Evaluation of the role of peroxisome-proliferator-activated receptor α in the regulation of cardiac pyruvate dehydrogenase kinase 4 protein expression in response to starvation, high-fat feeding and hyperthyroidism

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Inactivation of cardiac pyruvate dehydrogenase complex (PDC) after prolonged starvation and in response to hyperthyroidism is associated with enhanced protein expression of pyruvate dehydrogenase kinase (PDK) isoform 4. The present study examined the potential role of peroxisome-proliferator-activated receptor α (PPARα) in adaptive modification of cardiac PDK4 protein expression after starvation and in hyperthyroidism. PDK4 protein expression was analysed by immunoblotting in homogenates of hearts from fed or 48 h-starved rats, rats rendered hyperthyroid by subcutaneous injection of tri-iodothyronine and a subgroup of euthyroid rats maintained on a high-fat/low-carbohydrate diet, with or without treatment with the PPARα agonist WY14,643. In addition, PDK4 protein expression was analysed in hearts from fed, 24 h-starved or 6 h-refed wild-type or PPARα-null mice. PPARα activation by WY14,643 *in vivo* over the timescale of the response to starvation failed to up-regulate cardiac PDK4 protein expression in rats maintained on standard diet (WY14,643, 1.1-fold increase; starvation, 1.8-fold increase) or influence the cardiac PDK4 response to starvation. By contrast, PPARα activation by WY14,643 *in vivo* significantly enhanced cardiac PDK4 protein expression in rats maintained on a high-fat diet, which itself increased cardiac PDK4 protein expression. PPARα deficiency did not abolish up-regulation of cardiac PDK4 protein expression in response to starvation (2.9-fold increases in both wild-type and PPARα-null mice). Starvation and hyperthyroidism exerted additive effects on cardiac PDK4 protein expression, but PPARα activation by WY14,643 did not influence the response of cardiac PDK4 protein expression to hyperthyroidism in either the fed or starved state. Our data support the hypothesis that cardiac PDK4 protein expression is regulated, at least in part, by a fatty acid-dependent, PPARα-independent mechanism and strongly implicate a fall in insulin in either initiating or facilitating the response of cardiac PDK4 protein expression to starvation.

Key words: fatty acid, insulin, peroxisome-proliferator-activated receptor α (PPARα), PPARα-null mice, pyruvate dehydrogenase complex.

INTRODUCTION

Inhibitory phosphorylation of the pyruvate dehydrogenase complex (PDC) is catalysed by the pyruvate dehydrogenase kinases (PDKs 1–4; [1,2], and reviewed in [3]). PDK activity in oxidative tissues, including the heart, increases stably in response to insulin deficiency (diabetes, prolonged starvation) [4–6], insulin resistance (high-fat feeding) [7,8] and hyperthyroidism [5,9,10]. These increases are independent of the acute effects of low-molecular-mass effectors. The heart contains three PDK isoforms (PDK1, PDK2, PDK4) [2,6,10]. However, increased cardiac PDK activity in diabetes [6], after prolonged starvation [6] and in the fed state in response to a high-saturated-fat diet [10] or hyperthyroidism [10] is concomitant with marked increases in PDK4 protein expression only. Although both experimental diabetes and starvation lead to insulin deficiency, enhanced cardiac PDK4 protein expression in the fed state in response to high-fat feeding [10] or hyperthyroidism [10] occurs despite high insulin levels [9–11]. Both high-fat feeding [8] and hyperthyroidism [9] lead to cardiac insulin resistance, suggesting that insulin deficiency is not obligatory for cardiac PDK4 up-regulation, but that, if insulin levels remain high, insulin resistance may be important.

In rat gastrocnemius muscle, activation of peroxisome-proliferator-activated receptor α (PPARα) by the inclusion of the selective agonist WY14,643 in the diet for 3 days lead to selective increases in PDK4 mRNA and protein [12]. PPARα is expressed in the heart [13–16] and PPARα-null mice accumulate myocardial lipid in response to a high-fat diet [17] and after starvation [17,18]. These latter data emphasize that PPARα-linked functions are important for cardiac disposal of fatty acids when their systemic delivery is increased. Cardiac PDK4 mRNA and protein expression is also enhanced in response to dietary administration of WY14,643 for 3 days in wild-type mice but not in PPARα-deficient mice [19]. Closer analysis, however, revealed a lack of complete attenuation of the enhancement of PDK4 protein expression elicited by starvation in hearts of PPARα-null mice. This was taken to indicate that additional mechanisms might contribute to regulation of cardiac PDK4 protein expression in starvation [19]. Importantly, both prolonged starvation and experimental diabetes, conditions associated with sustained increases in cardiac fatty acid utilization, suppress cardiac PPARα expression [20]. Thus whereas increased signalling through PPARα could potentially trigger increased cardiac PDK4 protein expression, the impact of PPARα signalling is, by necessity, suppressed once starvation or diabetes is established.

Thyroid hormone receptors (TRs) and PPARs both heterodimerize with retinoid X receptors (RXRs) [15]. Unlike PPARs, TRs also bind to DNA as monomers and as heterodimers to

Abbreviations used: FAO, fatty acid oxidation; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PPARα, peroxisome-proliferator-activated receptor α; NEFA, non-esterified fatty acids; TR, thyroid hormone receptor; RXR, retinoid X receptor; TAG, triacylglycerol; T3, tri-iodothyronine.

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direct repeats and to inverted palindromes spaced by between four and six nucleotides [21–23]. There are several potential mechanisms by which hyperthyroidism might influence cardiac PDK4 protein expression. Direct effects of hyperthyroidism may be mediated by binding of the TR to DNA via a mechanism independent of PPARα or the activated TR may heterodimerize with the RXR through a mechanism that may involve competition with or substitution for PPARα. In this regard it is of interest that, while both high-fat feeding and starvation suppress glucose utilization (uptake and phosphorylation) *in vivo* [8,24], rates of glucose utilization are maintained in the hyperthyroid heart in the fed state and elevated, compared with the euthyroid heart, after starvation [25]. This pattern of glucose utilization would be predicted to occur if activation of PPARα-linked functions in response to starvation were suppressed by hyperthyroidism. Indirect mechanisms whereby hyperthyroidism might influence cardiac PDK4 protein expression are also possible. Hyperthyroidism augments adipose-tissue lipolysis [26], even in the fed state, increasing fatty acid delivery to the heart [9,25], and increases cardiac fatty acid oxidation (FAO) [25]. Thus hyperthyroidism may modulate cardiac PDK4 protein expression secondary to increased adipose-tissue lipolysis, with a direct regulatory action of fatty acids or a lipid metabolite derived from fatty acids, and/or to increased myocardial lipid oxidation. Alternatively, since hyperthyroidism elicits cardiac insulin resistance, impaired cardiac insulin action could lead to the removal of a restraint on cardiac PDK4 protein expression imposed by insulin.

In the present study, we evaluated the involvement of PPARα activation in the response of cardiac PDK4 protein expression to starvation and high-fat feeding and elucidated the extent to which PPARα might be involved in the cardiac response to hyperthyroidism. To test the hypothesis that cardiac PDK4 up-regulation was dependent on increased cardiac fatty acid delivery, we compared the responses of cardiac PDK4 protein expression with PPARα activation by WY14,643 in fed and starved euthyroid rats, and in fed euthyroid rats maintained on a high-fat diet. We administered WY14,643 via injection, rather than as a component of the diet, to ensure that food intake was not compromised (and thus lipolysis was not activated) in the fed state, and to permit direct comparison of its actions in the fed and starved states. Using the PPARα-null mouse as a model where suppression of cardiac PPARα signalling by prolonged elevation of fatty acids in response to starvation is absent, we investigated whether the response of cardiac PDK4 protein expression to refeeding was modified by PPARα deficiency. Comparison was made with the effect of PPARα deficiency on cardiac PDK4 protein expression in the fed state, as well as after starvation. We also examined whether starvation and hyperthyroidism were associated with additive or non-additive effects on cardiac PDK4 protein expression to evaluate the involvement of impaired insulin signalling in the response of the cardiac PDK isoform profile to hyperthyroidism. Finally, to examine whether competition might exist between PPARα and TR with respect to RXR binding for regulation of cardiac PDK4 expression, we examined whether activation of PPARα by WY14,643 could modify the response to hyperthyroidism.

MATERIALS AND METHODS

Materials

General laboratory reagents were from Roche Diagnostics (Lewes, East Sussex, U.K.) or Sigma (Gillingham, Dorset, U.K.), with the following exceptions. ECL® reagents, hyperfilm and secondary antibodies were purchased from Amersham Bioscience (Little Chalfont, Bucks., U.K.). Anti-PDK4 antibodies were generated in rabbits against individual recombinant rat PDK4 proteins and were provided generously by Professor R. A. Harris (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.) [2]. These antibodies have been validated for use previously to detect PDK4 protein in mouse tissue [19]. WY14,643 was purchased from Sigma. Bradford reagents for protein estimation were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). Kits for determination of non-esterified fatty acid (NEFA) and triacylglycerol (TAG) concentrations were purchased from Alpha Labs (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:12 h light/dark cycle. Rats were allowed access *ad libitum* to water and standard high-carbohydrate/low-fat rodent laboratory diet [52% carbohydrate, 15% protein, 3% lipid and 30% non-digestible residue (by weight); 2.61 kcal (1 cal ≡ 4.184 J) of metabolizable energy/g] purchased from Special Diet Services (Witham, Essex, U.K.) or, in some studies, provided with a low-carbohydrate/high-saturated-fat diet (33% carbohydrate, 20% protein, 47% lipid by energy; 4.19 kcal of metabolizable energy/g), containing predominantly saturated fat (lard) as the major source of lipid (43% of total energy) and corn oil (4% of total energy) to prevent essential fatty acid deficiency [27]. 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 affect daily caloric intake. For studies of the effects of starvation, food was removed at the end of the dark phase and animals were sampled after 48 h. Rats were maintained on the high-fat diet for 4 weeks.

Rats were rendered hyperthyroid by subcutaneous injection of tri-iodothyronine (T3; 1 mg/kg body weight per day for 3 days) and the age-matched controls injected with hormone solvent (10 mM NaOH/0.03% BSA) [9,26]. WY14,643 was administered to euthyroid or hyperthyroid rats as a single intraperitoneal injection (50 mg/kg body weight) at 24 h before sampling [28]. WY14,643-treated 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. Daily food intakes did not differ between euthyroid control and euthyroid WY14,643-treated rats (control, 19.7 ± 1.1 g; WY14,643-treated, 20.5 ± 0.7 g) or hyperthyroid and WY14,643-treated hyperthyroid rats (hyperthyroid, 23.3 ± 1.2 g; WY14,643-treated hyperthyroid, 20.7 ± 1.5 g). Male PPARα-null mice bred on to an Sv/129 genetic background were provided kindly by Dr Jeffrey M. Peters and Dr Frank J. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.). Wild-type male Sv/129 mice were used as controls. Mice were used between the ages of 14 and 20 weeks. To facilitate the sampling process, mice were maintained on a 12 h:12 h light/dark cycle, with the 12 h light period beginning at 03:00 h. Mice were either fed *ad libitum*, fasted for 24 h or refed for 6 h *ad libitum* after fasting for 24 h. The diet consisted of fat (4.3%), carbohydrate (mainly starch, 51.2%), protein (22.3%), fibre (4.5%) and ash (7.7%). Fasted mice had food removed at the mid-point of the dark phase of the diurnal cycle. They were either killed 24 h later (starved group) or refed for 6 h during the remainder of the dark phase, whereby they were killed at the beginning of the light phase (refed group). Mice fed *ad libitum* were also killed at the beginning of

the light phase (fed group). For the mouse studies, a starvation period of 24 h was selected as PPARα-null mice develop hypoglycaemia even after 24 h of starvation [17].

Tissue and blood sampling

Rats and mice 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, freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen and stored in liquid nitrogen. Blood was sampled from the chest cavities of rats or from the aortas of mice after removal of the hearts, centrifuged for 5 min at 12000 ***g*** and plasma stored at −20 °C.

Immunoblotting

Hearts 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 $CaCl_2$, 10% glycerol, 1% Igepal, 45 mM sodium orthovanadate, 0.2 mM PMSF, 10 μg/ml leupeptin, 1.5 mg/ml benzamidine, 50 μg/ml aprotinin and 50 μg/ml pepstatin A in DMSO, pH 8.0). Homogenates were placed on ice for 20 min, centrifuged (10000 ***g***, 20 min, 4 °C) and the supernatants stored at −20 °C until analysis. Protein concentrations were determined using the method of Bradford with BSA as a standard. The assay was linear over the range of protein concentrations used. Samples (up to 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 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 recombinant rat PDK4, washed with TBST (three times for 5 min each) and incubated with horseradish peroxidase-linked anti-rabbit IgG secondary antibody (1:2000, in 1% 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, and the signals quantified by scanning densitometry and analysed with Molecular Analyst 1.5 software (Bio-Rad). For each panel in each Figure, the results are from a single gel exposed for a uniform duration. To correct for possible changes in total PDC expression, protein expression of the PDC component E1α was also analysed routinely. There were no statistically significant changes in E1α protein expression as a result of the *in vivo* manipulations undertaken (results not shown).

Statistical analysis

Results are presented as means ± S.E.M. 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, Berkeley, CA, U.S.A.). A P value of < 0.05 was considered to be statistically significant.

RESULTS

PPARα activation for 24 h *in vivo* fails to influence cardiac PDK4 protein expression in rats maintained on standard low-fat/high-carbohydrate diet, but increases its expression in rats on a high-fat/low-carbohydrate diet

A hypolipidaemic effect of WY14,643 was observed in euthyroid rats in the fed state (a 27% decline in TAG concentration, $P < 0.01$; Table 1) but exposure to this selective PPARα agonist for 24 h *in vivo* did not significantly alter cardiac PDK4 protein expression in rats maintained on standard (low-fat/high-carbohydrate) diet (Figure 1). We confirmed the effectiveness of WY14,643 treatment in these animals by demonstrating that PDK4 protein expression in hindlimb skeletal muscle (anterior tibialis) was significantly increased (by 1.8 ± 0.2-fold, $P < 0.01$; see also [3,12]). Thus the dose and period of exposure to WY14,643 selected for use in the present study were adequate to increase PDK4 protein expression in skeletal muscle, but not in heart, of rats maintained on standard low-fat/high-carbohydrate diet. This suggests differential tissue sensitivities to PPARα activators *in vivo*, with the heart being relatively unresponsive to PPARα activation.

Confirming previous observations [10], high-fat/low-carbohydrate feeding for 28 days elicited a 2.2-fold ($P < 0.001$) increase in cardiac PDK4 protein expression (Figure 1). Although WY14,643 treatment *in vivo* for 24 h in high-fat-fed rats only modestly decreased plasma TAG concentrations (Table 1), it evoked a further significant increase (1.5-fold, $P < 0.001$) in cardiac PDK4 protein expression (Figure 1). As a result, cardiac PDK4 protein expression was more than 3-fold higher in high-fat-fed rats treated with WY14,643 than in untreated control rats maintained on standard diet (Figure 1).

PPARα activation for 24 h *in vivo* fails to influence cardiac PDK4 protein expression in the starved state

Plasma NEFA concentrations in euthyroid rats were elevated after 48 h of starvation (by 3.1-fold, $P < 0.001$; Table 1). Starvation for 48 h markedly increased (1.8-fold, $P < 0.001$) cardiac PDK4 protein expression in euthyroid rats (Figure 2), confirming previous findings by others [6]. We administered WY14,643 to 24 h-starved rats and then extended the starvation period from 24 to 48 h. Consistent with its known effect of promoting FAO, activation of PPARα by WY14,643 treatment for 24 h decreased plasma NEFA concentrations in euthyroid rats in the starved state (by 27%, $P < 0.01$; Table 1); however, cardiac PDK4 protein expression was not significantly altered (Figure 2).

Impact of PPARα deficiency on cardiac PDK4 protein expression in the fed, starved and refed states

We compared levels of PDK4 protein expression in hearts of fed *ad libitum* wild-type and PPARα-null mice (Figure 3). Mice were sampled when diurnal insulin levels peaked [29]. Cardiac PDK4 protein expression was decreased significantly in *ad libitum*-fed PPARα-null mice (by 26%, $P < 0.05$) compared with the wild-type controls (Figure 3). There was no significant change in cardiac E1α protein levels in PPARα-null mice (results not shown). These observations suggest that PPARα signalling may contribute to cardiac PDK4 protein expression in the fed state. Most functional consequences of lowered PPARα gene expression are observed after starvation [17]. Increases in PDK4 protein expression elicited by starvation have been reported previously to be somewhat attenuated in hearts of PPARα-null mice (approx. 1.2-fold compared with approx. 1.4-fold increases, respectively; see [19]). In the present experiments, we observed a marked (2.9-fold, $P < 0.001$) increase in cardiac PDK4 protein in wild-type mice after 24 h of starvation (Figure 3). Although cardiac PDK4 protein expression was significantly lower in fed PPARα-null mice compared with fed wild-type mice, the effect of 24 h starvation to increase cardiac PDK4 protein expression was intact in the PPARα-null mice [a 2.9-fold increase ($P < 0.001$)

Table 1 Impact of PPARα activation on plasma lipid profiles in euthyroid and hyperthyroid rats in the fed and starved states or in rats fed a high-fat/low-carbohydrate diet for 28 days

Results are means ± S.E.M. for the numbers of rats indicated in parentheses.

	NEFA (mM)		TAG (mM)	
	Fed	Starved	Fed	Starved
Euthyroid	0.18 ± 0.04 (9)	0.56 ± 0.03*** (13)	0.87 ± 0.09 (11)	0.49 ± 0.05*** (12)
Euthyroid + WY14,643	0.18 ± 0.05 (9)	0.41 ± 0.04***‡‡ (11)	0.64 ± 0.08‡ (6)	0.43 ± 0.04 (8)
High-fat fed	0.10 ± 0.02 (8)	–	0.47 ± 0.06§§§ (10)	–
High-fat fed + WY14,643	0.11 ± 0.02 (5)	–	0.38 ± 0.05§ (11)	–
Hyperthyroid	0.35 ± 0.08† (5)	0.63 ± 0.02*** (6)	0.65 ± 0.05 (3)	0.49 ± 0.04 (3)
Hyperthyroid + WY14,643	0.31 ± 0.03† (6)	0.43 ± 0.05‡‡ (6)	0.56 ± 0.05 (3)	0.48 ± 0.12 (3)

*** $P < 0.001$, statistically significant effects of starvation.
† $P < 0.05$, statistically significant effects of hyperthyroidism.
‡ $P < 0.05$; ‡‡ $P < 0.01$, statistically significant effects of WY14,643 treatment.
§ $P < 0.05$; §§§ $P < 0.001$, statistically significant effects of high-fat feeding.

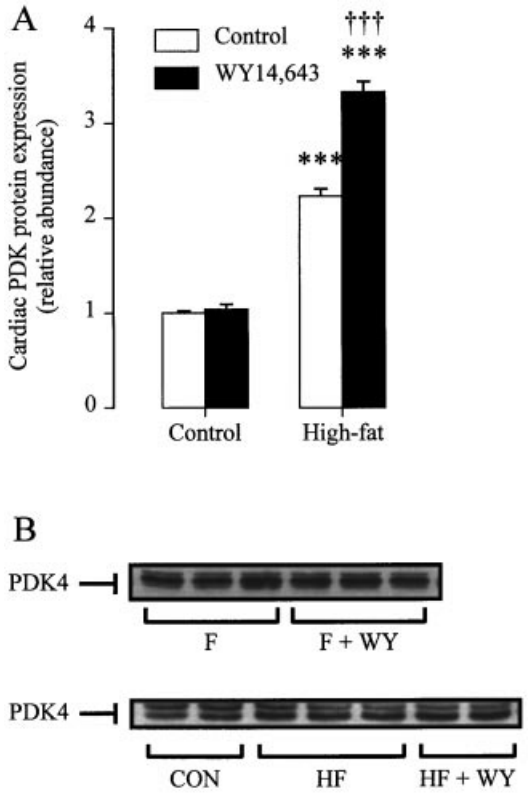

Figure 1 PPARα activation for 24 h *in vivo* fails to influence cardiac PDK4 protein expression in rats maintained on a standard low-fat/high-carbohydrate diet, but increases its expression in rats maintained on a high-fat/low-carbohydrate diet

Control rats maintained on a low-fat/high-carbohydrate diet or rats maintained on a high-fat/low-carbohydrate diet were treated with either WY14,643 for 24 h (solid bars) or vehicle (open bars). Rabbit polyclonal antisera raised against recombinant rat PDK4 protein was used to detect PDK4 protein using Western-blot analysis. Each lane corresponds to 25 μg of cardiac 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. from 12 [fed (F), CON], 10 [fed + WY14,643 (F + WY)], 3 [high-fat-fed (HF)] and 3 [high-fat-fed + WY14,643 (HF + WY)] heart preparations from individual rats in each experimental group. Statistically significant effects of high-fat feeding are indicated by ***($P < 0.001$). Statistically significant effects of WY14,643 treatment are indicated by †††($P < 0.001$).

identical with that observed in wild-type mice; Figure 3]. Nevertheless, cardiac PDK4 protein expression was 29% lower ($P < 0.001$) in PPARα-null mice compared with wild-type mice after starvation for 24 h by virtue of the lower level of PDK4 protein expression in PPARα-null mice in the fed state (Figure 3). We extended the study to examine the impact of a lack of PPARα signalling on the response of the cardiac PDK4 protein expression to refeeding (Figure 3). Refeeding for 6 h after 24 h of starvation elicited a marked 33% decline ($P < 0.001$) in cardiac PDK4 protein expression in wild-type mice, whereas the response to refeeding in PPARα-null mice was more muted (21% decline, $P < 0.001$; Figure 3). Nevertheless, because PDK4 protein expression was lower in the starved state in PPARα-null mice, cardiac PDK4 protein expression was 21% lower in refed PPARα-null mice compared with wild-type mice (Figure 3).

Impact of PPARα activation on plasma lipid profiles in fed and starved hyperthyroid rats

Plasma NEFA levels were higher in fed hyperthyroid rats than in fed euthyroid rats (Table 1). WY14,643 treatment of fed hyperthyroid rats failed to suppress plasma NEFA levels; as a result, plasma NEFA levels after WY14,643 treatment remained significantly higher in fed hyperthyroid rats than in fed euthyroid rats (Table 1). However, WY14,643 treatment significantly lowered plasma NEFA levels in starved hyperthyroid rats (32% decline, $P < 0.01$). Although plasma NEFA levels increased in response to starvation in hyperthyroid rats (2.5-fold increase, $P < 0.001$), this effect of starvation no longer achieved significance after WY14,643 treatment due to the effect of WY14,643 to lower plasma NEFA levels in starved hyperthyroid rats. Hyperthyroidism did not significantly affect plasma TAG concentrations in either the fed or starved state. In addition, the administration of WY14,643 did not alter plasma TAG concentrations in either fed or starved hyperthyroid rats (Table 1).

Starvation and hyperthyroidism exert approximately additive effects on cardiac PDK4 protein expression

We have demonstrated previously that the effects of starvation and hyperthyroidism on cardiac PDK activity are additive [9]. In the present study, we investigated whether starvation and hyperthyroidism elicited additive or non-additive effects on cardiac PDK4 protein expression. We have shown previously that experimental hyperthyroidism selectively increases cardiac PDK4 protein expression in the fed state [10]. In the present study,

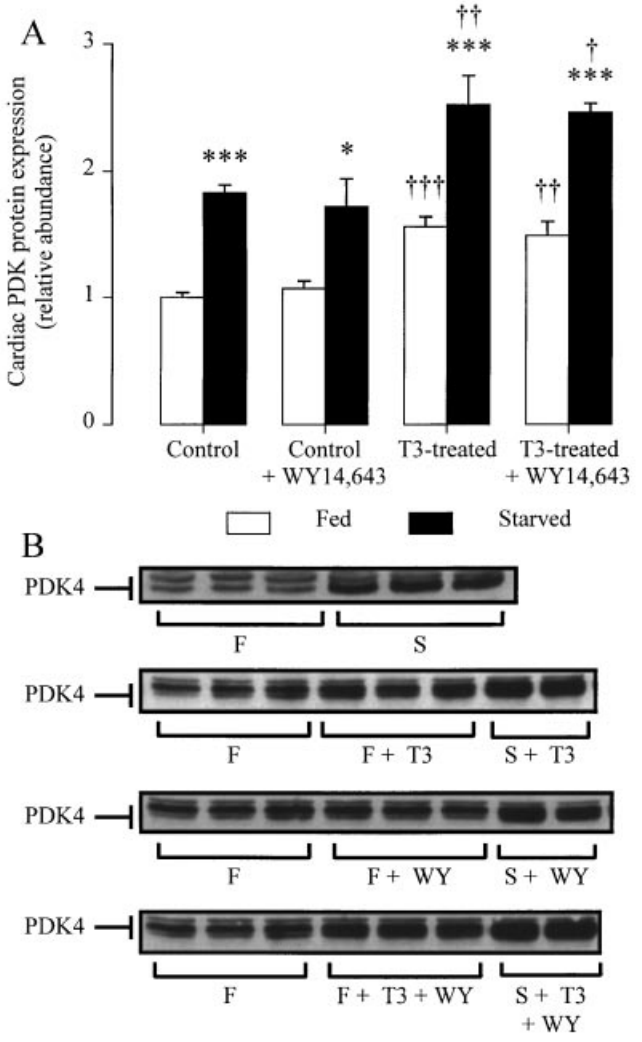

Figure 2 The response of the cardiac PDK4 protein expression to WY14,643 treatment or hyperthyroidism in the fed or starved state

Western-blot analysis of PDK4 protein expression was undertaken using hearts of fed (F; open bars) and starved (S; solid bars) rats. Each lane corresponds to 25 μg of heart protein. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software and expressed as 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. from 12 (fed), 4 (starved), 10 [fed + WY14,643 (F + WY)], 8 [starved + WY14,643 (S + WY)], 10 (fed + T3) and 8 (starved + T3) heart preparations from individual rats in each experimental group. Statistically significant effects of starvation are indicated by *($P < 0.05$) and ***($P < 0.001$). Statistically significant effects of hyperthyroidism are indicated by †($P < 0.05$), ††($P < 0.01$) and †††($P < 0.001$).

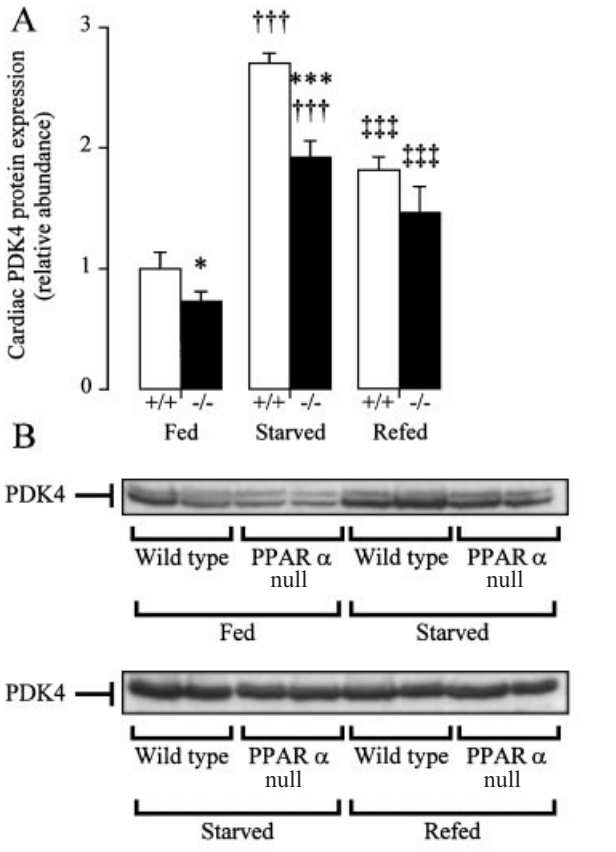

Figure 3 Effects of PPARα deficiency on the response of cardiac PDK4 protein expression to starvation and refeeding after starvation

Details of the starvation and refeeding protocols are given in the Materials and methods section. In brief, mice were starved for 24 h and refed for 6 h following the 24 h starvation. Rabbit polyclonal antisera raised against recombinant rat PDK4 were used to detect PDK4 protein using Western-blot analysis of hearts of wild-type mice and PPARα-null mice. Each lane corresponds to 25 μg of heart protein. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Quantification of Western analysis of cardiac PDK4 protein expression in fed, 24 h-starved or 6 h-refed wild-type (open bars) or PPARα-null (closed bars) mice is shown in (**A**). Data are means $\pm$ S.E.M. for 12 (fed), 16 (24 h-starved) and 4 (6 h refed) heart preparations from individual mice in each experimental group. Statistically significant effects of 24 h-starvation are indicated by *($P < 0.05$) and ***($P < 0.001$). Statistically significant differences between wild-type and PPARα-null mice are indicated by †††($P < 0.001$). Statistically significant effects of 6 h refeeding are indicated by ‡‡‡($P < 0.001$).

cardiac PDK4 protein expression increased 1.6-fold ($P < 0.001$) in fed hyperthyroid rats compared with fed euthyroid controls (Figure 2). In addition, starvation of hyperthyroid rats evoked a 1.6 $\pm$ 0.1-fold ($P < 0.001$) increase in cardiac PDK4 protein expression, comparable with the response observed in euthyroid rats (a 1.8-fold increase; Figure 2). Direct comparison revealed that cardiac PDK4 protein expression in starved hyperthyroid rats exceeded that in starved euthyroid rats (by 38 %, $P < 0.01$), suggesting a degree of additivity of the effects of starvation and hyperthyroidism on cardiac PDK4 protein expression (see Figure 2).

PPARα activation does not modulate the effect of hyperthyroidism on cardiac PDK4 protein expression

We administered WY14,643 to hyperthyroid rats during the last 24 h of the period of T3 treatment. PPARα activation did not abrogate the effect of hyperthyroidism to increase cardiac PDK4 protein expression in the fed state (Figure 2). We also investigated the effects of WY14,643 administration to starved T3-treated rats, administering WY14,643 at 24 h before sampling (i.e. between 24 and 48 h of starvation). Cardiac PDK4 protein levels in starved hyperthyroid rats were not significantly altered by administration of WY14,643 *in vivo* for 24 h (Figure 2).

DISCUSSION

PDC occupies a strategic role in cardiac intermediary metabolism [30,31]. Activation of PDC allows glucose oxidation, whereas

inactivation of PDC limits glucose oxidation. In the normal adult rat heart, PDC is active [32] and glucose utilization provides approx. 30% of total ATP requirement in the fed state, the remainder being provided by FAO [31]. The percentage contribution of FAO to ATP provision is increased further after starvation [31,33] and in hyperthyroidism [25], when PDC is inactivated. PPARα regulates basal and fatty acid-induced transcription of FAO genes and is critical to cardiac lipid handling after prolonged starvation (reviewed in [34]; see also [35]). The expression of mitochondrial and peroxisomal FAO enzymes is reduced in hearts of fasted PPARα-null mice, and such animals accumulate myocardial lipid [18]. PPARα-null mice maintained on a high-fat diet also accumulate myocardial lipid [17]. PPARα therefore has two potentially important roles in the heart. The first may be to enhance ATP supply by promoting FAO when glucose availability is limited. The second may be to enhance FAO to prevent cardiac lipid accumulation when fatty acid delivery exceeds the energetic requirement for FAO. Since we administered WY14,643 via injection, rather than as a dietary component, it was possible to vary dietary composition and to compare the effects of PPARα activation in the fed and starved states. We therefore evaluated and compared the possible involvement of PPARα activation in the responses of cardiac PDK4 protein expression to high-fat feeding and starvation. In addition, we limited the period of exposure to WY14,643 to 24 h, which is adequate to elicit up-regulation of cardiac PDK activity after food withdrawal.

When maintained on standard (low-fat/high-carbohydrate) diet, the exogenous fatty acid supply is low due to suppression of adipose-tissue lipolysis and, because the heart does not normally accumulate TAG and cardiac lipoprotein lipase activity is relatively low [36], other potential sources of fatty acids are limited. In addition, pharmacological activation of PPARα suppresses cardiac lipoprotein lipase activity, limiting the delivery of fatty acids from circulating lipoproteins [37]. By contrast, when rats are maintained on a high-fat diet, both dietary lipid delivery and cardiac lipoprotein lipase activity are increased [38,39]. PPARα activation by WY14,643 in fed rats over the timescale which elicits increased PDK4 protein expression in response to starvation failed to increase cardiac PDK4 protein expression in rats maintained on standard diet, although skeletal-muscle (anterior tibialis) PDK4 protein expression was increased. It has been noted recently that provision of WY14,643 for 4 days as a component of the diet increased rat heart PDK4 mRNA expression, but the effect was very much less than in skeletal muscle [20]. Similarly, provision of WY14,643 for 3 days as a component of the diet increases PDK4 mRNA expression by only 2–3-fold in the mouse heart, compared with > 4-fold in the mouse kidney [19]. Thus the apparent lack of impact of WY14,643 treatment for 24 h, compared with 3–4 days, on cardiac PDK4 protein expression may reflect the fact that, in the euthyroid fed state, the heart is a relatively poor target for PPARα activators compared with other tissues. Alternatively, PDK4 protein expression in the adult rat heart is already maximally activated by mechanisms operating through PPARα signalling in the fed state, such that further pharmacological stimulation of PPARα by exogenous agonist has little further effect on PDK4 protein expression. If so, then reduced PPARα signalling would be predicted to reduce cardiac PDK4 protein expression. In support of the latter, our data indicated modest but statistically significant suppression of cardiac PDK4 protein expression in the PPARα-null mouse in the fed state. However, provision of dietary lipid both itself increased cardiac PDK4 protein expression and facilitated a response of cardiac PDK4 protein expression to PPARα activation by WY14,643 administration. Taken together, the data suggest that increased fatty acid delivery is required for PPARα activation to enhance cardiac PDK4 protein expression. Thus high-fat feeding may enhance cardiac PPARα expression, thereby increasing the capacity for PPARα signalling if adequate agonist (e.g. WY14,643) is provided. Although outside the scope of the present study, use of a recently identified inhibitor of PPARα [40] would permit direct assessment of the role of PPARα in the response of cardiac PDK4 protein expression to high-fat feeding.

Most functional consequences of lowered PPARα gene expression are observed after starvation [17], where circulating insulin levels are low and fatty acid levels are elevated because of the absence of insulin's anti-lipolytic action. However, activation of PPARα by WY14,643 during the last 24 h of a 48 h period of starvation, while enhancing FAO (as demonstrated by a significant decline in plasma fatty acid levels), failed to augment the enhancement of cardiac PDK4 protein expression observed in response to starvation in euthyroid rats. It is possible that the concentrations of the endogenous natural PPARα ligands (including certain fatty acids [41]) after starvation are sufficient to elicit maximal activation of cardiac PPARα and therefore the administration of an exogenous PPARα ligand (WY14,643) would be incapable of further activating PPARα. This would support the contention that the effect of starvation to increase cardiac PDK4 protein expression is mediated via fatty acid signalling through PPARα. However, a direct comparison of PDK4 protein expression in hearts of fed WY14,643-treated rats versus starved WY14,643-treated rats revealed that the magnitude of the response to starvation was retained, although it was somewhat less after pharmacological PPARα activation. Prolonged starvation has been reported to decrease cardiac PPARα expression [20,42], implying that PPARα has a limited involvement in the maintenance of high cardiac PDK4 protein expression after prolonged starvation. This is supported by our demonstration that up-regulation of cardiac PDK4 protein expression after 24 h of starvation is intact in PPARα-deficient mice. Others have detected only partial impairment of PDK4 protein expression up-regulation in response to starvation in PPARα-null mice, albeit under conditions where only a modest (50%) increase in cardiac PDK4 protein expression was observed after starvation even in the wild-type group [19]. Taken together, the data indicate that PPARα activation can indirectly elicit, but is not obligatory for, the response of cardiac PDK4 protein expression to starvation.

We also reasoned that the normal down-regulation of cardiac PPARα observed in response to starvation [20] would limit the impact of changes in PPARα signalling on the response to refeeding. We therefore analysed the response of cardiac PDK4 protein expression to refeeding in PPARα-null mice and age-matched wild-type controls. The response of cardiac PDK4 protein expression to refeeding in PPARα-null mice was retained, albeit more muted than in wild-type mice. Nevertheless, cardiac PDK4 protein expression was lower in refed PPARα-null mice compared with wild-type mice, due to lower PDK4 protein expression in the starved state.

Thyroid hormones exert an important influence on cardiac function and induce cardiac hypertrophy [43,44]. Both PPARα and TR bind to the RXR [21], but, unlike PPARs, TRs can also bind to DNA as monomers and heterodimers [21–23]. Consequently, potential mechanisms by which hyperthyroidism might influence cardiac PDK4 protein expression are via direct binding of the TR to DNA via a mechanism independent of PPARα or heterodimerization of the activated TR with the RXR through a mechanism that may involve competition with or substitution for PPARα. We therefore examined the extent to which PPARα activation might modify the response of cardiac PDK4 protein

expression to hyperthyroidism. We confirmed previous studies [10] demonstrating that experimental hyperthyroidism enhances both fatty acid levels and cardiac PDK4 protein expression in the fed state. Although PPARα has been implicated in the pathology of diabetes [44,45], we failed to detect any further change in cardiac PDK4 protein expression in fed hyperthyroid rats in response to PPARα activation by WY14,643. Thus whereas hyperthyroidism leads to increased adipose-tissue lipolysis, and therefore increased NEFA delivery to the heart, the lack of response of PDK4 protein expression to PPARα activation in the hyperthyroid heart *in vivo* resembled that of euthyroid rats maintained on standard low-fat diet, rather than those maintained on high-fat diet. This suggests that, as in euthyroid rats, PPARα may be fully activated by endogenous agonists in the fed hyperthyroid state.

Cardiac PPARα expression and activity are suppressed in cardiac hypertrophy [46], whereas cardiac glucose utilization is increased (reviewed in [47]). If PDK4 protein expression in starvation was mediated predominantly via PPARα signalling, it would be predicted that hypertrophied hyperthyroid heart would exhibit impaired up-regulation of cardiac PDK4 protein expression in response to starvation. However, the present data did not demonstrate that hyperthyroidism impairs the response of cardiac PDK4 protein expression to starvation, rather that starvation and experimental hyperthyroidism exert approximately additive effects on cardiac PDK4 protein expression. Thus hyperthyroidism increases cardiac PDK4 protein expression not only in the fed state, but also after starvation. As for euthyroid rats, PPARα activation for 24 h *in vivo* had no effect on cardiac PDK4 protein expression in hyperthyroid rats in the starved state. Both hyperthyroid rats [11] and PPARα-null mice [48] respond to starvation with a lowering of plasma insulin levels and a rise in plasma NEFA levels. Our data therefore support the hypothesis that cardiac PDK4 protein expression is regulated, at least in part, by a fatty acid-dependent, PPARα-independent mechanism and strongly implicate a fall in insulin in either initiating or facilitating the response of cardiac PDK4 protein expression to starvation.

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