Up-regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) protein expression in oxidative skeletal muscle does not require the obligatory participation of peroxisome-proliferator-activated receptor α (PPARα)

Mark J. HOLNESS*, Karen BULMER*, Geoffrey F. GIBBONS† and Mary C. SUGDEN*[1]

*Department of Diabetes and Metabolic Medicine, Barts & the London, Queen Mary's School of Medicine and Dentistry, University of London, London E1 4NS, U.K., and †Oxford Lipid Metabolism Group, Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

In insulin deficiency, increased lipid delivery and oxidation suppress skeletal-muscle glucose oxidation by inhibiting pyruvate dehydrogenase complex (PDC) activity via enhanced protein expression of pyruvate dehydrogenase kinase (PDK) isoform 4, which phosphorylates (and inactivates) PDC. Signalling via peroxisome-proliferator-activated receptor α (PPARα) is an important component of the mechanism enhancing hepatic and renal PDK4 protein expression. Activation of PPARα in gastrocnemius, a predominantly fast glycolytic (FG) muscle, also increases PDK4 expression, an effect that, if extended to all muscles, would be predicted to drastically restrict whole-body glucose disposal. Paradoxically, chronic activation of PPARα by WY14,643 treatment improves glucose utilization by muscles of insulin-resistant high-fat-fed rats. In the resting state, oxidative skeletal muscles are quantitatively more important for glucose disposal than FG muscles. We evaluated the participation of PPARα in regulating PDK4 protein expression in slow oxidative (SO) skeletal muscle (soleus) and fast oxidative-glycolytic (FOG) skeletal muscle (anterior tibialis) containing a high proportion of oxidative fibres. In the fed state, acute (24 h) activation of PPARα by WY14,643 *in vivo* failed to modify PDK4 protein expression in soleus, but modestly enhanced PDK4 protein expression in anterior tibialis. Starvation enhanced PDK4 protein expression in both muscles, with the greater response in anterior tibialis. WY14,643 treatment *in vivo* during starvation did not further enhance upregulation of PDK4 protein expression in either muscle type. Enhanced PDK4 protein expression after starvation was retained in SO and FOG skeletal muscles of PPARα-deficient mice. Our data indicate that PDK4 protein expression in oxidative skeletal muscle is regulated by a lipid-dependent mechanism that is not obligatorily dependent on signalling via PPARα.

Key words: fatty acids, pyruvate dehydrogenase complex.

INTRODUCTION

The skeletal muscle mass is of major quantitative importance for glucose disposal, particularly under conditions of insulin stimulation [1]. However, when insulin levels decline (as after starvation) or insulin action is impaired (insulin resistance), whole-body glucose utilization declines, largely due to a decline in glucose utilization by oxidative slow-twitch skeletal muscle and, to a lesser extent, oxidative fast-twitch skeletal muscle [2,3]. In these oxidative muscles, decreased glucose utilization is compensated by increased supply and utilization of available lipid-derived substrates [non-esterified fatty acids (NEFA), triglyceride (TAG) and ketone bodies]. These observations support the concept that substrate competition, with preferential oxidation of lipid rather than glucose, underlies the development of insulin resistance and decreases glucose utilization [4].

The pyruvate dehydrogenase complex (PDC) is classically viewed as a major target for substrate competition, particularly in tissues such as skeletal muscle where PDC has a purely energetic role (reviewed in [5]). PDC catalyses the physiologically irreversible oxidative decarboxylation of pyruvate and hence loss of glucose carbon as CO_2. Consequently, regulation of skeletal muscle PDC activity is a potentially important component of the regulation of whole-body glucose homeostasis. PDC is rendered inactive by phosphorylation of the α-subunit of its pyruvate dehydrogenase (PDH) component by pyruvate dehydrogenase kinase (PDK) (reviewed in [5] and [6]). Four PDK isoenzymes (PDK1–4) have been identified in mammalian tissues [7]. The PDKs are activated by acetyl-CoA and NADH, the products of fatty acid (FA) oxidation (reviewed in [8]). Thus, the PDKs constitute the molecular targets through which the capacity for glucose oxidation can be diminished when FA oxidation is increased.

Skeletal-muscle PDK4 protein expression is selectively increased in insulin deficient [9,10] and insulin resistant states, both in rat [11] and man [12]. The potential significance of this observation is that PDK4 is of higher specific activity and more markedly activated by products of FA oxidation, NADH and acetyl-CoA, than is PDK2 [7], the second PDK isoform present in skeletal muscle [9]. In addition, administration of the systemic lipid-lowering agent WY14,643 [a peroxisome-proliferator-activated receptor α (PPARα) agonist] as a component of the

Abbreviations used: DCA, dichloroacetate; FA, fatty acid; FG, fast-twitch glycolytic; FOG, fast oxidative-glycolytic; NEFA, non-esterified fatty acids; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PPARα, peroxisome-proliferator-activated receptor α; SO, slow-twitch oxidative; TAG, triglyceride; TBS, Tris-buffered saline; TBST, TBS supplemented with 0.05% Tween; UCP, uncoupling protein.

[1] To whom correspondence should be addressed (e-mail m.c.sugden@qmul.ac.uk).

diet for 3 days leads to selective up-regulation of PDK4 in rat gastrocnemius muscle [9]. This suggests that signalling via PPARα could represent an important determinant of the level of PDK4 protein expression in skeletal muscle. Up-regulation of PDK4 expression by activation of PPARα would therefore be predicted to limit glucose oxidation. This, in the fed state, where glucose is readily available, would in turn be predicted to impair glucose clearance, limiting the beneficial systemic lipid-lowering effects of PPARα activation. Paradoxically, pharmacological activation of PPARα by WY14,643 for 2 weeks enhances muscle insulin sensitivity at the level of glucose uptake in insulin-resistant high-fat-fed rats [13]. In addition, Guerre-Millo and colleagues have demonstrated that PPARα activation by fenofibrate, ciprofibrate and GW9578 markedly lowered hyperinsulinaemia and, when present, hyperglycaemia in two rodent models of high-fat diet-induced (C57BL/6 mice) or genetic (obese Zucker rats) insulin resistance [14]. Conversely, PPARα deficiency appears to prevent high-fat diet-induced insulin resistance [15] and elicit greater suppression of endogenous glucose production in hyperinsulinaemic clamp experiments, reflecting less insulin-resistance in the absence of PPARα [16]. One possibility is that, via its effects to increase the capacity of skeletal muscle for FA oxidation, activation of PPARα by WY14,643 may up-regulate skeletal-muscle PDK4 protein expression without impairing insulin-mediated skeletal-muscle glucose uptake and, therefore, whole-body glucose homeostasis. Alternatively, it is possible that the mechanisms modulating PDK4 protein expression vary between different tissues, such that despite enhanced PDK4 expression in some tissues, there is nevertheless a net overall improvement in whole-body insulin action because PDK4 up-regulation does not occur in all tissues.

Studies in Morris hepatoma 7800 C1 cells have demonstrated that PPARα activation by WY14,643 and dexamethasone independently increase PDK4 mRNA and protein levels [17]. Incubation of Morris hepatoma 7800 C1 cells with FA (palmitate or oleate) also increases PDK4 mRNA levels. We have recently demonstrated that up-regulation of hepatic and renal PDK4 protein expression in response to prolonged starvation is markedly impaired in PPARα-deficient mice [18–20]. However, the enhancement of PDK4 protein expression elicited by starvation is only modestly attenuated in hearts of PPARα-null mice, suggesting that additional mechanisms might contribute to regulation of cardiac PDK4 protein expression in starvation [20–22]. The possibility is therefore raised that the mechanisms governing PDK4 protein expression vary between different skeletal muscle types, namely slow oxidative (SO) versus fast-oxidative-glycolytic (FOG) versus fast glycolytic (FG). If this is so, then suppression of glucose oxidation through PPARα-linked PDK4 up-regulation may have only a limited impact on peripheral glucose uptake under conditions of insulin stimulation.

Here we evaluate the importance of the involvement of PPARα activation in enhancing PDK4 protein expression in response to increased lipid supply/utilization in a representative SO skeletal muscle (soleus) and a representative FOG skeletal muscle containing a relatively high proportion of oxidative fast-twitch fibres (anterior tibialis). Having established that 24 h was the minimum period required to increase PDK4 protein expression in muscle, we limited the period of PPARα activation to 24 h. We administered WY14,643 via injection, rather than as a component of the diet, allowing direct comparison of its effects in the fed state, where adipose tissue lipolysis is suppressed, and after starvation, where lipolysis and ketogenesis are active. Using PPARα-null mice, we investigated the impact of PPARα-deficiency on the control of PDK4 protein expression in SO and FOG muscles in the fed state and in response to the elevated NEFA supply elicited by starvation. This latter approach allowed discrimination between the direct effects of PPARα activation and those of altered lipid delivery on PDK4 protein expression in skeletal muscle, in the absence of potentially confounding effects of modulation of PPARα signalling by endogenous ligands.

MATERIALS AND METHODS

Materials

General Laboratory reagents were from Roche Diagnostics (Lewes, East Sussex, U.K.) or from Sigma (Poole, Dorset, U.K.). Enhanced chemiluminescence (ECL®) reagents, Hyperfilm and secondary antibodies were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Anti-PDK4 antibodies were generated in rabbits against recombinant rat PDK4 protein [7] and were generously provided by Professor Bob Harris (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, U.S.A.). These antibodies have been used previously by ourselves [19,22] and others [20] to detect PDK4 protein in mouse tissue. WY14,643 was purchased from Sigma. Bradford reagents for protein estimation were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). Arylamine acetyltransferase was purified from pigeon liver acetone powder (purchased from Europa Bioproducts, Ely, Cambs., U.K.). Kits for determination of plasma insulin and blood 3-hydroxybutyrate concentrations were purchased from Mercodia (Uppsala, Sweden) and Sigma respectively. Kits for determination of plasma NEFA and 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 light/12 h 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 mass; 2.61 kcal metabolizable energy/g) purchased from Special Diet Services (Witham, Essex, U.K.) or starved for 48 h. WY14,643 was administered as a single intraperitoneal injection (50 mg/kg body mass) at 24 h before sampling [23]. 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. Male PPARα-null mice bred on to an Sv/129 genetic background were kindly provided by Dr J. M. Peters and Dr F. 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. Mice were maintained on a reverse light/dark cycle (12 h dark phase 03:00–15:00, 12 h light phase 15:00–03:00), and were either fed *ad libitum* (standard high-carbohydrate/low-fat rodent laboratory diet) and sampled at the end of the dark (feeding) phase, or starved for 24 h. A starvation period of 24 h was selected for the mouse studies; a more prolonged period of starvation was considered to be inappropriate since the PPARα-null mice may develop severe hypoglycaemia even after 24 h starvation [24].

Tissue sampling

Rats and mice were anaesthetized by injection of sodium pentobarbital [60 mg/ml in 0.9 % (w/v) NaCl; 1 ml/kg body mass,

intraperitoneal] and, once locomotor activity had ceased, skeletal muscles were rapidly excised and freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen. Frozen muscles were stored in liquid nitrogen. The fibre profiles (FOG: FG: SO) of the muscles selected for study are as follows: soleus, 0: 0: 100; anterior tibialis, 66: 32: 2; gastrocnemius, 37: 58: 5 [25]. In some experiments, blood was sampled from the chest cavity after the removal of the hearts. Blood samples were centrifuged for 5 min at 10000 ***g*** and plasma was stored at −20 °C.

Immunoblotting

Samples (25 μg of total protein) were subjected to SDS/PAGE using a 12 % resolving gel, with a 6 % stacking gel as previously described [11,26]. Following SDS/PAGE, resolved proteins were transferred electrophoretically to nitrocellulose membranes, and then blocked for 2 h at 20 ± 1 °C with Tris-buffered saline (TBS) 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. They were then washed with 0.05 % Tween in TBS (3 × 5 min) and incubated with horseradish peroxidase-linked secondary antibody IgG anti-rabbit [diluted at 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 exposed to Hyperfilm, the signals quantified by scanning densitometry and analysed with Molecular Analyst software (Bio-Rad). The amounts of extracts loaded on to gels were varied to establish that the relative densities of the bands corresponding to PDK4 were linear with concentration. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range. For each panel in each figure, the results are from a single gel exposed for a uniform duration.

PDH activities

In a limited series of experiments, soleus, anterior tibialis and gastrocnemius muscles were freeze-clamped for estimation of PDC active and total activities, as described previously [27]. To assess the impact of PDK inhibition on the percentage of active PDC (% total), rats were treated with dichloroacetate [DCA; 0.25 ml of a 5 % (w/v) solution in 0.9 % (w/v) NaCl] at 30 min intervals for 2 h, as described previously [28].

Statistical analysis

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

RESULTS AND DISCUSSION

PPARα activation for 24 h *in vivo* increases PDK4 protein expression in FOG but not SO skeletal muscle in the fed state

A previous study demonstrated that activation of PPARα by administration of WY14,643 as a component of the diet for 3 days selectively up-regulates protein expression of PDK4 in rat gastrocnemius muscle (comprising approximately two-thirds FG fibres and one-third FOG fibres) [9]. In the present experiments, WY14,643 was administrated by injection (as described in [22,23]), and PDK4 protein expression was analysed in anterior tibialis (comprising two-thirds FOG fibres and one-third FG fibres) and soleus, composed entirely of SO fibres. Muscles were sampled after a 24 h period of exposure, a time scale that is comparable to that period over which starvation increases tissue PDK activity. Acute PPARα activation *in vivo* by WY14,643 did not influence food intake [control, 19.7 ± 1.1 g ($n = 8$); WY14,643-treated, 20.5 ± 0.7 g ($n = 6$)] and steady-state plasma insulin concentrations were maintained [control, 31 ± 3 μ-units/ml ($n = 20$); WY14,643-treated, 29 ± 6 μ-units/ml ($n = 6$)]. However, as expected from its known hypolidaemic action, WY14,643 treatment resulted in a 32 % decline in plasma TAG concentration ($P < 0.05$) [control, 0.87 ± 0.09 mM ($n = 11$); WY14,643-treated, 0.59 ± 0.08 mM ($n = 15$)]. PPARα activation in fed rats was not associated with any significant modification of plasma NEFA or 3-hydroxybutyrate concentrations (Figure 1), showing that the restraint imposed by insulin on lipolysis (and therefore ketogenesis) remains intact.

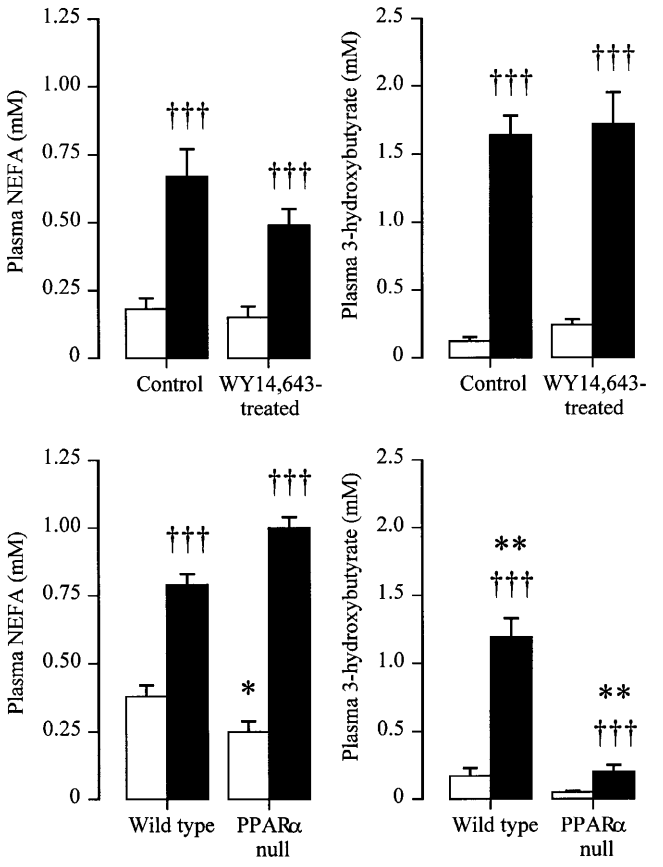

Figure 1 Effects of starvation on plasma NEFA and 3-hydroxybutyrate concentrations in control rats and WY14,643-treated rats and in wild-type mice and PPARα-null mice

Blood was sampled from control rats or rats treated with WY14,643 for 24 h *in vivo* in either the fed state (white bars) or after starvation for 48 h (black bars). Blood was also sampled from wild-type mice and PPARα-null mice in either the fed state (white bars) or after starvation for 24 h (black bars). Data are means ± S.E.M. for 9 (fed), 21 (starved), 5 (fed + WY14,643) and 10 (starved + WY14,643) individual rats and for 5 (fed, wild-type), 4 (starved, wild-type), 4 (fed, PPARα-null) and 4 (starved, PPARα-null) individual mice. Statistically-significant effects of 48 h-starvation are indicated by: ††† $P < 0.001$. Statistically-significant differences between wild-type and PPARα-null mice are shown by * $P < 0.05$; ** $P < 0.01$.

Analysis of the response of PDK4 protein expression in anterior tibialis revealed a significant (54 %; $P < 0.001$) increase

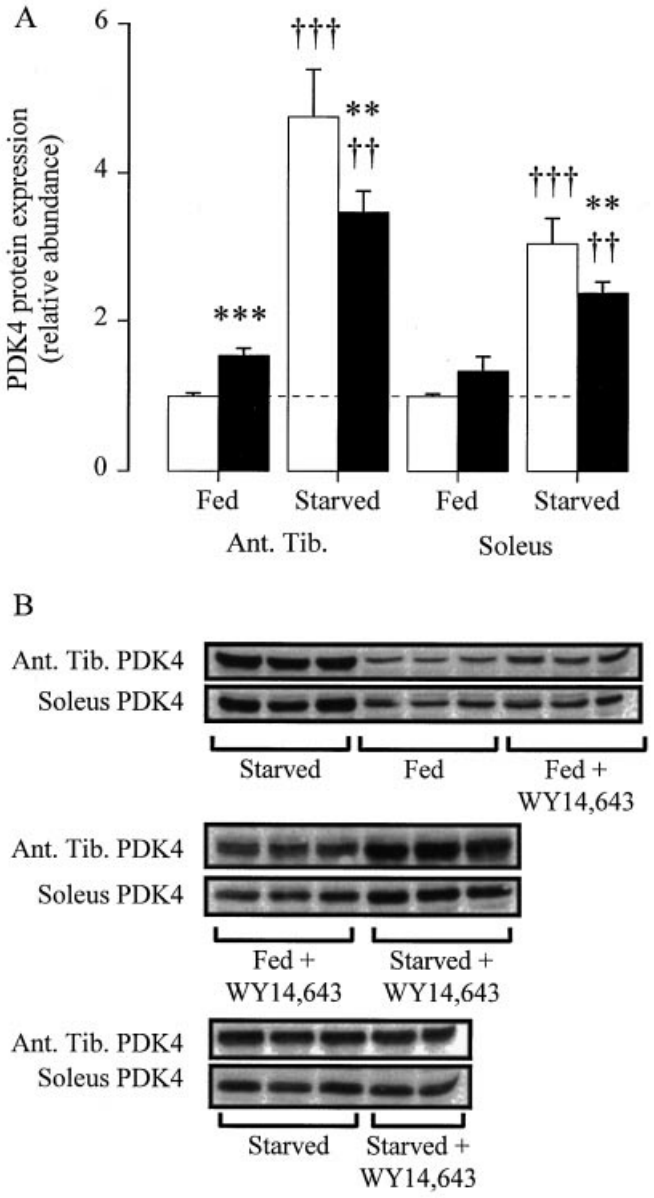

Figure 2 Effects of acute (24 h) *in vivo* activation of PPARα by WY14,643 on the PDK4 protein expression in SO (soleus) and FOG (anterior tibialis) skeletal muscles of fed or 48 h starved rats

Muscles were sampled from control rats (white bars) or rats treated with WY14,643 for 24 h *in vivo* (black bars) in either the fed state or after starvation for 48 h, as indicated. Rabbit polyclonal antisera raised against recombinant rat PDK4 were used to detect PDK4 protein using Western-blot analysis. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Quantification of Western analysis of PDK4 protein expression is shown for anterior tibialis (Ant. Tib.) and for soleus in (**A**). Data are means ± S.E.M. for muscles from 12 (fed), 8 (starved), 10 (fed + WY14,643) and 5 (starved + WY14,643) individual rats. Typical immunoblots for PDK4 protein expression in anterior tibialis and soleus skeletal muscles of control rats, or rats treated with WY14,643 for 24 h and sampled in either the fed state or after starvation for 48 h, are shown in (**B**). Each lane corresponds to 25 μg of protein from muscle from a different individual rat. Statistically-significant effects of 48 h-starvation are indicated by: ††$P < 0.01$; †††$P < 0.001$. Statistically-significant effects of WY14,643 treatment are shown by **$P < 0.01$; ***$P < 0.001$.

in PDK4 protein expression in response to exposure to the selective PPARα agonist WY14,643 for 24 h *in vivo* (Figure 2). Protein expression of PDH E1α in anterior tibialis was unchanged by WY14,643 treatment [relative protein expression, control, 1.00 ± 0.04 ($n = 8$); WY14,643-treated, 1.10 ± 0.04 ($n = 8$); not significant]. In the same animals, PPARα activation by WY14,643 treatment did not significantly increase PDK4 protein expression in soleus (Figure 2). Again, exposure to WY14,643 for 24 h did not affect protein expression of E1α in soleus in the fed state [relative protein expression, control, 1.00 ± 0.02 ($n = 8$); WY14,643-treated, 1.00 ± 0.06 ($n = 8$); not significant]. The present study therefore extends previous observations of enhanced PDK4 protein and mRNA expression in rat FG skeletal muscle after prolonged activation of PPARα with WY14,643 over a 3 day timescale [9] by demonstrating that activation of PPARα with WY14,643 *in vivo* can also increase PDK4 protein expression in a predominately FOG skeletal muscle. This response cannot be attributed to insulin deficiency, nor to any increase in lipid delivery. In addition, we demonstrate, for the first time, that the PDK4 response to PPARα activation is absolutely selective for fast-twitch skeletal muscle. Thus, acute (24 h) PPARα activation *in vivo* did not significantly increase PDK4 protein expression in a SO skeletal muscle (soleus) although PDK4 protein expression significantly increased in a FOG skeletal muscle (anterior tibialis) of the same animal.

PPARα activation for 24 h *in vivo* does not increase PDK4 protein expression in FOG or SO skeletal muscle in the starved state

We studied rats after starvation for 48 h to investigate the influence of increased lipid fuel supply/oxidation on muscle PDK4 protein expression. As expected, starvation significantly increased the plasma concentration of NEFA (3.7-fold; $P < 0.001$) and 3-hydroxybutyrate (13.7-fold; $P < 0.001$) (Figure 1), and evoked a significant decline (97%; $P < 0.001$) in ambient insulin concentrations [control fed, 31 ± 3 μ-units/ml ($n = 20$); control starved, 1 ± 0.2 μ-units/ml ($n = 5$)]. These starvation-induced changes were associated with a marked (4.8-fold; $P < 0.001$) increase in PDK4 protein expression in rat anterior tibialis (Figure 2A), confirming previous findings [10]. Starvation also elicited a 25% increase in E1α protein expression, which was significant [relative protein expression, control, 1.00 ± 0.04 ($n = 8$); starved, 1.25 ± 0.03 ($n = 3$); $P < 0.001$] in anterior tibialis. The effect of starvation to increase PDK4 protein expression in the SO soleus muscle (a 3.1-fold increase; $P < 0.001$) was less than in the FOG anterior tibialis muscle (Figure 2A). Protein expression of E1α in soleus [relative protein expression, control, 1.00 ± 0.02 ($n = 8$); starved, 1.01 ± 0.03 ($n = 3$)] was unaffected by starvation.

We subsequently analysed the extent to which PPARα activation might, either directly or indirectly, influence the overall response of PDK4 protein expression in anterior tibialis and soleus to increased circulating lipid fuels elicited by starvation by directly comparing muscles of fed WY14,643-treated rats and 48 h-starved WY14,643-treated rats on the same immunoblot. WY14,643 was administered to 24 h-starved rats and then the starvation period extended from 24 h to 48 h. Although a trend towards lowered ambient NEFA concentrations after starvation was apparent in the WY14,643-treated rats, this did not attain significance (Figure 1). However, 3-hydroxybutyrate levels also tended to be elevated, and an increased 3-hydroxybutyrate to NEFA concentration ratio suggests increased NEFA oxidation. Moreover, the hypotriglyceridaemic effect of WY14,643 treatment was retained after starvation, with a 22% decline in circulating TAG concentrations (results not shown). Under conditions of PPARα activation, starvation for 48 h elicited a

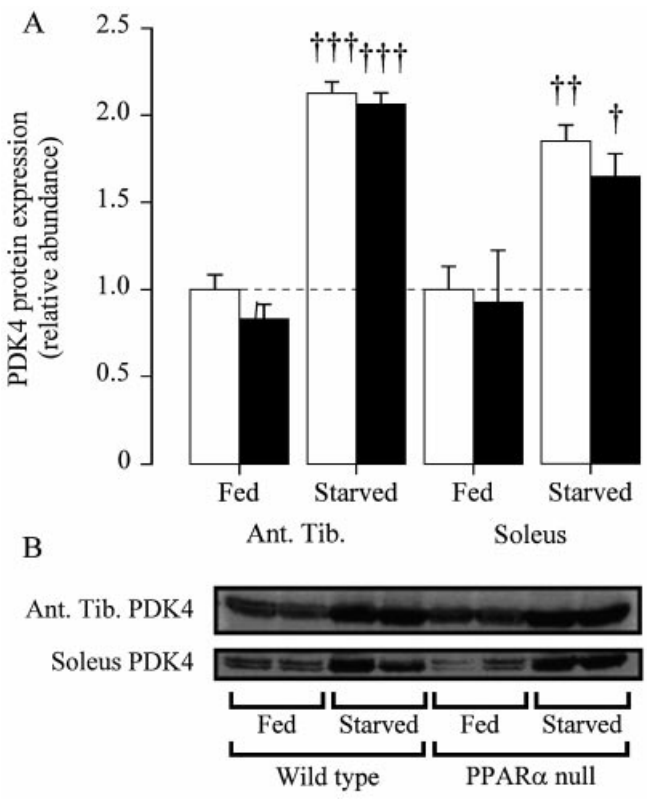

Figure 3 Effects of 24 h-starvation on PDK4 protein expression in anterior tibialis (Ant. Tib.) and soleus muscles of wild-type mice (white bars) and PPARα-null mice (black bars)

Rabbit polyclonal antisera raised against recombinant rat PDK4 were used to detect PDK4 protein using Western blot analysis. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Quantification of Western analysis of PDK4 protein expression in anterior tibialis (Ant. Tib.) and soleus is shown in (**A**). Data are means ± S.E.M. for 5 preparations from individual mice in each experimental group. Typical immunoblots for PDK4 protein expression in anterior tibialis and soleus muscles of wild-type mice or PPARα-null mice sampled in the fed state or after starvation for 24 h, as indicated, are shown in (**B**). Each lane corresponds to 25 μg of muscle protein from a different mouse. Statistically-significant effects of 24 h-starvation are indicated by: †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$. There were no statistically-significant differences between wild-type mice and PPARα-null mice.

marked 2.31 ± 0.13-fold increase ($P < 0.01$) in PDK4 protein expression in anterior tibialis (Figure 2), together with a 31% decline in E1α protein expression. However, PDK4 protein expression was actually slightly lower (27% lower; $P < 0.01$) in anterior tibialis after WY14,643 treatment from 24 h to 48 h of starvation compared with 48 h-starved controls (Figure 2). Thus, PDK4 protein expression is enhanced in anterior tibialis muscle when WY14,643-treated rats are starved, but the PDK4 response to starvation in anterior tibialis is not further enhanced by activation of PPARα *in vivo* for the 24 h immediately preceding tissue sampling.

Direct comparison of PDK4 protein expression in soleus muscles of fed WY14,643-treated rats and starved WY14,643-treated rats on the same immunoblot revealed that, under conditions of PPARα activation, starvation markedly increased soleus PDK4 protein expression (1.91-fold; $P < 0.01$) (Figure 2). However, as for the FOG muscle, PPARα activation during starvation did not further enhance PDK4 protein expression in this SO muscle, but again led to a modest (22%; $P < 0.01$) decline in PDK4 protein expression (Figure 2), while failing to influence the protein expression of E1α (results not shown).

In summary, therefore, our data demonstrate that the responses of PDK4 protein expression in FOG muscle to PPARα activation in the fed state and to prolonged starvation are qualitatively similar, but that the effect of starvation is quantitatively much greater. Furthermore, although prolonged starvation markedly increases PDK4 protein expression in SO muscle, PPARα activation does not significantly alter PDK4 protein expression in SO muscle in the fed state. Finally, our data demonstrate that activation of PPARα by WY14,643 during prolonged starvation does not further enhance up-regulation of PDK4 protein expression in either FOG or SO skeletal muscle. However, the failure to observe an effect of activation of PPARα by WY14,643 on PDK4 expression in FO muscle in the fasted state does not exclude a possible effect of starvation to suppress PPARα expression and/or activity. To address this issue, studies were conducted using PPARα-null mice.

PPARα deficiency has a limited impact on PDK4 protein expression in FOG and SO skeletal muscle in the fed state

We compared levels of PDK4 protein expression in anterior tibialis and soleus skeletal muscles of PPARα-null and wild-type mice. PDK4 protein expression in anterior tibialis and soleus muscles was not significantly affected by PPARα deficiency in the fed state (Figure 3), although a non-significant trend towards lower PDK4 protein expression was observed in anterior tibialis. In addition, there were no significant changes in skeletal muscle E1α protein levels in fed PPARα-null mice compared with fed wild-type mice (results not shown). Our data therefore demonstrate, for the first time, that although activation of PPARα by WY14,643 treatment modestly enhances PDK4 protein expression in FOG skeletal muscle in the fed state, signalling through PPARα is not a vital component required for normal PDK4 protein expression in FOG skeletal muscle in the fed state. In addition, consistent with the failure of activation of PPARα by WY14,643 to modulate PDK4 protein expression in SO muscle, signalling through PPARα is not required for normal PDK4 protein expression in SO skeletal muscle in the fed state.

Enhanced PDK4 protein expression in FOG and SO skeletal muscles after starvation does not require PPARα signalling

To discriminate between direct effects of PPARα activation and those of altered lipid delivery on PDK4 protein expression in FOG and SO skeletal muscles, we investigated the impact of PPARα deficiency on the response of PDK4 protein expression to prolonged (24 h) starvation. As we have noted previously [19], plasma NEFA and ketone body concentrations significantly increase with starvation in the wild-type mice (Figure 1). Starvation elicited a significant (2.1-fold; $P < 0.001$) increase in PDK4 protein expression in anterior tibialis muscles of wild-type mice (Figure 3). The effect of 24-h starvation to increase PDK4 protein expression in anterior tibialis was intact in PPARα-null mice (a 2.1-fold increase; $P < 0.001$) and similar in magnitude to that observed in wild-type mice (Figure 3). Starvation also elicited a significant (1.9-fold; $P < 0.01$) increase in PDK4 protein expression in soleus muscles of wild-type mice and, again, this response was unaffected by PPARα deficiency (Figure 3). There were no effects of PPARα deficiency on E1α protein expression (results not shown) in either skeletal muscle in the starved state. Up-regulation of PDK4 protein expression in FOG and SO

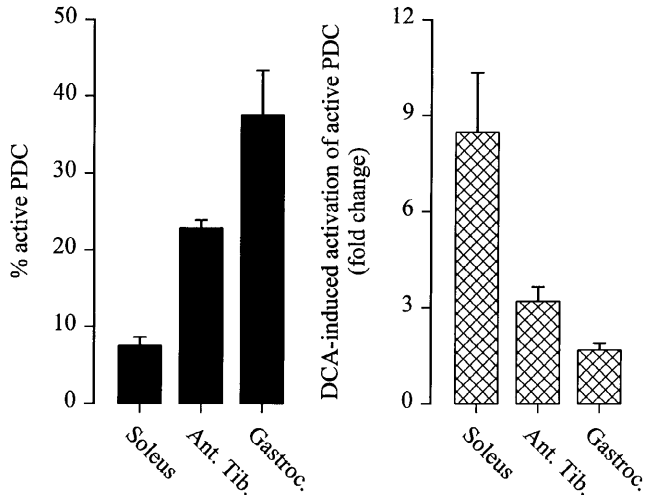

Figure 4 Active pyruvate dehydrogenase complex (PDHa) activities and effects of inhibition of PDK by DCA in soleus, anterior tibialis (Ant. Tib.) and gastrocnemius (Gastroc.) muscles of *ad libitum* fed rats

(**A**) Estimation of PDC active and total activities were measured as described previously [27], and data are expressed as % active PDC. (**B**) Effects of DCA treatment. Data are means ± S.E.M. for muscles from 5 individual rats in each experimental group.

muscles of PPARα-null mice was observed in conjunction with significantly increased NEFA delivery (Figure 1). By contrast, as reported previously [19], starvation-induced increases in ketonaemia are greatly attenuated (Figure 1) due to impaired hepatic ketogenesis [29]. The present data categorically demonstrate, for the first time, that enhanced PDK4 protein expression in SO skeletal-muscle in response to starvation can occur in association with an increased NEFA supply, with no obligatory participation of either increased ketone body utilization or signalling via PPARα. Furthermore, since anterior tibialis comprises approximately one-third FG fibres, the response of PDK4 protein expression to PPARα in this muscle may reflect a response of the FG component. It thus remains entirely possible that up-regulation of PDK4 protein expression in the gastrocnemius, where FG fibres predominate, reflects direct signalling through PPARα.

The impact of PDK inhibition on active pyruvate dehydrogenase complex (PDHa) activities in skeletal muscles shows fibre-type-specific variation

As shown in Figure 4(A), the percentage of active PDC is relatively low in SO (soleus) muscle in the fed state, indicating that a significant proportion of the ATP requirement of this SO muscle is obtained via FA oxidation even in the fed state. By contrast, FOG muscles are normally reliant on glucose and glycogen utilization and do not normally oxidize lipid-derived fuels at high rates in the fed state, switching towards lipid oxidation only after prolonged starvation. As shown in Figure 4(A), the percentage of active PDC in the FOG anterior tibialis muscle in the fed state is higher than in SO soleus muscle, consistent with a lesser dependency of this muscle type on lipids as fuel substrate in the fed state. DCA is a known inhibitor of all PDK isoforms [7]. It is noteworthy that the response of PDC activity to pharmacological inhibition of PDK with DCA *in vivo* is less in FOG anterior tibialis than in SO soleus, suggesting a lower functional PDK activity in anterior tibialis compared with soleus in the fed state (Figure 4B). We evaluated previous data showing effects of PPARα activation on PDK4 expression in gastrocnemius [9], predominantly FG, in relation to the effects of PPARα activation and deficiency on PDK4 expression in FOG and SO muscle. As shown in Figure 4(A), the percentage of active PDC in gastrocnemius muscle in the fed state is higher than that of either soleus or anterior tibialis and, strikingly, gastrocnemius exhibits a relatively refractory response to pharmacological inhibition of PDK with DCA *in vivo* (Figure 4B). Our findings suggest that, in the fed state, the relative influence of PDK on PDC activity *in vivo* in individual skeletal muscles closely reflects their relative use of lipids as fuel substrate.

Evidence of muscle fibre-type specific regulation of expression has been observed previously for uncoupling protein 3 (*UCP3*) gene regulation [30]. These important studies demonstrated that a more pronounced fasting-induced up-regulation of *UCP3* gene expression in FOG skeletal muscles occurs in parallel with more pronounced up-regulation of key regulators of lipid oxidation in FOG than in SO skeletal muscle. These authors also showed that *UCP3* gene expression in SO muscle is primarily modulated by changes in FA delivery whereas *UCP3* gene expression in FOG muscle is primarily responsive to changes in the mitochondrial β-oxidation pathway. By contrast, *UCP3* gene expression in FG gastrocnemius muscle is unresponsive to changes in delivery and flux of lipid substrates [30]. By analogy, we propose that PDK4 protein expression in oxidative skeletal muscle fibres predominantly reflects the flux of lipid substrates (circulatory delivery and/or mitochondrial oxidation). Supporting such a proposal, we show that starvation (which leads to increased adipose-tissue lipolysis and FA delivery) markedly up-regulates PDK4 protein expression in SO skeletal-muscle of PPARα-deficient mice, which exhibit the normal fasting-induced elevation in plasma NEFAs (Figure 1 and [19]). By contrast, the more pronounced response of PDK4 protein expression to fasting in the anterior tibialis, a skeletal muscle containing proportionally more FOG fibres, is likely to reflect the greater forced shift between glucose and lipids as fuel substrate. The failure of WY14,643 to activate PPARα during the last 24 h of a 48 h period of starvation, and thereby to enhance PDK4 protein expression further in oxidative fast-twitch skeletal muscle in response to starvation, is consistent with activation of lipid oxidation during starvation, with no further effect of WY14,643. Our data do not exclude the possibility that direct signalling through PPARα may have a more vital role for regulation of PDK4 expression in FG muscles.

Concluding remarks

Experimental (streptozotocin) diabetes enhances PDK4 protein expression in FG gastrocnemius muscle [9]. Furthermore, insulin resistance [31] enhances PDK4 protein expression in both SO soleus and FOG anterior tibialis muscles [11,31]. Taken together with the present findings, it is implied that starvation may remove a restraint on up-regulation of PDK4 expression imposed, either directly or indirectly, by insulin. Given that relatively high ambient insulin levels were maintained in the present experiments with 24 h exposure to WY14,643 *in vivo* in the absorptive state, and PPARα-null mice show a starvation-induced decline in plasma insulin concentration [19], our data support the hypothesis that PDK4 protein expression in oxidative (SO and FOG) skeletal muscle is regulated, at least in part, by NEFA

supply, possibly initiated or facilitated by insulin deficiency or other hormonal changes induced by starvation. They also show unequivocally that the influence of these lipid fuels does not require the obligatory participation of signalling through PPARα. The failure of PPARα activation to up-regulate PDK4 protein expression in SO muscle and the somewhat modest effect of PPARα activation to up-regulate PDK4 protein expression in FOG muscle reconciles reported effects of PPARα activation to improve glucose homeostasis in rodent models of insulin resistance.

Previous studies have shown that acute (24 h) activation of PPARα by WY14,643 treatment does not increase PDK4 protein expression in the heart [21,22], which (like the soleus) is an oxidative working muscle which oxidizes lipid to provide approximately 70% of its total ATP requirement in the fed state [32]. By contrast, activation of PPARα by WY14,643 elicits increased PDK4 protein expression in the liver [21], where the predominant fate of incoming FA is re-esterification, rather than oxidation, in the fed state [33]. Furthermore, up-regulation of hepatic PDK4 protein expression in response to prolonged starvation is markedly impaired in PPARα-deficient mice [19] and PPARα activation by WY14,643 also increases PDK4 mRNA and protein levels in Morris hepatoma 7800 C1 cells, an effect mimicked by culture in the presence of the synthetic glucocorticoid dexamethasone [17]. It appears likely, therefore, that PDK4 protein expression in liver reflects a combination of effects of increased mitochondrial FA oxidation and direct activation of PPARα, but not FA delivery itself. The present study therefore extends previous studies that, taken together, provide strong evidence that the mechanisms regulating PDK4 protein expression differ significantly between tissues in a manner that may reflect individual tissue responses to altered lipid supply and/or oxidation.

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