments an artificial elevation of NEFA concentrations was achieved by the administration of 5 ml of corn oil at the time of food removal, followed by heparin

treatment (for details, see [30]). A subgroup of rats maintained on standard diet was rendered hyperthyroid by subcutaneous injection of T_3 (1 mg/day per kg body wt.; 3 days) [22,31]. WY14,643 was administered as a single intraperitoneal injection (50 mg/kg body wt.) at 24 h before sampling [32]. WY14,643-treated euthyroid and hyperthyroid rats were sampled in the fed state or after starvation for 48 h, with WY14,643 treatment for the last 24 h of the 48 h starvation period and/or the last 24 h of T_3 treatment. Daily food intakes did not differ between euthyroid control and euthyroid WY14,643-treated rats (control, 21.9 ± 1.0 g; WY14,643-treated, 20.6 ± 0.7 g) or hyperthyroid and WY14,643-treated hyperthyroid rats (hyperthyroid, 25.6 ± 2.4 g; WY14,643-treated hyperthyroid, 22.6 ± 0.7 g).

Tissue and blood sampling

Rats were anaesthetised by injection of sodium pentobarbital (60 mg/ml in 0.9 % NaCl; 1 ml/kg body wt. intraperitoneally) and, once locomotor activity had ceased, livers were rapidly excised. A portion of liver was freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen and stored in liquid nitrogen until analysis. A further portion was used for preparation of mitochondria. Blood was sampled from the chest cavity. An aliquot was immediately added to ice-cold perchloric acid for metabolite analysis. A further aliquot was centrifuged for 5 min at 12000 ***g*** and plasma was stored at −20 °C.

Enzyme assays

Active pyruvate dehydrogenase (PDHa) activity was assayed both in freeze-clamped tissue extracts and in isolated mitochondria, prepared as described in [7]. PDHa was assayed spectrophotometrically by coupling it to arylamine acetyltransferase [33]. Total PDC activity was assayed after complete activation through the action of endogenous PDC phosphate phosphatase as active PDC in mitochondria incubated for 15 min in the absence of respiratory substrate and in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone [34]. PDK activities were determined in mitochondrial extracts at pH 7.0 by the rate of ATP-dependent inactivation of PDHa and computed as apparent first-order rate constants for ATP-dependent PDHa inactivation [7,35].

CPT analysis was performed using freshly prepared mitochondria by monitoring rates of [^{3}H]palmitoylcarnitine formation from [^{3}H]carnitine and palmitoyl-CoA as described in [36]. The mitochondrial protein concentration was standardized at 1 mg/ml, and carnitine, palmitoyl-CoA and BSA were present throughout at fixed concentrations of 0.5 mM, 135 μM and 10 mg/ml respectively. Where malonyl-CoA-sensitivity was estimated, mitochondria were pre-incubated (5 min, 37 °C) with malonyl-CoA at the concentrations indicated before starting the reaction by the addition of radiolabelled carnitine.

Immunoblotting

Liver samples (approx. 100 mg) were homogenized using a Polytron tissue homogenizer (PT 10 probe; position 5, 15 s) in 1 ml of ice-cold extraction buffer [20 mM Tris/137 mM NaCl/2.7 mM KCl/1 mM CaCl2/10 % (v/v) glycerol/1 % Igepal (≡ Nonidet P40)/45 mM sodium orthovanadate/0.2 mM PMSF/10 μg/ml leupeptin/1.5 mg/ml benzamidine/50 μg/ml aprotinin/50 μg/ml pepstatin A (in DMSO), pH 8.0]. Homogenates were placed on ice for 20 min, centrifuged in an Eppendorf centrifuge (12000 ***g*** for 20 min. at 4 °C) and the supernatants stored (−20 °C) until analysis. Protein concentrations were determined using the Bradford method and with BSA as standard. The assay was linear over the range of protein concentrations routinely used. Samples (25–50 μg of total protein) were subjected to SDS/PAGE using a 12 % resolving gel with a 6 % stacking gel. Following SDS/PAGE, resolved proteins were transferred electrophoretically to nitrocellulose membranes and then blocked for 2 h at room temperature with Tris-buffered saline (TBS; 150 mM NaCl/10 mM Tris/HCl, pH 7.6) supplemented with 0.05 % Tween and 5 % (w/v) non-fat powdered milk. The nitrocellulose blots were incubated overnight at 4 °C with polyclonal antisera raised against specific recombinant PDK isoforms (PDK2 and PDK4), washed with 0.05 % Tween in TBS (3 × 5 min) and incubated with the horseradish peroxidase-linked secondary antibody anti-rabbit IgG [1:2000, in 1 % (w/v) non-fat milk in 0.05 % Tween in TBS] for 2 h at room temperature. Bound antibody was visualized using ECL® according to the manufacturer's (Amersham) instructions. The blots were then exposed to Hyperfilm and the signals quantified by scanning densitometry and analysed with Molecular Analyst software (Bio-Rad). The amounts of protein loaded on to the gel were varied to establish that the relative densities of the bands corresponding to the PDK isoforms were linear with concentration. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range. For each representative immunoblot presented, the results are from a single gel exposed for a uniform duration, and each lane represents a preparation from a different rat.

Metabolite analysis

Freeze-clamped liver was ground to a powder at −40 °C, and assayed for free and acylated carnitines as described in [37]. Free (non-esterified) carnitine was taken to be the carnitine found when the extracts were examined without prior exposure to alkali. $HClO_4$-insoluble carnitine was assumed to be long-chain esters (chain length more than ten carbon atoms) and the $HClO_4$-soluble fraction was assumed to contain short-chain derivatives and free carnitine [38]. Ketone-body (3-hydroxybutyrate + acetoacetate) concentrations were determined in KOH-neutralized $HClO_4$ extracts of whole blood and freeze-clamped livers as described previously [39]. NEFA and TAG concentrations were determined in plasma and liver samples using Wako NEFA-C and TAG kits from Alpha Laboratories Limited.

Statistical analysis

Results are presented as the means ± S.E.M., with the numbers of rats in parentheses. Statistical analysis was performed by ANOVA followed by Fisher's post-hoc tests for individual comparisons or Student's *t* test as appropriate (Statview; Abacus Concepts, Inc., Berkeley, CA, U.S.A.). A P value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Hepatic PDHa activity in relation to acute and long-term increases in lipid delivery and oxidation

We analysed the acute effects of an elevation of plasma NEFA supply on hepatic PDHa activities in control (euthyroid) rats with free access to standard low-fat/high-carbohydrate diet (Figure 1). Plasma NEFA concentrations were significantly elevated by the administration of corn oil + heparin. At 1 h after this treatment, plasma FA concentrations were 5-fold higher than control, and this degree of elevation of plasma FAs was

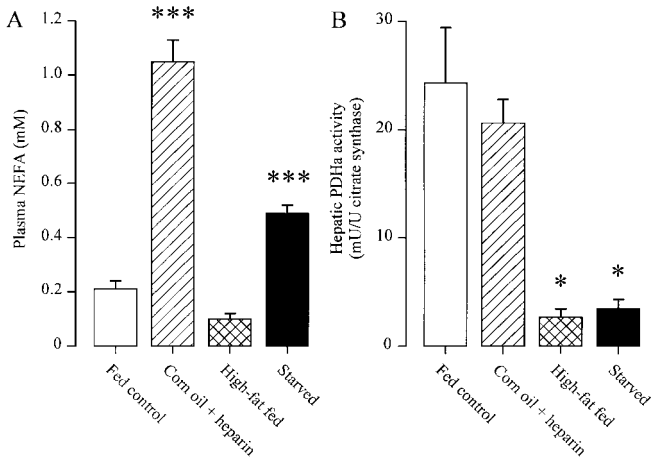

Figure 1 Plasma NEFA concentrations and hepatic PDHa activities in response to corn oil plus heparin, 28-day high-fat feeding or starvation for 48 h

NEFA concentrations were determined in plasma samples using a Wako NEFA-C-test kit. PDHa activities were assayed spectrophotometrically in freeze-clamped liver extracts by coupling to arylamine acetyltransferase. Data are means ± S.E.M. for at least five samples from individual rats in each experimental group. Statistically significant differences from control rats are indicated by: $^{*}P < 0.05$; $^{***}P < 0.001$. Abbreviation: U, unit.

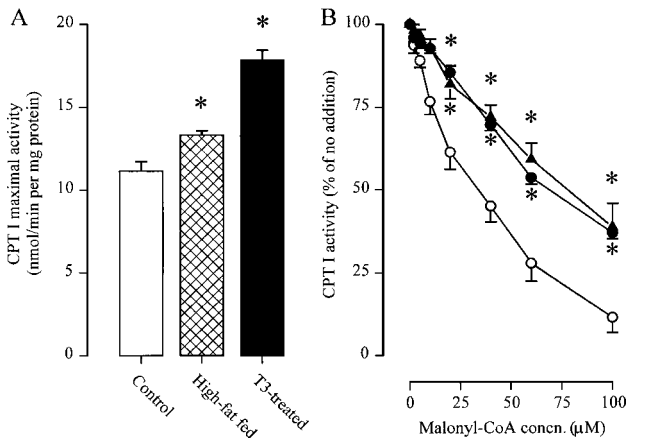

Figure 2 Effects of hyperthyroidism and high-fat feeding on hepatic malonyl-CoA-sensitivity in isolated liver mitochondria

CPT analysis was performed using liver mitochondria by monitoring the rate of [^{3}H]palmitoylcarnitine formation from [^{3}H]carnitine and palmitoyl-CoA as described in [36]. CPT analyses performed in the absence of malonyl-CoA are shown for euthyroid rats, hyperthyroid rats and high-fat-fed rats in (**A**). CPT analyses performed in the presence of malonyl-CoA are shown for euthyroid rats (○), hyperthyroid rats (●) and high-fat-fed rats (▲) are shown in (**B**). Further details are provided in the Materials and methods section. Results are means ± S.E.M. for ten euthyroid rats, seven hyperthyroid rats and five high-fat-fed rats. Statistically significant differences from control are indicated by: $^{*}P < 0.05$.

maintained for a further 1 h (Figure 1A). The acute elevation in FA supply did not lead to any significant change in hepatic PDHa activity (Figure 1B). By contrast, high-fat feeding (4 weeks) did not affect plasma non-esterified FA concentrations, but was associated with a marked suppression of hepatic PDHa activity in the fed state (Figure 1). As expected, starvation (48 h) both increased plasma non-esterified FA concentrations and suppressed hepatic PDHa activity (Figure 1).

Impact of mitochondrial long-chain FA oxidation on hepatic PDHa activity

In cultured hepatocytes, palmitate leads to a stable enhancement of PDK activity which is blocked by inhibition of CPT I [10,11,40]. We inhibited CPT I by the administration of TDG to examine to what extent suppression of PDHa activity in livers of high-fat fed rats might reflect increased mitochondrial long-chain FA oxidation. The effect of high-fat feeding to suppress PDHa activity was rapidly reversed by acute treatment of the high-fat-fed rats with TDG (high fat, 2.7 ± 0.7 munits/unit of citrate synthase; TDG-treated high fat, 13.0 ± 0.7 munits/unit of citrate synthase). TDG treatment of rats maintained on standard high-carbohydrate diet did not significantly affect hepatic PDHa activity (control fed, 20.7 ± 1.8 munits/unit of citrate synthase; TDG-treated control, 24.5 ± 2.0 munits/unit of citrate synthase. TDG treatment of 48 h-starved rats failed to reverse PDHa inactivation (48 h starved, 3.5 ± 0.8 munits/unit of citrate synthase; TDG-treated 48 h starved, 4.1 ± 0.6 munits/unit of citrate synthase). Taken together, these findings suggests that liver PDHa activity is refractory to an acute increase in FA delivery in the carbohydrate-fed state, probably due to a restraint imposed on mitochondrial long-chain FA oxidation by a low CPT I activity. They also indicate that a chronic, rather than an acute, elevation in FA supply is required for hepatic PDC inactivation and that high-fat feeding leads to metabolic changes that facilitate mitochondrial FA oxidation.

Regulatory influence of high-fat feeding on intrahepatic FA handling

Contrasting with the response to prolonged starvation, high-fat feeding was not associated with any change in CPT I activity measured in the absence of malonyl-CoA (Figure 2A), and ketonaemia (sum of 3-hydroxybutyrate + acetoacetate concentrations) was not increased (control, 0.12 ± 0.03 mM; high-fat-fed, 0.19 ± 0.05 mM). Nevertheless, high-fat feeding both decreased the sensitivity of hepatic CPT I to suppression by malonyl-CoA (Figure 2B) and elicited a significant (2.7-fold: $P < 0.001$) increase in hepatic TAG concentrations (control, 14.1 ± 1.7 mg/g wet wt.; high-fat fed, 38.4 ± 2.7 mg/g wet wt.). These data indicate that, during high-fat feeding, hepatic FA delivery exceeds its rate of disposal via oxidation and confirm that, in the absence of long-term increase in CPT I maximal activity, a significant fate of incoming FAs is esterification rather than oxidation. Nevertheless, as observed previously [7], high-fat feeding elicited a stable increase in hepatic PDK activity, measured in isolated liver mitochondria (control, 1.9 ± 0.7 min^{-1}; high fat, 4.7 ± 0.6 min^{-1}; $P < 0.05$).

An increased dietary lipid supply alone fails to enhance hepatic PDK4 protein expression

PDK [10] and CPT I [41] activity both decline in hepatocytes during culture. FAs themselves can act directly, or via PPARα, or by both mechanisms, to modulate gene expression [42–46]. Supplementation of the diet with the PPARα agonist WY14,643 for 3 days selectively increases hepatic PDK4 mRNA and protein expression, and incubation of Morris hepatoma 7800 C1 cells with WY14,643 selectively up-regulates PDK4, but not PDK2, mRNA and protein expression [15]. Hepatic steatosis in the absence of ketonaemia is observed in PPARα-null mice after starvation [47], which show impaired up-regulation of hepatic PDK4 protein expression after starvation [14] and in response to PPARα activation [48]. We have previously demonstrated that

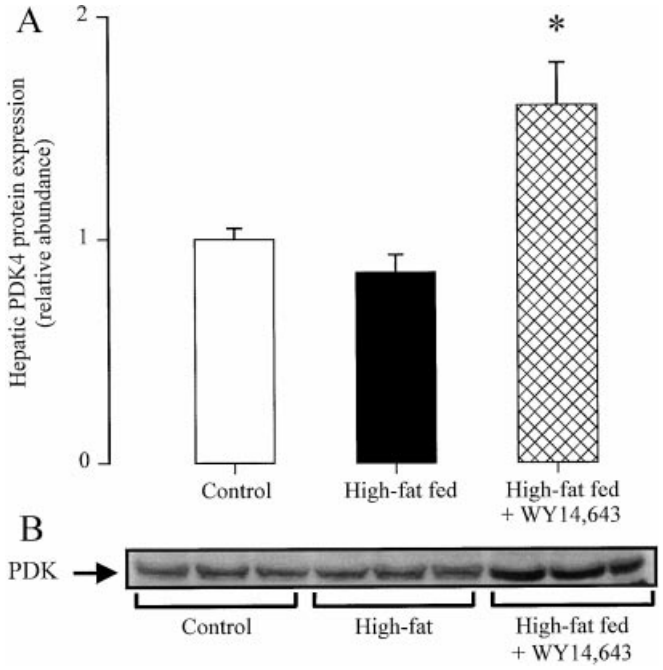

Figure 3 Response of the hepatic PDK4 protein expression to high-fat feeding in the absence or presence of WY14,643 treatment in the fed state

Western-blot analysis of PDK4 protein expression was undertaken using livers of fed rats (white bar), untreated high-fat-fed rats (black bar) and WY14,643-treated high-fat-fed rats (cross-hatched bar). Each lane corresponds to 50 μg of liver protein. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software and expressed as the relative abundance compared with corresponding results in fed rats (**A**). Representative immunoblots are shown in (**B**). Further details are given in the Materials and methods section. Data are means $\pm$ S.E.M. for five control (fed), six untreated high-fat-fed and six WY14,643-treated high-fat-fed liver preparations from individual rats. Statistically significant differences from control rats are indicated by: $^{*}P < 0.05$.

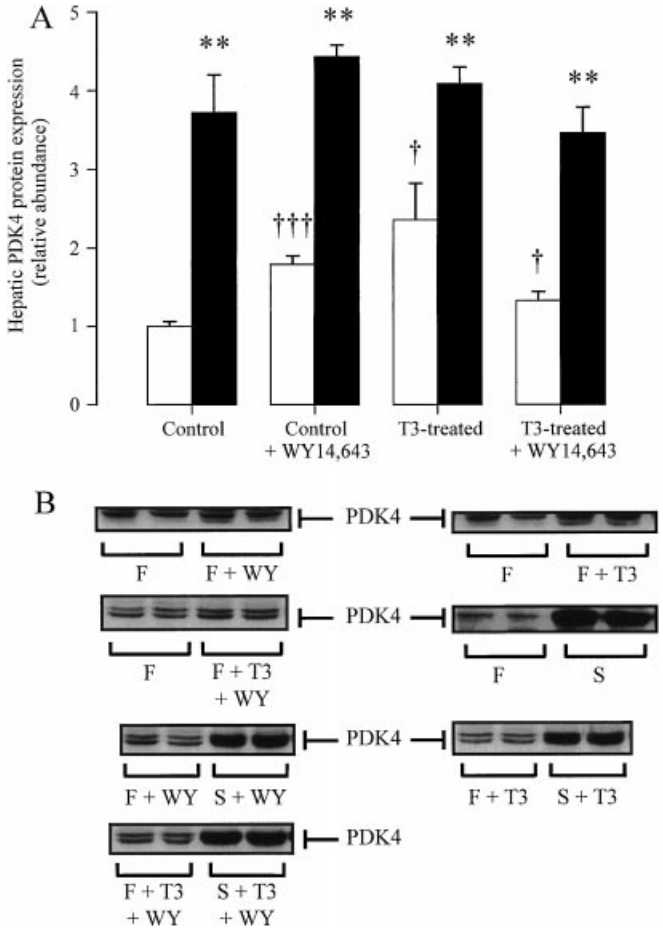

Figure 4 Response of the hepatic PDK4 protein expression to WY14,643 treatment and/or hyperthyroidism in the fed or starved state

Western-blot analysis of PDK4 protein expression was undertaken using livers of fed (white bars) and starved (black bars) rats. Each lane corresponds to 50 μg of liver protein. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software and expressed as the relative abundance compared with corresponding results in fed control rats (**A**). Representative immunoblots are shown in (**B**). Further details are given in the Materials and methods section. Data are means $\pm$ S.E.M. for 20 (fed, F), nine (starved, S), 20 (fed + WY14,643, F + WY), seven (starved + WY14,643, S + WY), six (fed + T_3, F + T3), seven (starved + T_3, S + T3), five (fed + T_3 + WY14,643, F + T3 + WY) and five (starved + T_3 + WY14,643, S + T3 + WY) liver preparations from individual rats in each experimental group. Statistically significant effects of starvation are indicated by: $^{**}P < 0.01$. Statistically significant differences from corresponding control fed or starved rats are indicated by: $\dagger P < 0.05$; $\dagger\dagger\dagger P < 0.001$.

high-fat feeding selectively increases PDK4 protein expression in heart [49] and oxidative skeletal muscle [50]. Analysis of hepatic PDK isoform expression in high-fat-fed rats revealed that, unlike starvation, which elicited a 3.7-fold ($P < 0.01$) increase in PDK4 protein expression, high-fat feeding was not associated with increased hepatic PDK4 protein expression (Figure 3). However, administration of the PPARα activator WY14,643 to high-fat-fed rats resulted in a significant (1.6-fold, $P < 0.05$) increase in PDK4 protein expression compared with untreated high-fat fed rats (Figure 3). The magnitude of the response of PDK4 protein expression to PPARα-activation in high fat-fed rats is comparable with that observed in control rats maintained with *ad libitum* access to standard (low-fat/high-carbohydrate) diet (1.8-fold increase; $P < 0.001$) (see Figure 4). It should be noted that the data presented in Figure 4 demonstrate two immunoreactive bands for PDK4. Both bands were used for quantification in those cases where two bands were detected. Multiple bands can also be observed in some earlier reports of PDK protein expression [13,51,52], although this was not commented on in these earlier reports. At present, we do not have any explanation for the multiple bands. Administration of WY14,643 to rats from 24 to 48 h of starvation did not significantly increase PDK4 protein expression compared with PDK4 protein expression in untreated 48 h starved rats (results not shown). These data therefore indicate that even if hepatic lipid delivery is increased, TAG accumulation in the absence of increased FA oxidation does not influence hepatic PDK4 protein expression, and that additional signalling via PPARα is required for up-regulation of PDK4 protein expression. Our data imply a restraint on hepatic PDK4 protein expression in the fed state that can be removed by PPARα activation.

Effects of hyperthyroidism on hepatic lipid supply and disposition

Hyperthyroidism elevates FA supply via increased adipocyte lipolysis [22]. In addition, it has been reported to increase hepatic CPT I maximal activity and reduce CPT I sensitivity to suppression by malonyl-CoA [23,53]. In the present experiments with *ad libitum* fed rats, hyperthyroidism significantly elevated plasma NEFA concentrations (3.5-fold; $P < 0.001$) (euthyroid, 0.18 ± 0.01 mM; hyperthyroid, 0.63 ± 0.04 mM). In addition, and supporting results of previous studies, hyperthyroidism

significantly increased (by 1.6-fold, $P < 0.001$) hepatic CPT I maximal activity (Figure 2A). In addition, hyperthyroidism impaired CPT I sensitivity to suppression by malonyl-CoA, even in rats with *ad libitum* access to food (Figure 2B). However, hepatic ketone-body concentrations (3-hydroxybutyrate + acetoacetate) concentrations were only modestly influenced by hyperthyroidism (euthyroid, 0.31 ± 0.07 μmol/g wet wt.; hyperthyroid, 0.46 ± 0.03 μmol/g wet wt.; not significant), whereas, as found after high-fat feeding, hepatic TAG concentrations were increased (euthyroid, 16.5 ± 2.7 mg/g wet wt.; hyperthyroid, 31.8 ± 3.4 mg/g wet wt.).

Hyperthyroidism increases hepatic PDK4 protein expression in the fed state

Hyperthyroidism evoked a marked (2.1-fold; $P < 0.01$) increase in hepatic PDK activity (euthyroid, 1.8 ± 0.3 min^{-1}; hyperthyroid 3.8 ± 0.5 min^{-1}; $P < 0.01$). This was associated with marked suppression (by 83%) of hepatic PDHa activity (euthyroid, 17.2 ± 1.5 m-units/unit of citrate synthase; hyperthyroid, 3.0 ± 0.5 m-units/unit of citrate synthase; $P < 0.001$). We have demonstrated previously that, like high-fat feeding, experimental hyperthyroidism increases hepatic PDK2 protein expression [24]. However, assuming that PDK2 specific activity is unchanged, this is insufficient to account for the increase in total PDK activity elicited by hyperthyroidism [7]. In the present study we observed a substantial (2.4-fold; $P < 0.001$) increase in hepatic PDK4 protein expression in response to hyperthyroidism in the fed state (Figure 4). Expression of the E1α subunit of PDC was unaffected by experimental hyperthyroidism in the fed state (euthyroid, 1.00 ± 0.01-fold; hyperthyroid, 1.08 ± 0.09-fold). Hence, the present study demonstrates that experimental hyperthyroidism up-regulates PDK4 protein expression, in addition to its previously demonstrated effect to increase PDK2 protein expression [24], in livers of *ad libitum*-fed rats. Enhanced protein expression of the high-specific-activity PDK4 isoform, demonstrated in the present study, is likely to account for the discrepancy between the overall increase in hepatic total PDK activity and the more limited enhancement of hepatic PDK2 protein expression observed in response to hyperthyroidism [11].

Effects of acute PPARα activation for 24 h *in vivo* on hepatic PDK4 protein expression in hyperthyroid rats

RXR forms the dimerization partner with both PPARα and the TR, and PPARα may have both thyromimetic and thyroid antagonistic actions. One thyromimetic action is activation of 'malic' enzyme gene transcription [54] and up-regulation of PDK4 expression by activation of PPARα *in vivo* in the euthyroid state can be considered as a further thyromimetic action. Conversely, the hepatic expression of the spot-14 (S14) gene product gene is markedly and rapidly induced by T_3 [55], whereas S14 expression is down-regulated by PPARα [56,57]. Finally, the induction of peroxisomal β-oxidation enzymes by peroxisome proliferators is inhibited when thyroid hormone is added simultaneously, implying competition between PPAR and TR for the RXR [58]. We therefore analysed interactions between the effects of hyperthyroidism and acute activation of PPARα by WY14,643 treatment on hepatic PDK4 protein expression. WY14,643 was administered to hyperthyroid rats for the last 24 h of T_3 treatment. Whereas acute (24 h) PPARα activation by WY14,643 treatment *in vivo* selectively up-regulated hepatic PDK4 protein expression by 1.8-fold ($P < 0.001$) in livers of euthyroid rats, the administration of WY14,643 to fed hyperthyroid rats failed to up-regulate hepatic PDK4 protein expression further and, instead, tended to reduce PDK4 protein expression (Figure 4). Since the effects of hyperthyroidism and PPARα activation to increase hepatic PDK4 expression are not additive, our data suggest that PDK4 up-regulation can be achieved by heterodimerization of either PPARα or TR with the common partner, the RXR receptor. The observation that WY14,643 tended to reduce PDK4 protein expression in fed hyperthyroid rats suggests that there may be antagonism between PPARα and TR at the level of the RXR. This mirrors the induction of peroxisomal β-oxidation enzymes by peroxisome proliferators, which is inhibited when thyroid hormone is added simultaneously [58]. The molecular basis for antagonism between PPARα and TR at the level of the RXR in relation to PDK4 protein expression remains unclear. It is possible that altered PDK4 protein levels in response to these compounds could result from altered PDK4 mRNA stability. However, available evidence suggests that PDK4 protein expression is not regulated at the level of mRNA stability, since changes in PDK4 mRNA stability are not responsible for increased PDK4 message and protein induced by WY14,643 or dexamethasone [15]. It is possible that PPARα competes more effectively for RXR than does TR, resulting in less significant up-regulation of PDK4 protein expression (PPARα activation by WY14,643 administration in euthyroid rats elicits less significant up-regulation of PDK4 protein expression than does T_3 treatment in the absence of WY14,643).

Complex interactions between starvation and RXR-mediated signalling in the regulation of hepatic PDK4 protein expression

Starvation-induced increases in hepatic PDK4 protein expression are attenuated, but not completely blocked, by PPARα deficiency [14]. Effects of starvation to increase hepatic PDK4 protein expression were significant both in rats treated with WY14,643 (2.5-fold; $P < 0.01$) and in hyperthyroid (1.7-fold; $P < 0.01$) rats, the latter contrasting with the failure of pharmacological PPARα activation to increase hepatic PDK4 protein expression in hyperthyroid rats. Furthermore, starvation also increased hepatic PDK4 protein expression in hyperthyroid rats that additionally had been treated with WY14,643 (2.6-fold; $P < 0.01$). These data indicate that effects of starvation to increase hepatic PDK4 expression can be achieved by mechanisms in addition to PPARα or TR heterodimerization with the RXR.

Common features of PDK4 up-regulation in response to hyperthyroidism and starvation

The data obtained with high-fat-fed rats treated with TDG suggest that a positive correlation exists between increases in PDK activity and acute changes in hepatic FA oxidation that may be achieved via modulation of CPT I activity by malonyl-CoA. The failure to up-regulate hepatic PDK4 protein expression in response to high-fat feeding was associated with impaired suppression of CPT I activity by malonyl-CoA, but not with increased CPT I activity when measured in the absence of malonyl-CoA or increased adipose-tissue lipolysis (as assessed from ambient NEFA concentrations). Both starvation and hyperthyroidism increase adipocyte lipolysis and hepatic CPT I maximal activity and decrease hepatic CPT I sensitivity to inhibition by malonyl-CoA (Figure 2; see also [17,18]). Taken together, the data appear to indicate that increased FA delivery together with a chronic stimulus to increase CPT I maximal activity, rather than an acute change in CPT I flux (as might be the case in high-fat-fed rats) may be required for long-term up-regulation of PDK4 expression. Although mRNA of the liver form of CPT I is up-regulated normally in response to starvation in livers of PPARα-null mice [19], FA can activate pre-existing,

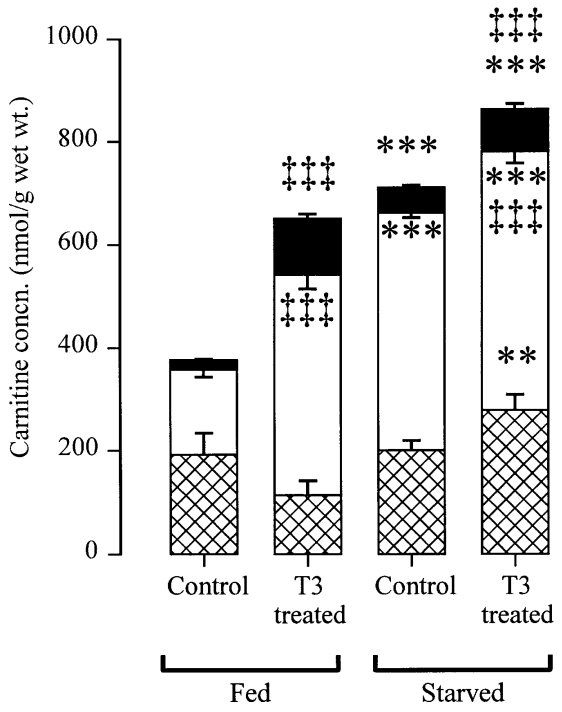

Figure 5 Effects of T_3 treatment on free, short-chain and long-chain carnitine in livers from fed or 48 h-starved rats

Free (cross-hatched portion of bars), short-chain (white portion of bars) and long-chain (black portion of bars) carnitine concentrations were measured as described in the Materials and methods section. The total height of the bar corresponds to the total carnitine concentration in each group. Data are means ± S.E.M. for at least five samples from individual rats in each experimental group. Statistically significant effects of starvation are indicated by: $^{**}P < 0.01$; $^{***}P < 0.01$. Statistically significant effects of T_3 treatment are indicated by: ‡‡‡$P < 0.001$.

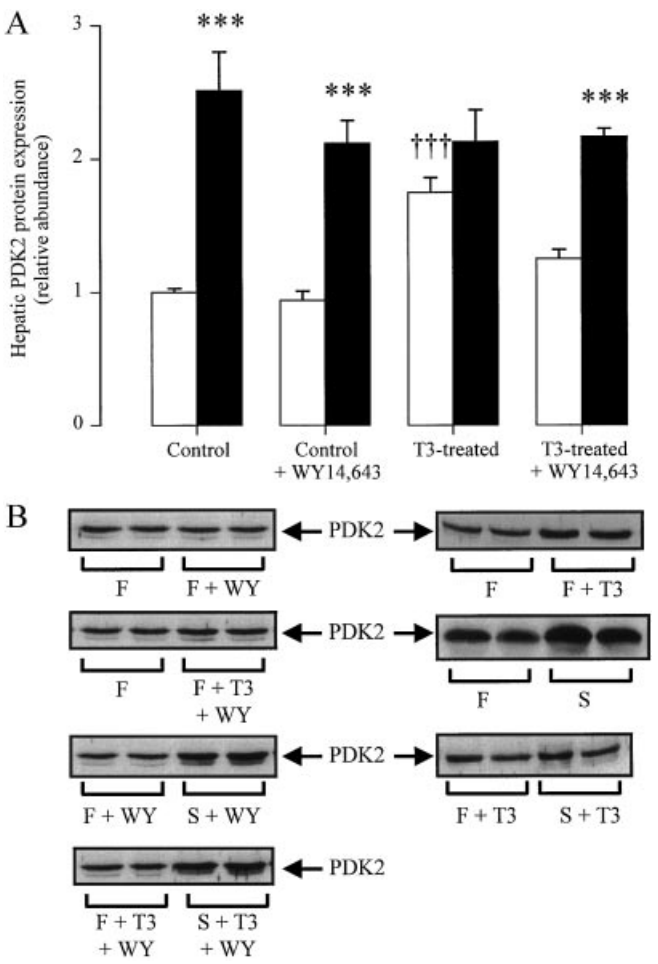

Figure 6 Response of hepatic PDK2 protein expression to WY14,643 treatment and/or hyperthyroidism in the fed or starved state

Western-blot analysis of PDK2 protein expression was undertaken using livers of fed (white bars) and starved (black bars) rats. Each lane corresponds to 50 μg of liver protein. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software and expressed as the relative abundance compared with corresponding results in fed control rats (**A**). Representative immunoblots are shown in (**B**). Further details are given in the Materials and methods section. Data are means ± S.E.M. for 20 (fed, F), eight (starved, S), 19 (fed + WY14,643, F + WY), seven (starved + WY14,643, S + WY), ten (fed + T_3, F + T3), seven (starved + T_3, S + T3), five (fed + T_3 + WY14,643, F + T3 + WY) and five (starved + T_3 + WY14,643, S + T3 + WY) liver preparations from individual rats in each experimental group. Statistically significant effects of starvation are indicated by: $^{***}P < 0.001$. Statistically significant differences from corresponding control fed or starved rats are indicated by: †††$P < 0.001$.

inactive CPT I without involvement of gene transcription and independently of malonyl-CoA [41]. We therefore compared free carnitine and long-chain acylcarnitine concentrations in freeze-clamped liver extracts from fed and starved euthyroid and hyperthyroid rats (Figure 5). In the fed state, hyperthyroidism decreased the hepatic concentration of free carnitine (by 41%). Although starvation did not affect hepatic free carnitine concentrations in euthyroid rats, starvation of hyperthyroid rats resulted in a 2.4-fold increase ($P < 0.01$) in hepatic free carnitine concentrations. As a consequence, free carnitine concentrations in the starved state were 39% higher in hyperthyroid rats compared with euthyroid rats (Figure 5). Both hyperthyroidism and starvation significantly increased long-chain acylcarnitine concentrations. The effect of hyperthyroidism (a 5.8-fold increase) actually exceeded that of starvation (a 2.6-fold increase). However, the effects of starvation and hyperthyroidism were not additive. Notably, both hyperthyroidism and starvation led to significant increases in hepatic total carnitine concentrations (1.7- and 1.9-fold respectively) through increased short-chain acylcarnitine (by 2.6- and 2.8-fold increases respectively). Furthermore, the effects of starvation and hyperthyroidism on hepatic total carnitine concentrations were additive, hyperthyroidism eliciting a 21% increase in total carnitine concentrations in the starved state. Our data are consistent with the concept that metabolites generated from adipocyte-derived FA via flux through CPT (which may include acylcarnitine) may facilitate up-regulation of hepatic PDK4 expression.

Factors regulating hepatic PDK2 expression

Data obtained in the present study provide insight into the differential regulation of hepatic PDK2 and PDK4 expression by hormonal and dietary manipulations. In the present study, we were unable to detect any effect of high-fat feeding to enhance hepatic PDK4 protein expression, although hepatic PDK2 protein expression was increased (results not shown; see [7]). The absolute selectivity of high-fat feeding to elevate hepatic PDK2, but not PDK4, protein expression, demonstrated for the first time in the present study, contrasts with the absolute selectivity of PPARα activation to enhance hepatic PDK4 protein expression (compare Figures 4 and 6). However, as shown in Figure 6, both PPARα activation and hyperthyroidism blunt

the response of hepatic PDK2 protein expression to starvation. This effect was particularly marked for hyperthyroidism. Thus, although 48 h starvation of hyperthyroid rats significantly (1.7-fold; $P < 0.01$) increased PDK4 protein expression (see above) (Figure 4), it failed to up-regulate PDK2 protein expression further (Figure 6). In addition, although hepatic PDK2 protein expression was unaffected by WY14,643 treatment in fed euthyroid rats (implying that up-regulation of hepatic PDK2 by thyroid hormone occurs via a mechanism that does not involve PPARα), PPARα activation *in vivo* antagonized the effect of hyperthyroidism to up-regulate hepatic PDK2 protein expression in the fed state (Figure 6). As for PDK4 protein expression (see above), there is no evidence to suggest that PDK2 protein expression is regulated at the level of mRNA stability; studies by Harris and co-workers [15] demonstrated that neither dexamethasone nor WY14,643 altered the stability of PDK2 mRNA. Again, it is possible that PPARα competes more effectively for RXR than does TR, thereby opposing any potential up-regulation of PDK2 protein expression by T_3. Thus it appears that PPARα activation leads to a switch in the hepatic PDK isoform profile to favour PDK4 protein expression rather than PDK2 protein expression, while hyperthyroidism leads to a more general up-regulation of hepatic PDK protein expression. The finding that PPARα activation suppresses the increase in hepatic PDK2 protein expression elicited by hyperthyroidism emphasizes that different mechanisms are likely to control the protein expression of PDK2 and PDK4 in the liver. However, our data strongly imply that PPARα elicits a switch in hepatic PDK isoform profile to enhance the relative protein expression of PDK4. The question remains whether a change in the relative protein expression of PDK4 and PDK2 has a physiological implication in addition to altered PDK total activity. Differences in the regulatory characteristics of PDK2 and PDK4 have been identified using the recombinant proteins [3]. Rapid re-activation of hepatic PDHa can be observed in response to inhibition of CPT I activity by TDG in high-fat-fed rats, where hepatic protein expression of PDK2, but not PDK4, is increased. Our data exclude enhanced hepatic protein expression of PDK2 as a mechanism to confer resistance of hepatic PDHa to re-activation.

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REFERENCES

1 Sugden, M. C. and Holness, M. J. (1994) Interactive regulation of the pyruvate dehydrogenase complex and the carnitine palmitoyltransferase system. FASEB J. **8**, 54–61
2 Harris, R. A., Popov, K. M. and Zhao, Y. (1995) Nutritional regulation of the protein kinases responsible for the phosphorylation of the α-ketoacid dehydrogenase complexes. J. Nutr. **125**, 1758S–1761S
3 Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A. and Popov, K. M. (1998) Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. Biochem. J. **329**, 191–196
4 Popov, K. M., Hawes, J. W. and Harris, R. A. (1997) Mitochondrial α-ketoacid dehydrogenase kinases: a new family of protein kinases. Adv. Second Messenger Phosphoprotein Res. **31**, 105–111
5 Roche, T. E., Baker, J. C., Yan, X., Hiromasa, Y., Gong, X., Peng, T., Dong, J., Turkan, A. and Kasten, S. A. (2001) Distinct regulatory properties of pyruvate dehydrogenase kinase and phosphatase isoforms. Prog. Nucleic Acid Res. Mol. Biol. **70**, 33–75
6 McGarry, J. D. and Brown, N. F. (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur. J. Biochem. **244**, 1–14
7 Sugden, M. C., Fryer, L. G., Orfali, K. A., Priestman, D. A., Donald, E. and Holness, M. J. (1998) Studies of the long-term regulation of hepatic pyruvate dehydrogenase kinase. Biochem. J. **329**, 89–94
8 Denyer, G. S., Kerbey, A. L. and Randle, P. J. (1986) Kinase activator protein mediates longer-term effects of starvation on activity of pyruvate dehydrogenase kinase in rat liver mitochondria. Biochem. J. **239**, 347–354
9 Fatania, H. R., Vary, T. C. and Randle, P. J. (1986) Modulation of pyruvate dehydrogenase kinase activity in cultured hepatocytes by glucagon and n-octanoate. Biochem. J. **234**, 233–236
10 Marchington, D. R., Kerbey, A. L., Giardina, M. G., Jones, A. E. and Randle, P. J. (1989) Longer-term regulation of pyruvate dehydrogenase kinase in cultured rat hepatocytes. Biochem. J. **257**, 487–491
11 Sugden, M. C., Fryer, L. G., Priestman, D. A., Orfali, K. A. and Holness, M. J. (1996) Increased hepatic pyruvate dehydrogenase kinase activity in fed hyperthyroid rats: studies *in vivo* and with cultured hepatocytes. Mol. Cell Endocrinol. **119**, 219–224
12 Jones, B. S., Yeaman, S. J., Sugden, M. C. and Holness, M. J. (1992) Hepatic pyruvate dehydrogenase kinase activities during the starved-to-fed transition. Biochim. Biophys. Acta **1134**, 164–168
13 Wu, P., Blair, P. V., Sato, J., Jaskiewicz, J., Popov, K. M. and Harris, R. A. (2000) Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. Arch. Biochem. Biophys. **381**, 1–7
14 Sugden, M. C., Bulmer, K., Gibbons, G. F., Knight, B. L. and Holness, M. J. (2002) Peroxisome-proliferator-activated receptor α (PPARα) deficiency leads to dysregulation of hepatic lipid and carbohydrate metabolism by fatty acids and insulin. Biochem. J. **364**, 361–368
15 Huang, B., Wu, P., Bowker-Kinley, M. M. and Harris, R. A. (2002) Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-α ligands, glucocorticoids and insulin. Diabetes **51**, 276–283
16 Sugden, M. C., Bulmer, K. and Holness, M. J. (2001) Fuel-sensing mechanisms integrating lipid and carbohydrate utilization. Biochem. Soc. Trans. **29**, 272–278
17 Grantham, B. D. and Zammit, V. A. (1986) Restoration of the properties of carnitine palmitoyltransferase I in liver mitochondria during re-feeding of starved rats. Biochem. J. **239**, 485–488
18 Zammit, V. A. (1984) Time-dependence of inhibition of carnitine palmitoyltransferase I by malonyl-CoA in mitochondria isolated from livers of fed or starved rats. Evidence for transition of the enzyme between states of low and high affinity for malonyl-CoA. Biochem. J. **218**, 379–386
19 Le May, C., Pineau, T., Bigot, K., Kohl, C., Girard, J. and Pegorier, J. P. (2000) Reduced hepatic fatty acid oxidation in fasting PPARα null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression. FEBS Lett. **475**, 163–166
20 Pineda, T. I., Gervois, P. and Staels, B. (1999) Peroxisome proliferator-activated receptor α in metabolic disease, inflammation, atherosclerosis and aging. Curr. Opin. Lipidol. **10**, 151–159
21 Kersten, S., Desvergne, B. and Wahli, W. (2000) Roles of PPARs in health and disease. Nature (London) **405**, 421–424
22 Fryer, L. G., Holness, M. J. and Sugden, M. C. (1997) Selective modification of insulin action in adipose tissue by hyperthyroidism. J. Endocrinol. **154**, 513–522
23 Stakkestad, J. A. and Bremer, J. (1983) The outer carnitine palmitoyltransferase and regulation of fatty acid metabolism in rat liver in different thyroid states. Biochim. Biophys. Acta **750**, 244–252
24 Priestman, D. A., Donald, E., Holness, M. J. and Sugden, M. C. (1997) Different mechanisms underlie the long-term regulation of pyruvate dehydrogenase kinase (PDHK) by tri-iodothyronine in heart and liver. FEBS Lett. **419**, 55–57
25 Sugden, M. C., Priestman, D. A., Orfali, K. A. and Holness, M. J. (1999) Hyperthyroidism facilitates cardiac fatty acid oxidation through altered regulation of cardiac carnitine palmitoyltransferase: studies *in vivo* and with cardiac myocytes. Horm. Metab. Res. **31**, 300–306
26 Wu, P., Sato, J., Zhao, Y., Jaskiewicz, J., Popov, K. M. and Harris, R. A. (1998) Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. Biochem. J. **329**, 197–201
27 Holness, M. J. and Sugden, M. C. (1999) Antecedent protein restriction exacerbates development of impaired insulin action after high-fat feeding. Am. J. Physiol. **276**, E85–E93
28 Kraegen, E. W., James, D. E., Jenkins, A. B., Chisholm, D. J. and Storlien, L. H. (1989) A potent *in vivo* effect of ciglitazone on muscle insulin resistance induced by high fat feeding of rats. Metab. Clin. Exp. **38**, 1089–1093
29 Sugden, M. C. and Holness, M. J. (1993) Control of muscle pyruvate oxidation during late pregnancy. FEBS Lett. **321**, 121–126
30 Rennie, M. J., Winder, W. W. and Holloszy, J. O. (1976) A sparing effect of increased plasma fatty acids on muscle and liver glycogen content in the exercising rat. Biochem. J. **156**, 647–655
31 Orfali, K. A., Fryer, L. G., Holness, M. J. and Sugden, M. C. (1995) Interactive effects of insulin and triiodothyronine on pyruvate dehydrogenase kinase activity in cardiac myocytes. J. Mol. Cell Cardiol. **27**, 901–908

32 Brun, S., Carmona, M. C., Mampel, T., Vinas, O., Giralt, M., Iglesias, R. and Villarroya, F. (1999) Activators of peroxisome proliferator-activated receptor-α induce the expression of the uncoupling protein-3 gene in skeletal muscle: a potential mechanism for the lipid intake-dependent activation of uncoupling protein-3 gene expression at birth. Diabetes **48**, 1217–1222

33 Caterson, I. D., Fuller, S. J. and Randle, P. J. (1982) Effect of the fatty acid oxidation inhibitor 2-tetradecylglycidic acid on pyruvate dehydrogenase complex activity in starved and alloxan-diabetic rats. Biochem. J. **208**, 53–60

34 Kerbey, A. L., Radcliffe, P. M. and Randle, P. J. (1977) Diabetes and the control of pyruvate dehydrogenase in rat heart mitochondria by concentration ratios of adenosine triphosphate/adenosine diphosphate, of reduced/oxidized nicotinamide–adenine dinucleotide and of acetyl-coenzyme A/coenzyme A. Biochem. J. **164**, 509–519

35 Kerbey, A. L. and Randle, P. J. (1982) Pyruvate dehydrogenase kinase/activator in rat heart mitochondria, Assay, effect of starvation and effect of protein-synthesis inhibitors of starvation. Biochem. J. **206**, 103–111

36 Drynan, L., Quant, P. A. and Zammit, V. A. (1996) The role of changes in the sensitivity of hepatic mitochondrial overt carnitine palmitoyltransferase in determining the onset of the ketosis of starvation in the rat. Biochem. J. **318**, 767–770

37 Schofield, P. S., French, T. J., Goode, A. W. and Sugden, M. C. (1985) Liver carnitine metabolism after partial hepatectomy in the rat. Effects of nutritional status and inhibition of carnitine palmitoyltransferase. FEBS Lett. **184**, 214–220

38 Brass, E. P. and Hoppel, C. L. (1978) Carnitine metabolism in the fasting rat. J. Biol. Chem. **253**, 2688–2693

39 Holness, M. J., MacLennan, P. A., Palmer, T. N. and Sugden, M. C. (1988) The disposition of carbohydrate between glycogenesis, lipogenesis and oxidation in liver during the starved-to-fed transition. Biochem. J. **252**, 325–330

40 Priestman, D. A., Mistry, S. C., Halsall, A. and Randle, P. J. (1994) Role of protein synthesis and of fatty acid metabolism in the longer-term regulation of pyruvate dehydrogenase kinase. Biochem. J. **300**, 659–664

41 Sleboda, J., Risan, K. A., Spydevold, O. and Bremer, J. (1999) Short-term regulation of carnitine palmitoyltransferase I in cultured rat hepatocytes: spontaneous inactivation and reactivation by fatty acids. Biochim. Biophys. Acta **1436**, 541–549

42 Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M. and Lazar, M. A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem. **270**, 23975–23983

43 Forman, B. M., Chen, J. and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc. Natl. Acad. Sci. U.S.A. **94**, 4312–4317

44 Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1993) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc. Natl. Acad. Sci. U.S.A. **90**, 2160–2164

45 Gearing, K. L., Gottlicher, M., Widmark, E., Banner, C. D., Tollet, P., Stromstedt, M., Rafter, J. J., Berge, R. K. and Gustafsson, J. A. (1994) Fatty acid activation of the peroxisome proliferator activated receptor, a member of the nuclear receptor gene superfamily. J. Nutr. **124**, 1284S–1288S

46 Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M. and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc. Natl. Acad. Sci. U.S.A. **94**, 4318–4323

47 Leone, T. C., Weinheimer, C. J. and Kelly, D. P. (1999) A critical role for the peroxisome proliferator-activated receptor α (PPARα) in the cellular fasting response: the PPARα-null mouse as a model of fatty acid oxidation disorders. Proc. Natl. Acad. Sci. U.S.A. **96**, 7473–7478

48 Harris, R. A., Huang, B. and Wu, P. (2001) Control of pyruvate dehydrogenase kinase gene expression. Adv. Enzyme Regul. **41**, 269–288

49 Sugden, M. C., Langdown, M. L., Harris, R. A. and Holness, M. J. (2000) Expression and regulation of pyruvate dehydrogenase kinase isoforms in the developing rat heart and in adulthood: role of thyroid hormone status and lipid supply. Biochem. J. **352**, 731–738

50 Holness, M. J., Kraus, A., Harris, R. A. and Sugden, M. C. (2000) Targeted upregulation of pyruvate dehydrogenase kinase (PDK)-4 in slow-twitch skeletal muscle underlies the stable modification of the regulatory characteristics of PDK induced by high-fat feeding. Diabetes **49**, 775–781

51 Wu, P., Peters, J. M. and Harris, R. A. (2001) Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor α. Biochem. Biophys. Res. Commun. **287**, 391–396

52 Peters, S. J., Harris, R. A., Heigenhauser, G. J. and Spriet, L. L. (2001) Muscle fiber type comparison of PDH kinase activity and isoform expression in fed and fasted rats. Am. J. Physiol. **280**, R661–R668

53 Mynatt, R. L., Park, E. A., Thorngate, F. E., Das, H. K. and Cook, G. A. (1994) Changes in carnitine palmitoyltransferase-I mRNA abundance produced by hyperthyroidism and hypothyroidism parallel changes in activity. Biochem. Biophys. Res. Commun. **201**, 932–937

54 Hertz, R., Nikodem, V., Ben Ishai, A., Berman, I. and Bar-Tana, J. (1996) Thyromimetic mode of action of peroxisome proliferators: activation of 'malic' enzyme gene transcription. Biochem. J. **319**, 241–248

55 Liu, H. C. and Towle, H. C. (1994) Functional synergism between multiple thyroid hormone response elements regulates hepatic expression of the rat S14 gene. Mol. Endocrinol. **8**, 1021–1037

56 Ren, B., Thelen, A. and Jump, D. B. (1996) Peroxisome proliferator-activated receptor-α inhibits hepatic S14 gene transcription. Evidence against the peroxisome proliferator-activated receptor-α as the mediator of polyunsaturated fatty acid regulation of s14 gene transcription. J. Biol. Chem. **271**, 17167–17173

57 Ren, B., Thelen, A. P., Peters, J. M., Gonzalez, F. J. and Jump, D. B. (1997) Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor-α. J. Biol. Chem. **272**, 26827–26832

58 Chu, R., Madison, L. D., Lin, Y., Kopp, P., Rao, M. S., Jameson, J. L. and Reddy, J. K. (1995) Thyroid hormone (T_3) inhibits ciprofibrate-induced transcription of genes encoding β-oxidation enzymes: cross talk between peroxisome proliferator and T_3 signaling pathways. Proc. Natl. Acad. Sci. U.S.A. **92**, 11593–11597

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