# Expression and regulation of pyruvate dehydrogenase kinase isoforms in the developing rat heart and in adulthood: role of thyroid hormone status and lipid supply

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Activation of the pyruvate dehydrogenase (PDH) complex (PDHC) promotes glucose disposal, whereas inactivation conserves glucose. The PDH kinases (PDHKs) regulate glucose oxidation through inhibitory phosphorylation of PDHC. The adult rat heart contains three PDHK isoforms PDHK1, PDHK2 and PDHK4. Using Western-blot analysis, with specific antibodies raised against individual recombinant PDHK1, PDHK2 and PDHK4, the present study investigated PDHK isoform expression in the developing rat heart and adulthood. We identified clear differences in the patterns of protein expression of each of these PDHK isoforms during the first 3 weeks of post-natal development, with most marked up-regulation of isoforms PDHK1 and PDHK4. Distinctions between the three cardiac PDHK isoforms were also demonstrated with respect to post-neonatal maturational up-regulation; with greatest up-regulation of PDHK1 and least up-regulation of PDHK4 from the post-neonatal period until maturity. The study also examined the role of thyroid hormone status and lipid supply on PDHK isoform expression. We observed marked selective increases in the amount of PDHK4 protein present relative to total cardiac protein in both hyperthyroidism and high-fat feeding. Overall, our data identify PDHK isoform PDHK1 as being of more potential regulatory importance for glucose oxidation in the adult compared with the neonatal heart, and cardiac PDHK4 as a PDHK isoform whose expression is specifically responsive to changes in lipid supply, suggesting that its up-regulation during early post-natal life may be the perinatal switch to use fatty acids as the energy source. We also identify regulation of pyruvate sensitivity of cardiac PDHK as a physiological variable, a change in which requires factors in addition to a change in lipid supply.

Key words: pyruvate dehydrogenase complex, hyperthyroidism, high-fat diet.

## INTRODUCTION

Under resting conditions, the adult heart derives approx. 70 % of its energy from the oxidation of lipids, and the remainder primarily from glycolysis and glucose oxidation [1]. However, specific situations exist that are associated with increased cardiac glucose utilization: these include late fetal life [2], myocardial ischaemia [3] and pressure-overload-induced cardiac hypertrophy [4–6]. Others (for example, the early post-natal period, starvation and diabetes) are associated with increased rates of lipid oxidation and suppression of myocardial glucose utilization [7–9]. The pyruvate dehydrogenase (PDH) complex (PDHC) catalyses the physiologically irreversible step committing glucose carbon to oxidation, and is a major target for substrate competition between glucose and fatty acids, which influences the percentage of total complex present in the active non-phosphorylated form [10,11]. This is achieved via opposing acute effects of intermediates of lipid and glucose metabolism on the activity of the PDH kinases (PDHKs), which specifically phosphorylate PDHC (reviewed in [10]). NADH and acetyl-CoA, generated by increased rates of fatty acid $\beta$-oxidation, activate the PDHKs, whereas pyruvate (produced by glycolysis) suppresses their activity (reviewed in [10]). Three of the four members of the PDHK family identified to date are found in the adult rat heart, namely PDHK1, PDHK2 and PDHK4 [12]. Experimental diabetes enhances PDHK activity in the adult rat heart, and is observed in association with specific up-regulation of the protein expression of only one of these isoforms, namely PDHK4 [13]. Starvation, another insulin-deficient state, also up-regulates cardiac PDHK4 expression [13]. Thus in the adult rat heart, PDHK4 expression appears to reflect insulin status, either by virtue of a direct effect of insulin or via the modulation of fuel utilization (decreased glucose utilization and increased lipid utilization) that is associated with altered insulin status.

The perinatal and neonatal periods are associated with marked changes in the pattern of isoform expression of a number of key regulatory enzymes controlling glucose and fatty acid oxidation. Glucose enters the heart via two facilitative glucose transporters, GLUT1 and GLUT4 [14]. GLUT1 is a major mediator of basal cardiac glucose uptake [15,16], and normally accounts for approx. 30 % of the total cardiac glucose transporters in the adult heart [17]. However, the contribution of GLUT1 to total cardiac glucose-transporter number is even higher in late fetal life and early neonatal life, when the protein expression of GLUT4, the insulin-regulable isoform, is relatively low [18,19]. However, the relative contribution of the two glucose transporters changes during early post-natal development when GLUT1 protein expression is down-regulated [18,19], but GLUT4 protein expression is up-regulated and, as a consequence, glucose uptake by the heart becomes more insulin sensitive [18,19]. The activity of the outer mitochondrial membrane carnitine palmitoyltransferase (CPT) I is a major determinant of rates of mitochondrial oxidation of long-chain fatty acids [20,21]. Control of long-chain fatty acid oxidation is thought to be exerted via

Abbreviations used: CPT, carnitine palmitoyltransferase; PDH, pyruvate dehydrogenase; PDHa, active PDH; PDHC, PDH complex; PDHK, PDH kinase; TBST, Tris-buffered saline supplemented with 0.05 % Tween.

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modulation of CPT I activity by a change in malonyl-CoA concentration [20,21]. The rat cardiac myocyte expresses two kinetically distinct isoforms of CPT I, L (liver)-type and M (muscle)-type, which exhibit different kinetic properties, particularly with respect to inhibition of activity by malonyl-CoA [20,22]. In the neonatal heart the L-isoform, which is less sensitive to malonyl-CoA, contributes a substantially greater fraction to the total CPT I activity than in the adult heart [22]. This would be predicted to render cardiac fatty acid oxidation less sensitive to external factors that influence malonyl-CoA content (e.g. fluctuations in glycaemia; see [23]), and thereby to favour fatty acid oxidation. The expression of L-type CPT I is down-regulated during the neonatal period [22].

Regulation of cardiac GLUT1 and GLUT4 expression during early post-natal development has been attributed in part to the post-natal surge of thyroid hormones [2], an event considered to be critical to the normal maturation of the heart [24,25]. GLUT1 protein expression in the adult heart is decreased by hyperthyroidism, reiterating the pattern of expression seen during the early neonatal period when thyroid hormone concentrations rise [26]. Similarly, in adulthood, experimental hyperthyroidism leads to altered regulatory characteristics of cardiac CPT activity, such that suppression of its activity by increasing malonyl-CoA concentrations is diminished [27]. Previous studies have shown that experimental hyperthyroidism leads to a stable enhancement of PDHK activity in the absence of any change in PDHK2 protein expression in the adult rat heart [28]. The heart switches from carbohydrates to fatty acids (provided in maternal milk) as the main energy source at birth [29]. High-fat feeding *in vivo* – like hyperthyroidism – leads to a stable enhancement of PDHK activity in the adult rat heart in the absence of any change in PDHK2 protein expression [30]. Studies with cultured cardiac myocytes have shown that fatty acids up-regulate cardiac PDHK activity, particularly when in combination with dibutyryl cAMP [31].

Given that it is clearly apparent that there are distinct 'predominantly fetal' versus 'predominantly adult' isoforms of key regulatory enzymes of cardiac fuel utilization, several important questions are raised. First, are there distinct 'predominantly fetal' versus 'predominantly adult' PDHK isoforms? Secondly, does the ontogeny of protein expression of one or more of the individual PDHK isoforms change during early development, and do such putative changes parallel those of the glucose transporters (implying a role for thyroid hormones) or reflect cardiac substrate preference (carbohydrate compared with lipid)? Finally, does either hyperthyroidism or high-fat feeding in adulthood lead to reiteration of the PDHK isoform expression profile seen during early life? The present study therefore examined the presence and patterns of protein expression of each of the individual PDHK isoforms in the rat heart during late fetal and early post-natal development, making comparisons with the pattern of changes in cardiac GLUT1 and GLUT4 protein expression. In addition, it investigated the direct effects of hyperthyroidism and high-fat feeding on the cardiac PDHK expression profile in adulthood.

## MATERIALS AND METHODS

### Materials

Female Wistar rats were purchased from Charles River Ltd. (Margate, Kent, U.K.). Arylamine acetyltransferase was purified from pigeon liver acetone powder purchased from Europa Bioproducts Ltd. (Ely, Cambs., U.K.). Organic solvents were of analytical grade and were obtained from BDH (Poole, Dorset, U.K.). General laboratory reagents were purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.) or from Sigma (Poole, Dorset, U.K.), with the following exceptions. ECL® reagents, Hyperfilm and secondary antibodies were purchased from Amersham International (Little Chalfont, Bucks., U.K.). Anti-GLUT1 and anti-GLUT4 polyclonal IgG antibodies were purchased from Insight Biotechnology Ltd. (Wembley, Middx., U.K.). Anti-PDHK antibodies were generated in the authors' laboratories against individual recombinant proteins [13]. Bradford reagents were purchased from Bio-Rad Ltd. (Hemel Hempstead, Herts., U.K.).

### Animals

All studies were conducted in adherence to the regulations of the United Kingdom Animal Scientific Procedures Act (1986). Female albino Wistar rats (200–220 g) were maintained at a temperature of $22 \pm 2$ °C, subjected to a 12-h light/dark cycle and provided with free access to standard, pelleted rodent diet [72 % carbohydrate, 20 % protein and 8 % lipid, by energy; 2.61 kcal metabolizable energy/g (1 cal $\equiv$ 4.184 J)] purchased from Special Diet Services (Witham, Essex, U.K.). One group of rats was rendered hyperthyroid by injection of tri-iodothyronine (1 mg/kg body weight per day, for 3 days) and the age-matched controls were injected with hormone solvent (10 mM NaOH/0.03 % BSA) [32]. Experimental hyperthyroidism did not suppress food intake (results not shown). In a parallel series of studies, rats were provided with a low-carbohydrate/high-saturated-fat diet (33 % carbohydrate, 20 % protein and 47 % lipid, by energy; 4.19 kcal metabolizable energy/g), containing predominantly saturated fat (lard) as the major source of lipid (43 % of the total energy), and corn oil (4 % of the total energy) to prevent essential-fatty-acid deficiency [33]. Although rats provided with the high-fat diet consumed less diet (by weight), this diet was of higher energy density, and transfer of rats to the high-fat diet did not significantly reduce daily caloric intake.

### Tissue and blood sampling

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/ml in 0.9 % NaCl; 1 ml/kg body weight) and, once locomotor activity had ceased, hearts were rapidly excised and freeze-clamped using aluminium clamps precooled in liquid nitrogen. Frozen hearts were stored in liquid nitrogen. Blood was sampled from the chest cavity after the removal of the heart. Blood samples were centrifuged for 5 min at 12000 ***g*** and the plasma was stored at $-20$ °C.

### Enzyme assays

Active PDH (PDHa) activity was assayed both in freeze-clamped tissue extracts and in isolated mitochondria, prepared as described previously [34–36]. PDHa was assayed spectrophotometrically by coupling its activity to arylamine acetyltransferase [34,35]. Total PDHC activity was assayed as active PDHC after complete activation through the action of endogenous PDHC phosphate phosphatase in mitochondria incubated for 15 min in the absence of respiratory substrate and in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone [37]. PDHK activities were determined in mitochondrial extracts at pH 7.0 by monitoring the rate of ATP-dependent inactivation of PDHa, and were computed as apparent first-order rate constants for ATP-dependent PDHa inactivation [38,39]. To test the effects of pyruvate, freshly prepared mitochondria were incubated at 30 °C in KCl medium (100 mM KCl, 20 mM Tris/HCl, 5 mM $KH_2PO_4$ and 2 mM EGTA, pH 7.4) in the presence of respiratory substrate (5 mM 2-oxoglutarate/0.5 mM L-malate) together with

the concentrations of pyruvate (added as sodium salt) indicated [31]. Incubations were terminated by centrifugation after 5 min, and mitochondrial extracts were assayed for PDHa activity [34,35].

### Immunoblotting

Cardiac samples (approx. 100 mg) were homogenized using a Polytron tissue homogenizer (PT 10 probe; position 5 for 15 s) in 1 ml of ice-cold extraction buffer [20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 10 % (v/v) glycerol, 1 % (v/v) Igepal, 45 mM sodium orthovanadate, 0.2 mM PMSF, 10 $\mu$g/ml leupeptin, 1.5 mg/ml benzamidine, 50 $\mu$g/ml aprotinin and 50 $\mu$g/ml pepstatin A (in DMSO), pH 8.0]. Homogenates were placed on ice for 20 min, centrifuged in an Eppendorf centrifuge (12000 rev./min for 20 min at 4 °C), and the supernatants were stored at −20 °C until analysis. Protein concentrations were determined using the method of Bradford [40], using BSA as a standard. The assay was linear over the range of protein concentrations routinely used. Samples (20–50 $\mu$g of total protein) were subjected to SDS/PAGE using a 12 % resolving gel and a 6 % stacking gel. After SDS/PAGE, resolved proteins were transferred electrophoretically on to nitrocellulose membranes, which were subsequently blocked for 2 h at room temperature with Tris-buffered saline supplemented with 0.05 % Tween (TBST) and 5 % (w/v) non-fat powdered milk. The nitrocellulose blots were incubated overnight at 4 °C with polyclonal antisera raised against specific recombinant PDHK isoforms or GLUT isoforms. The blots were subsequently washed three times with TBST (5 min/wash) and incubated with the horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (diluted 1:2000, in 1 % (w/v) non-fat milk in TBST) for 2 h at room temperature. Bound antibody was visualized using ECL®, according to the manufacturer's instructions. The blots were then exposed to Hyperfilm. The signals were quantified by scanning densitometry and analysed with Molecular Analyst software (Bio-Rad Ltd). The amounts of mitochondrial extracts loaded on to the gel were varied to establish that the relative densities of the bands corresponding to the PDHK isoforms were linear with concentration. For each panel in each Figure, the results are from a single gel exposed for a uniform duration.

### Statistical analysis

Results are presented as means ± S.E.M., with the numbers of rats in parentheses. Curve fitting was carried out using Fig P software (Biosoft, Cambridge, U.K.). Statistical analysis was performed using 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

### Cardiac glucose-transporter expression during late fetal and early post-natal life

Levels of protein expression of GLUT1 and GLUT4 in rat hearts during late fetal and early post-natal development, as quantified by Western-blot analysis, and expressed relative to levels of protein expression found in hearts of 21-day-old fetuses (term = 23 days), are shown in Figure 1(A). Representative immunoblots are shown in Figure 2. Confirming previous studies [18,19], we observed a post-natal decline in cardiac GLUT1 protein expression. Cardiac GLUT1 protein expression was significantly suppressed (by approx. 30 %; $P < 0.01$), relative to the fetal day 21 level, by post-natal day 7 and continued to decline progressively between post-natal days 7 and 15 (to 46 % of the fetal day 21 level). No further significant decline in cardiac GLUT1 protein expression was observed during the third post-natal week, but continued suppression of GLUT1 protein expression occurred from post-natal day 21 to adulthood (Figure 1B). Thus at 6 months of age, cardiac GLUT1 protein expression had significantly ($P < 0.01$) declined to 46 % of the post-natal day 21 level. Cardiac GLUT4 protein expression was modestly increased at post-natal day 7 (by 45 %; not significant), but subsequently increased dramatically (to 2.9-fold of the fetal day 21 value; $P < 0.001$) between post-natal days 7 and 15. Cardiac GLUT4 protein expression was maintained, although it did not increase further with age, from post-natal day 21 to adulthood (Figure 1B).

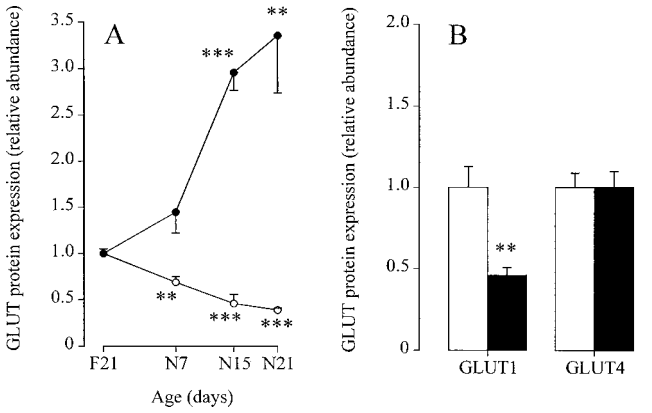


**Figure 1 Ontogeny of cardiac GLUT1 and GLUT4 protein expression during peri-natal and early post-natal life, and in adulthood**

Western-blot analysis was undertaken using GLUT isoform-specific antibodies as described in the Materials and methods section. (**A**) Relative protein expression of GLUT1 (○) and GLUT4 (●) is shown for hearts of 21-day-old fetal rats (F21), and of rats sampled at approx. weekly intervals during the first 3 weeks of post-natal development (7 days, N7; 15 days, N15; and 21 days, N21). (**B**) Relative protein expression of GLUT1 and GLUT4 in hearts of 21-day-old rats (open bars) and adult rats (solid bars). Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Further details are provided in the Materials and methods section. Data are presented relative to values in 21-day-old fetuses (**A**) or 21-day-old rats (**B**). Values are means ± S.E.M. for 5–7 preparations from individual rats. Statistically significant differences from 21-day-old fetuses (**A**) or 21-day-old rats (**B**) are indicated by: $^{**}P < 0.01$; $^{***}P < 0.001$.

### Temporal correlation of PDHK isoform expression with glucose-transporter expression during late fetal and early post-natal life

Levels of protein expression of PDHK1, PDHK2 and PDHK4 in hearts during early post-natal development, as quantified by Western-blot analysis, and expressed relative to levels of expression found in hearts of 21-day-old fetuses, together with representative immunoblots, are shown in Figure 3. Protein expression of all three PDHK isoforms was immunodetectable in the 21-day-old fetal heart. All three PDHK isoforms were also immunodetectable in the rat heart during early post-natal life, and their abundances (relative to total cardiac protein) increased with age. Cardiac PDHK1 protein expression was modestly enhanced to a level of approx. 1.6-fold ($P < 0.05$) of the fetal day 21 level at post-natal day 7 (Figure 3A). However, a more dramatic increase in cardiac PDHK1 protein expression was observed between post-natal days 7 and 15, at which time PDHK1 expression reached approx. 4.4-fold higher levels ($P < 0.001$) than those found in late fetal life (Figure 3A). This developmental profile thus closely parallels that of GLUT4. A

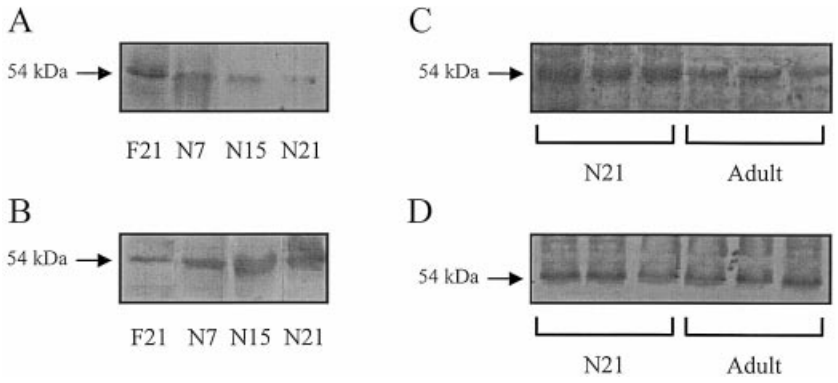


**Figure 2 Representative immunoblots of cardiac GLUT1 and GLUT4 protein expression during peri-natal and early post-natal life, and in adulthood**

Representative immunoblots of GLUT1 and GLUT4 in hearts of 21-day-old fetuses (F21) and of rats sampled at approx. weekly intervals during the first 3 weeks of post-natal development (7 days, N7; 15 days, N15; and 21 days, N21) are shown in (**A**) and (**B**) respectively. Representative immunoblots of GLUT1 and GLUT4 in hearts of 21-day-old rats and adult rats are shown in (**C**) and (**D**) respectively. Further details are provided in the Materials and methods section.

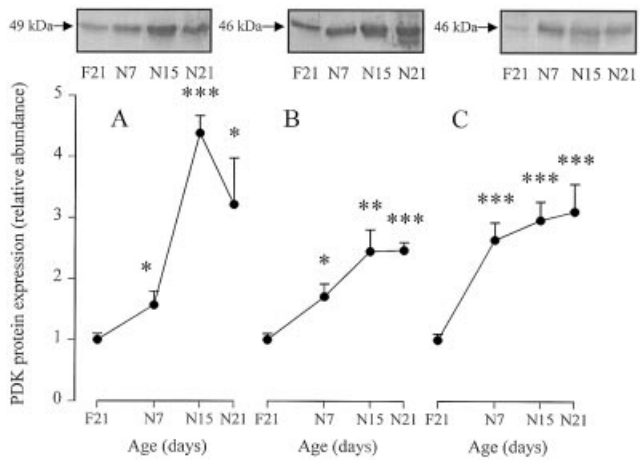


**Figure 3 Ontogeny of cardiac PDHK1, PDHK2 and PDHK4 protein expression during peri-natal and early post-natal life**

Western-blot analysis was undertaken using PDHK isoform-specific antibodies as described in the Materials and methods section. Relative protein expression of PDHK1 (**A**), PDHK2 (**B**) and PDHK4 (**C**) is shown for hearts of 21-day-old fetuses (F21) and of rats sampled at approx. weekly intervals during the first 3 weeks of post-natal development (7 days, N7; 15 days, N15; and 21 days, N21). Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Further details are provided in the Materials and methods section. Data are presented relative to values in 21-day-old fetuses. Representative immunoblots are shown. Values are means ± S.E.M. for 5–7 preparations from individual rats. Statistically significant differences from 21-day-old fetuses are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. PDK, PDHK.

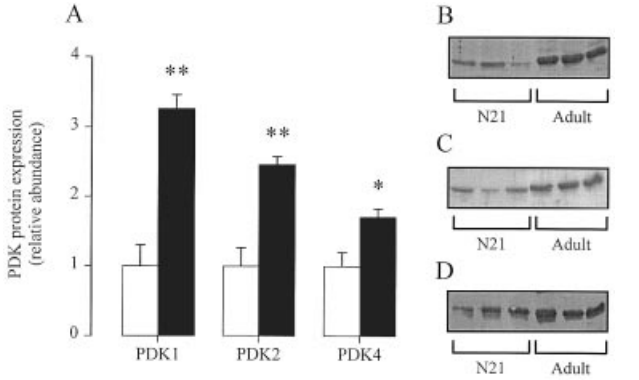


**Figure 4 Relative protein expression of PDHK1, PDHK2 and PDHK4 in hearts of 21-day-old rats and adult rats**

Western-blot analysis was undertaken using PDHK isoform-specific antibodies as described in the Materials and methods section. (**A**) Relative protein expression of PDHK1, PDHK2 and PDHK4 in hearts of 21-day-old rats (open bars) and adult rats (solid bars). Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Further details are provided in the Materials and Methods section. Data are presented relative to values in 21-day-old rats (N21). Representative immunoblots are shown for PDHK1 (**B**), PDHK2 (**C**) and PDHK4 (**D**). Values are means ± S.E.M. for 5–7 preparations from individual rats. Statistically significant differences from 21-day-old rats are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$. PDK, PDHK.

modest (non-significant) decline in PDHK1 protein expression was seen between post-natal days 15 and 21 (Figure 3A). Cardiac protein expression of PDHK2 increased by approx. 1.7-fold ($P < 0.05$) between fetal day 21 and post-natal day 7, and continued to increase progressively between post-natal days 7 and 15, reaching approx. 2.5-fold ($P < 0.001$) of the fetal day 21 level by post-natal day 15 (Figure 3B). No further change in protein expression was observed between post-natal day 15 and post-natal day 21 (Figure 3B). Thus the profile of PDHK2 up-regulation appears to be temporally related to GLUT1 down-regulation. The protein expression of PDHK4, the PDHK isoform whose expression in adulthood appears to be specifically linked to insulin deficiency (or the metabolic changes associated with insulin deficiency), exhibited a unique pattern of post-natal expression, in that the major increase in relative abundance occurred during the first 7 days of post-natal life (Figure 3C). The ontogeny of PDHK4 protein expression thus appears unrelated to that of either GLUT1 or GLUT4. Furthermore, cardiac PDHK4 protein expression over the first 7 days of post-natal life (a 2.6-fold increase; $P < 0.001$) was more dramatically up-regulated than that of either PDHK1 (a 1.6-fold increase) or PDHK2 (a 1.7-fold increase).

### Maturational changes in PDHK protein expression

The protein expression of all three PDHK isoforms continued to increase during maturation to adulthood (Figure 4). In particular,

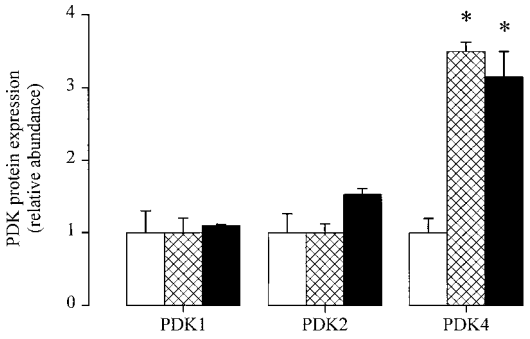


**Figure 5 Relative protein expression of PDHK1, PDHK2 and PDHK4 in the hearts of control rats, hyperthyroid rats and rats fed a high-fat diet**

Western-blot analysis of PDHK isoform protein expression was undertaken using hearts of control rats (open bars), hyperthyroid rats (crosshatched bars) and rats fed a high-fat diet (solid bars). Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Further details are given in the Materials and Methods section. Values are means ± S.E.M. for 5–7 preparations from individual rats. Statistically-significant differences from control rats are indicated by: $^{*}P < 0.001$. PDK, PDHK.

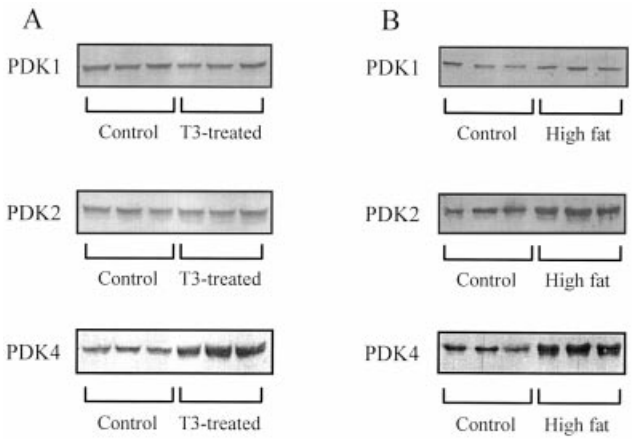


**Figure 6 Representative immunoblots of PDHK1, PDHK2 and PDHK4 protein expression in the hearts of control rats, hyperthyroid rats and rats fed a high-fat diet**

Representative immunoblots of PDHK1, PDHK2 and PDHK4 in hearts of hyperthyroid rats and rats fed a high-fat diet are shown in (**A**) and (**B**) respectively. Further details are provided in the Materials and methods section. T3, tri-iodothyronine. PDK, PDHK.

PDHK1 protein expression increased dramatically by 3.5-fold between post-natal day 21 and adulthood (6 months), suggesting that cardiac PDHK1 protein expression may be of greater relative importance to the function of the adult heart than that of the fetal or neonatal heart. This maturational up-regulation of PDHK1 expression contrasts with the absence of change in the protein expression of the insulin-sensitive glucose transporter, GLUT4, which is essentially complete during the first 3 weeks of post-natal life. Although the relative abundance of PDHK2 also increased between neonatal day 21 and adulthood, the maturational increase was more modest (2.5-fold) than that of PDHK1 (Figure 4). Thus maturational up-regulation of PDHK2 is of approximately the same magnitude as maturational down-regulation of GLUT1. Of the three PDHK isoforms found in the heart, PDHK4 showed the least maturational increase in protein expression (a 1.7-fold increase; Figure 4).

### Effects of hyperthyroidism and a high-fat diet on cardiac PDHK isoform expression in adulthood

As previously demonstrated [28,30], both hyperthyroidism and high-fat feeding lead to significant 2.1- and 1.8-fold increases in PDHK activity (from $0.46 \pm 0.07$ min$^{-1}$ to $0.97 \pm 0.20$ min$^{-1}$ and $0.82 \pm 0.13$ min$^{-1}$ respectively; $P < 0.05$ in both instances) that are stable to mitochondrial preparation, and therefore do not reflect changes in small-molecular-mass effectors. Western-blot analysis was used to determine whether these effects were associated with general or isoform-specific changes in cardiac PDHK1, PDHK2 and/or PDHK4 protein expression (Figures 5 and 6). We observed marked increases in the amount of PDHK4 protein present relative to total cardiac protein in both instances [3.5-fold ($P < 0.001$) and 3.2-fold ($P < 0.001$) for hyperthyroidism and high-fat feeding respectively]. In hyperthyroidism, this effect was observed in the absence of any significant change in the expression of either PDHK1 or PDHK2. By contrast, high-fat feeding modestly, but not significantly, increased PDHK2 protein expression (by 1.5-fold) but did not alter PDHK1 protein expression. Thus the amount of PDHK4 protein relative to total PDHK protein (PDHK1 + PDHK2 + PDHK4) in cardiac mitochondria is specifically increased by both hyperthyroidism and high-fat feeding.

### Hyperthyroidism and high-fat feeding decrease PDHa activities, but do not affect PDHK pyruvate sensitivity, in hearts of fed adult rats

We measured PDHa activities to determine whether increased PDHK4 protein expression in hyperthyroidism and after high-fat feeding was associated with altered PDHC phosphorylation (i.e. activity) status. Hearts were freeze-clamped from control and experimental animals in the absorptive state. Both hyperthyroidism and high-fat feeding were associated with significant suppression of cardiac PDHa activity (Figure 7A). Since, as in previous studies, total PDHC activities (expressed relative to the

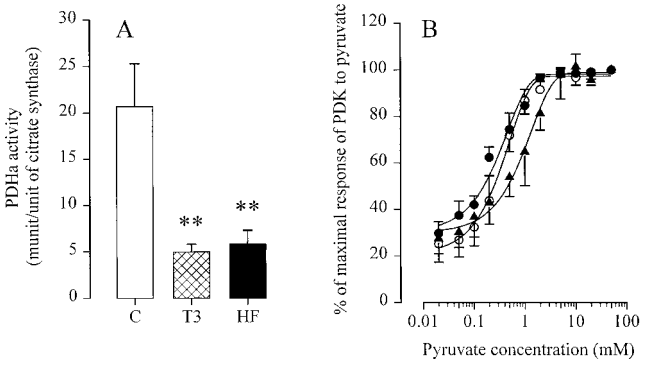


**Figure 7 Effects of hyperthyroidism and high-fat feeding on cardiac PDHa activity, and the sensitivity of PDHK activity to suppression by pyruvate**

(**A**) PDHa activities were measured in extracts of freeze-clamped heart of control rats (C), hyperthyroid rats (T3) and rats fed a high-fat diet (HF). (**B**) Sensitivity of PDHK activity to suppression by pyruvate, measured in freshly-isolated mitochondria from hearts of control rats (○), hyperthyroid rats (▲) and rats fed a high-fat diet (●). The results of the effects of pyruvate on PDHK activity are expressed as percentages of the maximal response to pyruvate observed with each individual mitochondrial preparation. Further details are given in the Materials and methods section. Values are means ± S.E.M. for 5–7 mitochondrial preparations from individual rats. Statistically significant differences from control rats are indicated by: $^{**}P < 0.01$. PDK, PDHK.

mitochondrial marker citrate synthase) were unchanged by these manipulations, effects that lower the concentration of active PDHC can be attributed to increased net phosphorylation of PDHC, i.e. an increased PDHK/PDHC phosphate phosphatase activity ratio.

We also examined whether the increases in the expression of the less pyruvate-sensitive PDHK isoform PDHK4 evoked by hyperthyroidism and high-fat feeding were accompanied by impaired suppression of PDHK activity by pyruvate. When rat heart mitochondria are incubated in the absence of respiratory substrate, the ATP concentration is low and $> 90$–$100\,\%$ of the extracted PDHC is in the active form [41,42]. The addition of respiratory substrates increases the ATP concentration, and this allows phosphorylation of PDHC by PDHK, with subsequent inactivation. By suppressing PDHK activity, pyruvate attenuates this inactivation. PDHa activities were measured in isolated cardiac mitochondria incubated with respiratory substrate (2-oxoglutarate/L-malate) in the presence or absence of pyruvate. The effects of increasing concentrations of sodium pyruvate on PDHa activities are shown in Figure 7(B). As observed previously [38,41,43,44], sodium pyruvate inhibited PDHK in rat heart mitochondria with activation of PDHC. The sodium pyruvate concentration giving $50\,\%$ active complex (i.e. $EC_{50}$) in mitochondria from fed control rats was approx. 0.25 mM. The $EC_{50}$ for PDHC activation was slightly increased by hyperthyroidism (to 0.80 mM), but was slightly decreased by high-fat feeding (to 0.09 mM). In contrast, the sodium pyruvate concentration giving $50\,\%$ active complex in heart mitochondria prepared from 48-h starved rats was 10.5 mM (results not shown).

## DISCUSSION

Suppression of PDHC activity by phosphorylation is achieved by the structurally related PDHK isoforms (PDHK1–4). Previous studies have shown that the adult rat heart contains three PDHK isoforms PDHK1, PDHK2 and PDHK4 [13]. Using Western-blot analysis, with specific antibodies raised against individual recombinant PDHK isoforms, the present study identified clear differences in the patterns of protein expression of each of these PDHK isoforms during the first 3 weeks of post-natal development, with most marked up-regulation of isoforms PDHK1 and PDHK4. Distinctions between the three cardiac PDHK isoforms were also demonstrated with respect to post-neonatal maturational up-regulation, with greatest up-regulation of PDHK1 and least up-regulation of PDHK4 from the post-neonatal period until maturity. Finally, we demonstrated isoform-selective regulation of PDHK isoform expression by thyroid status and fatty acid supply. Overall, our data identify PDHK isoform PDHK1 as being of more potential regulatory importance for glucose oxidation in the adult compared with the neonatal heart, and cardiac PDHK4 as a PDHK isoform whose expression is specifically responsive to changes in lipid supply, suggesting that its up-regulation during early post-natal life may underlie the perinatal switch to the use of fatty acids as the energy source. Finally, we identify regulation of pyruvate sensitivity of cardiac PDHK as a physiological variable, a change in which requires factors in addition to a change in lipid supply/utilization.

Until the present study, no information had been available regarding whether the pattern of expression of the individual PDHK isoforms in the heart, like those of the individual glucose transporters, varies during early post-natal life during the period of the thyroid hormone surge and cardiac substrate preference change. Thyroid hormones regulate many of the developmental shifts that characterize cardiac maturation during the early post-natal period, including the reciprocal changes in glucose transporter expression – up-regulation of GLUT4 and down-regulation of GLUT1 – observed during the first 3 weeks of life [18,19]. We have previously shown that tri-iodothyronine directly increases total PDHK activity in cultured cardiac myocytes from adult rats [45]. In view of this finding and the important involvement of thyroid hormones in cardiac maturation during the early post-natal period, we compared the patterns of protein expression of the individual PDHK isoforms with those of the glucose transporters. The overall pattern of up-regulation of two of the three PDHK isoforms, namely PDHK1 and PDHK2, follows the general pattern of change in thyroid hormone concentrations found post-natally, which peak during the third week of post-natal life [46]. The developmental increases in cardiac PDHK1 and PDHK2 protein expression also parallel the developmental increase in GLUT4 protein expression and decline in GLUT1 protein expression. Thus although the present study did not address directly the impact of the neonatal thyroid surge on the ontogeny of cardiac PDHK1 and PDHK2 protein expression during early post-natal development, our data are compatible with a facilitative role for thyroid hormones in the ontogeny of cardiac PDHK1 and PDHK2 protein expression during early post-natal development. It is notable that although thyroid hormones are essential for the maintenance of post-natal induction of GLUT4 and the repression of GLUT1 in the rat heart [47], hyperthyroidism down-regulates cardiac GLUT1 protein expression [26], and is without effect on cardiac GLUT4 protein expression in adulthood [26,32]. Age dependence of the effects of thyroid status may similarly underlie the present finding that the protein expression of PDHK1 and PDHK2 is not responsive to hyperthyroidism in the adult heart, even though the level of thyroid hormones in the blood of adult rats is only approx. one-half of that in 3-week-old animals [46]. The significance of the marked enhancement of relative abundance of PDHK1 and PDHK2 in adult compared with 21-day-old neonatal hearts is not known, but may be related to altered myocardial energy requirements or cardiac remodelling over this period. In particular, PDHK1 is uniquely expressed in the heart [12], suggesting a role related to its essential function as a rhythmically contracting pump.

The cardiac protein expression of PDHK4 has been shown previously to respond to insulin, increasing in insulin deficient states (starvation and diabetes) [13]. We therefore anticipated that the protein expression of PDHK4 would parallel that of the insulin-regulable glucose transporter GLUT4 during early life, thereby making pyruvate oxidation sensitive to insulin. However, the developmental profile of protein expression of PDHK4 was quite distinct from that of GLUT4 since the major increase in the relative abundance of PDHK4 occurred over the first 7 days of post-natal life, i.e. prior to the post-natal thyroid surge and the period of greatest up-regulation of cardiac GLUT4 protein expression. During the fetal period, glucose and lactate serve as the chief myocardial energy substrates. After birth and during the early post-natal period, myocardial energy is derived increasingly from reducing equivalents generated by the mitochondrial oxidation of long-chain fatty acids. In the present experiments, a high-fat diet led to specific up-regulation of the cardiac protein expression of PDHK4 in adulthood. Although the fatty acid composition of milk fat and the high-saturated-fat diet used in the present study differ, increased PDHK4 protein expression in response to an increased dietary fatty acid supply suggests that up-regulation of PDHK4 during the first week of post-natal life may be achieved in response to increased fatty acid provision via maternal milk. In support of this suggestion, both starvation and diabetes, situations associated with cardiac

PDHK4 up-regulation in adulthood, are associated with greatly enhanced fatty acid supply and rates of myocardial fatty acid oxidation. Up-regulation of PDHK4 protein expression in response to increased fatty acid supply/oxidation does not, nevertheless, exclude a role for thyroid hormone status in maintaining cardiac PDHK4 protein expression between post-natal days 15 and 21, when the pups start to ingest the high-carbohydrate/low-fat rodent diet and become less reliant on dietary fat (maternal milk) as the predominant fuel substrate.

Entry and exit of pyruvate from the tricarboxylic acid cycle via non-oxidative pathways are components of normal cardiac metabolism, and carbohydrate depletion can impair aerobic energy metabolism by reducing the level of tricarboxylic acid cycle intermediates. Thus for example, in heart muscle, depletion of tricarboxylic acid cycle intermediates through a block of 2-oxoglutarate dehydrogenase results in a rapid decline in contractile function, which is reversed by the addition of substrates promoting flux through the carboxylating enzymes, pyruvate carboxylase, malic enzyme and propionyl-CoA carboxylase [48]. The rapid up-regulation of cardiac PDHK4 protein expression in response to an increased lipid supply during the early neonatal period, when the protein expression of PDHK1 and PDHK2 remains relatively low, is associated with relatively low cardiac PDHa activities compared with those found in adulthood [neonate day 7, $8 \pm 1$ m-units/unit of citrate synthase ($n = 5$); adult, $21 \pm 5$ m-units/unit of citrate synthase ($n = 7$)]. Low PDHa activities would ensure that a significant proportion of available pyruvate is 'spared' for oxaloacetate formation (rather than oxidized via PDHC) to support the complete oxidation (via the tricarboxylic acid cycle) of acetyl-CoA derived from fatty acid $\beta$-oxidation. In addition, by maintaining tricarboxylic acid cycle flux, the accumulation of citrate – the immediate precursor of the cytoplasmic acetyl-CoA that is used for malonyl-CoA synthesis – will be impaired, and the resultant constraint on the production of malonyl-CoA will facilitate uptake of long-chain fatty acids into the mitochondria for oxidation.

The increased relative expression of PDHK4 protein relative to PDHK1 and PDHK2 protein in the heart in early neonatal life and in states associated with increased myocardial fatty acid utilization (hyperthyroidism, high-fat feeding, diabetes and starvation) may also have a regulatory function. PDHK in isolated cardiac mitochondria is activated acutely by increased NADH/$NAD^+$ and acetyl-CoA/CoA concentration ratios [41]. *In vivo* increased NADH/$NAD^+$ ratios are produced by high rates of $\beta$-oxidation; however, in the heart, carnitine acetylcarnitine transferase activity is high and changes in acetyl-CoA/CoA ratios may be buffered by the formation of acetylcarnitine [49]. Studies with recombinant PDHK proteins indicate that PDHK4 is relatively responsive to an increased mitochondrial NADH/$NAD^+$ concentration ratio compared with PDHK1 and PDHK2 [12]. However, the further addition of acetyl-CoA activates PDHK1 and PDHK2, but not PDHK4. Thus selective up-regulation of cardiac PDHK4 protein expression might sensitize cardiac glucose oxidation via PDHC to changes in rates of $\beta$-oxidation and render cardiac glucose oxidation increasingly sensitive to overall energy demand, as reflected by NADH accumulation.

Pyruvate is an allosteric inhibitor of PDHK: increasing pyruvate concentrations activate PDHC in respiring heart mitochondria [50], and administration of the pyruvate analogue dichloroacetate increases the percentage of active PDHC in heart in the fed state *in vivo* [51,52]. Starvation greatly decreases the susceptibility of cardiac PDHK activity to inhibition by pyruvate [31,43,44,53]. Studies with recombinant PDHK proteins have indicated that, compared with the other recombinant PDHK proteins, PDHK4 has a relatively high specific activity, but its activity is relatively insensitive to suppression by dichloroacetate [12]. In the present study, the degree of up-regulation of PDHK4 protein expression observed in the adult rat heart in response to hyperthyroidism and high-fat feeding is comparable to that observed in starvation (see [13]) and, again, is specific for PDHK4. Since the protein expression of PDHK1 and PDHK2 is unchanged, it is implied that the increase in PDHK activity elicited by these manipulations is due to targeted up-regulation of PDHK4. However, up-regulation of PDHK4 in the adult heart in hyperthyroidism and high-fat feeding occurs in the absence of any change in the sensitivity of PDHK activity to inhibition by pyruvate. Thus it can be concluded that up-regulation of PDHK4 isoform expression alone is insufficient to elicit changes in pyruvate sensitivity of cardiac PDHK activity, and although up-regulation of PDHK4 protein expression may be a prerequisite for changes in pyruvate sensitivity in starvation and diabetes, the mechanisms that control fluctuations of pyruvate sensitivity of PDHK are different from the mechanisms that control PDHK4 protein expression. In cardiac myocytes, in culture, the presence of a fatty acid and dibutyryl cAMP is sufficient both to up-regulate PDHK activity and to induce changes in pyruvate sensitivity analogous to those observed in starvation [31], but neither hyperthyroidism [27] nor high-fat feeding [30] led to altered steady-state cardiac cAMP concentrations *in vivo*. Thus available data are compatible with the concept that specific up-regulation of PDHK4 protein expression in the heart may occur via a mechanism linked with increased lipid supply and/or oxidation and, furthermore, that post-translational modification of one of the PDHK isoforms (possibly PDHK4) may influence cardiac PDHK pyruvate sensitivity through a mechanism linked in some way to cAMP-dependent protein phosphorylation or gene expression.

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