Fibre-type specific modification of the activity and regulation of skeletal muscle pyruvate dehydrogenase kinase (PDK) by prolonged starvation and refeeding is associated with targeted regulation of PDK isoenzyme 4 expression

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Using immunoblot analysis with antibodies raised against recombinant pyruvate dehydrogenase kinase (PDK) isoenzymes PDK2 and PDK4, we demonstrate selective changes in PDK isoenzyme expression in slow-twitch versus fast-twitch skeletal muscle types in response to prolonged (48 h) starvation and refeeding after starvation. Starvation increased PDK activity in both slow-twitch (soleus) and fast-twitch (anterior tibialis) skeletal muscle and was associated with loss of sensitivity of PDK to inhibition by pyruvate, with a greater effect in anterior tibialis. Starvation significantly increased PDK4 protein expression in both soleus and anterior tibialis, with a greater response in anterior tibialis. Starvation did not effect PDK2 protein expression in soleus, but modestly increased PDK2 expression in anterior tibialis. Refeeding for 4 h partially reversed the effect of 48-h starvation on PDK activity and PDK4 expression in both soleus and anterior tibialis, but the response was more marked in soleus than in anterior tibialis. Pyruvate sensitivity of PDK activity was also partially restored by refeeding, again with the greater response in soleus. It is concluded that targeted regulation of PDK4 isoenzyme expression in skeletal muscle in response to starvation and refeeding underlies the modulation of the regulatory characteristics of PDK *in vivo*. We propose that switching from a pyruvate-sensitive to a pyruvate-insensitive PDK isoenzyme in starvation (a) maintains a sufficiently high pyruvate concentration to ensure that the glucose → alanine → glucose cycle is not impaired, and (b) may 'spare' pyruvate for anaplerotic entry into the tricarboxylic acid cycle to support the entry of acetyl-CoA derived from fatty acid (FA) oxidation into the tricarboxylic acid cycle. We further speculate that FA oxidation by skeletal muscle is both forced and facilitated by upregulation of PDK4, which is perceived as an essential component of the operation of the glucose-FA cycle in starvation.

Key words: pyruvate dehydrogenase complex, lactate, fatty acids.

INTRODUCTION

Regulation of the activity of the pyruvate dehydrogenase complex (PDC) is an important component of the regulation of glucose homeostasis. Activation of PDC promotes glucose disposal, whereas suppression of PDC activity is crucial to glucose conservation in prolonged starvation, when three-carbon compounds (including pyruvate) are required for gluconeogenesis to maintain glycaemia. PDC is rendered inactive by phosphorylation of the α-subunit of its pyruvate dehydrogenase (PDH) component by pyruvate dehydrogenase kinase (PDK) (reviewed in [1,2]). Acute low-molecular-weight effectors of PDK include pyruvate, NADH and acetyl-CoA. Pyruvate, generated via glycolysis or from circulating lactate, suppresses PDK activity, whereas NADH and acetyl-CoA, generated by increased rates of fatty acid (FA) β-oxidation, activate PDK. Four PDK isoenzymes (PDK1–4) have been identified in mammalian tissues [3]. In previous studies in the rat, stable effects of prolonged starvation to enhance PDK activity in the heart (which expresses PDK1, PDK2 and PDK4 [3]) have been demonstrated to occur in association with specific upregulation of the protein expression of only one PDK isoenzyme, namely PDK4 [4]. In contrast, increased PDK activity after starvation in rat liver [5] and rat kidney [6] is associated with upregulation of PDK2 protein expression. The physiological significance of PDK-isoenzyme shifts has not been clearly established. However, studies with the individual recombinant PDK isoenzymes expressed in *Escherichia coli* have demonstrated potentially important differences in their specific activities and in their acute regulation by metabolites [3]. For example, PDK4 is a relatively high-specific-activity PDK isoenzyme whose activity is relatively insensitive to suppression by dichloroacetate [3] (a specific pharmacological allosteric inhibitor of PDK that is believed to mimic the effect of the physiological inhibitor pyruvate [7,8]), but relatively responsive to an increased mitochondrial $NADH/NAD^+$ concentration ratio compared with PDK2 [3]. Thus it is possible that changes in the relative expression of different native PDK isoenzymes may be associated with concomitant changes in PDK

Abbreviations: FA, fatty acids; GUI, glucose utilization index; MCT, monocarboxylate transporters; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDHa, active PDC; PDK, pyruvate dehydrogenase kinase; V_{O_2}, oxygen uptake.

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activity and/or characteristics of regulation by metabolite effectors *in vivo*. Up to now, this has not been investigated.

In terms of whole-body glucose disposal, the skeletal muscle mass is quantitatively of major importance [9]. During rest, approx. 40 % of oxygen uptake ($V\mathrm{O}_2$) is used for carbohydrate oxidation, and the remainder, approx. 60 %, is used for fat oxidation [10]. Although skeletal muscle can utilize many different substrates (including, in addition to glucose and FA, exogenous lactate [11,12]), it is metabolically heterogeneous. In the fed state, oxidative slow-twitch muscles (such as soleus), display higher rates of glucose utilization [13] than muscles containing predominantly fast-twitch fibres (such as the anterior tibialis), which tend to be recruited during exercise. Furthermore, slow-twitch muscles oxidize FA more avidly [13] and, although they are intrinsically more insulin sensitive in terms of glucose utilization [14], glucose utilization is markedly decreased in these muscles to a greater extent than the decrease in whole-body glucose disposal after high-fat feeding [15]. It has recently emerged that individual skeletal muscle types also differ with respect to lactate handling. The transport of lactate across the plasma membrane of muscle fibres involves proton-linked monocarboxylate transporters (MCTs), eight of which have been cloned and sequenced. Skeletal muscle contains MCT1 (present in most cells) and MCT4 (formerly termed MCT3) [16]. MCT4 is expressed in all muscle fibres. However, the expression of MCT1 is higher in slow-twitch oxidative muscles such as soleus, whereas MCT4 expression is greater in fast-twitch muscles [16–18]. It has been proposed that MCT1 is involved in lactate uptake into slow-twitch oxidative muscles for oxidation [19], whereas lactate efflux from fast-twitch skeletal muscle is catalysed by MCT4 (see [16]).

It has been demonstrated previously that PDK activity increases in response to prolonged starvation in hindlimb skeletal-muscle (mixed muscle) [20] and in soleus (slow twitch) [21]. PDK2 and PDK4 mRNA expression has been demonstrated in rat gastrocnemius (fast twitch) muscle [3]. It is not known whether the increased PDK activity elicited by prolonged starvation in the slow-twitch soleus muscle represents upregulation of the protein expression of PDK2 or PDK4 isoenzymes, or a combination of the two. Furthermore, it is not known whether PDK activity increases in response to prolonged starvation in fast-twitch muscle and, if so, what mechanism(s) are involved.

In the present study, we examined whether individual muscle types respond differently to prolonged (48 h) starvation, and refeeding (4 h) after prolonged starvation, with respect to changes in expression of specific PDK isoenzymes. In addition, since it has been shown recently that exogenous lactate is readily oxidized by rat skeletal muscle, even during contraction [11], we investigated whether changes in PDK isoenzyme profile in individual skeletal muscles are reflected in altered sensitivity of PDK activity to suppression by pyruvate. Finally, we evaluated whether PDK isoenzyme switching in individual skeletal muscles correlates with the propensity with which the muscle oxidizes lipid-derived fuels. The results indicate that PDK4 expression is specifically upregulated both in slow-twitch and in fast-twitch skeletal muscle after prolonged starvation and that this response to starvation is both greater and more persistent in fast-twitch muscle. Refeeding (4 h) after starvation suppressed PDK activity in both skeletal muscle types, and suppression of PDK activity was associated with a decline in PDK4 protein expression. Changes in PDK4 protein expression correlate well with changes in PDK pyruvate sensitivity. We propose that PDK isoenzyme switching is physiologically important for the control of skeletal-muscle glucose oxidation by changes in pyruvate supply *in vivo*. In addition, we propose that the impairment in the ability of skeletal muscle to oxidize pyruvate, introduced by targeted upregulation of PDK4, may be important for forcing and facilitating FA oxidation in starvation through 'sparing' pyruvate for anaplerotic entry into the tricarboxylic acid cycle, and for aiding lactate, H^+ and nitrogen export from skeletal muscle.

MATERIALS AND METHODS

Materials

Radiochemicals and ECL® reagents were purchased from Amersham International. Arylamine acetyltransferase was purified from pigeon liver acetone powder purchased from Europa Bioproducts Ltd. (Ely, Cambridgeshire, U.K.). The remaining chemicals were purchased from Bio-Rad, Boehringer Mannheim and Sigma. Female Wistar rats were purchased from Charles River (Margate, Kent, U.K.).

Rats

Adult female Wistar rats (initial weights 230–250 g) with free access to food (standard laboratory diet) and water, were housed in individual cages in a temperature-controlled (21 ± 2 °C) and light-controlled room (a 12-h light–dark cycle). After an acclimatization period of 7 days, food was withdrawn from one group of rats. The fed and starved groups were sampled after a further 48 h. Some rats were provided with access to food *ad libitum* after a 48-h starvation period. These rats, termed 'refed', were sampled after 4 h of refeeding. In all the experimental protocols utilized, the two individual muscles (soleus and anterior tibialis) were sampled from the same animal and analysed in parallel.

In vivo glucose utilization in individual muscles

Estimations of *in vivo* glucose utilization by individual skeletal muscles in conscious unrestrained rats were obtained by measuring the accumulation of 2-deoxy-D-[1-^{3}H]glucose 6-phosphate in the tissue after the bolus intravenous injection of tracer amounts (30 μCi) of 2-deoxy-D-[1-^{3}H]glucose via a chronic indwelling cannula inserted 5–7 days previously [13,22]. Blood samples (100 μl) for determination of blood glucose and plasma tracer concentrations were obtained at 1, 3, 5, 10, 20, 40 and 60 min after 2-deoxy-D-[1-^{3}H]glucose bolus administration. Throughout the study, the rats were awake and moving freely, with the connecting tubing suspended overhead. At the end of the 60-min study, a final blood sample (500 μl) was added to a heparin-treated tube, immediately centrifuged at 4 °C and plasma was frozen at -20 °C for subsequent insulin determinations. Rats were killed by the intravenous injection of pentobarbitone (60 mg/kg) via the indwelling cannula. Soleus and anterior tibialis muscles were freeze-clamped when locomotor activity had ceased (within 5 s). The fibre profiles (percentage of fast oxidative glycolytic:fast glycolytic:slow oxidative) of soleus and anterior tibialis in the rat are 0:0:100 and 66:32:2 respectively [23]. The freeze-clamped muscles were stored in liquid nitrogen until analysis, as described in [13,22]. No correction has been made for possible discrimination against 2-deoxyglucose versus glucose with respect to glucose transport and phosphorylation, and hence rates of tissue accumulation of 2-deoxy-D-[^{3}H]glucose 6-phosphate are referred to as glucose utilization indices (GUI values).

Enzyme assays

Active PDC (PDHa) activity was assayed spectrophotometrically by coupling the generation of acetyl-CoA to the acetylation of *p*-

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(p-aminophenylazo)benzenesulphonic acid by arylamine acetyltransferase [24,25]. Mitochondria were prepared from soleus and anterior tibialis, as described in [20], from rats anaesthetized by the intraperitoneal injection of pentobarbitone (60 mg/kg). PDHa activities were expressed relative to citrate synthase to correct for possible differences in mitochondrial extraction [24,25]. Total PDC activity was assayed after complete activation through the action of endogenous PDC phosphate phosphatase as active PDC in mitochondria incubated for 15 min in the absence of respiratory substrate and in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone [26]. PDK activities were determined at 30 °C in mitochondrial extracts at pH 7.0 by the rate of ATP-dependent inactivation of PDHa [27,28]. PDK activities were expressed as first-order rate constants for ATP-dependent PDHa inactivation. To evaluate functional PDK activity, freshly-prepared mitochondria were incubated at 30 °C in KCl medium (100 mM KCl, 20 mM Tris/HCl, 5 mM KH_2PO_4, 2 mM EGTA, pH 7.4) in the presence of respiratory substrate (5 mM 2-oxoglutarate/0.5 mM malate). To test the effects of pyruvate, freshly-prepared mitochondria were incubated at 30 °C in KCl medium with 5 mM 2-oxoglutarate/0.5 mM malate and the concentrations of pyruvate (added as sodium salt) indicated. Incubations were terminated by centrifugation after 5 min, and mitochondrial extracts assayed for PDHa activity [20,29].

Western blotting analysis

Mitochondria were prepared from soleus and anterior tibialis, as described in [20], from rats anaesthetized by the intraperitoneal injection of pentobarbitone (60 mg/kg), and stored at −70 °C until analysis (within 1 week). Mitochondria were extracted in 50 mM KH_2PO_4, 50 mM K_2HPO_4, 10 mM EGTA, 1 mM benzamidine, 50 μM aprotinin, 50 μM pepstatin, and 10 μM leupeptin. Samples of mitochondrial extracts were denatured by heating to 60 °C with a 1:2 dilution of Laemmli electrophoresis buffer [0.25 M Tris/HCl (pH 6.8), 10 % glycerol, 0.01 % Bromophenol Blue, 2 % β-mercaptoethanol, 2 % sodium dodecyl sulphate, 0.01 M dithiothreitol]. 3 μg of mitochondrial protein was then separated by discontinuous SDS/PAGE electrophoresis and subsequently transferred electrophoretically to nitrocellulose membranes using the electrophoresis semi-dry apparatus (Bio-Rad). Nitrocellulose filters were then blocked overnight at 4 °C with Tris-buffered saline (150 mM NaCl, 10 mM Tris/HCl, pH 7.6) supplemented with 0.05 % Tween 20 and 5 % (w/v) non-fat powdered milk, and incubated for 3 h at room temperature with polyclonal antisera raised against specific recombinant PDK isoenzymes. After washing (3 × 5 min) with Tris-buffered saline containing 0.05 % Tween 20, membranes were incubated with horseradish peroxidase-linked secondary antibody IgG anti-rabbit [1:2000, in 1 % (w/v) non-fat milk in Tris-buffered saline containing 0.05 % Tween 20] for 2 h at room temperature. The blots were then extensively washed in Tris-buffered saline containing 0.05 % Tween 20, and bound antibody was visualized using ECL® (following 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).

Statistical analysis

Experimental data are expressed as means ± S.E.M. One-way analysis of variance was performed on all sets of results, with individual comparisons by Fisher post-hoc test. Statistical comparisons were made with StatView (Abacus Concepts Inc., Berkeley, CA, U.S.A.). Curve fitting was carried out using Fig P software (Biosoft, Cambrdige, U.K.).

RESULTS

Effects of 48 h starvation and refeeding after 48 h starvation on glucose utilization in individual skeletal muscles *in vivo*

We measured glucose utilization (transport plus phosphorylation) in soleus and anterior tibialis *in vivo* in conscious, unrestrained rats in the fed, starved and refed states to validate our selection of these muscles as representative of slow-twitch and fast-twitch skeletal muscle. In *ad libitum* fed rats, glucose utilization (transport plus phosphorylation) rates were significantly higher in soleus (by 3.2-fold; $P < 0.001$) than in anterior tibialis. In addition, the absolute decline in glucose utilization observed in response to 48-h starvation was 3.2-fold greater in soleus than in anterior tibialis (Table 1). Nevertheless, GUI values in the 48-h starved state remained 3.5-fold greater in soleus than in anterior tibialis (Table 1). Refeeding for 4 h after 48-h starvation restored GUI values in soleus to 89 % of the fed *ad libitum* values. GUI values in anterior tibialis after 4 h of refeeding were 111 % of those found in the fed *ad libitum* state (Table 1). The patterns of responses of soleus and anterior tibialis to starvation and refeeding after starvation are typical of slow-twitch and fast-twitch muscle respectively [30].

Effect of 48 h starvation on PDK activities and the protein expression of individual PDK isoenzymes in slow-twitch and fast-twitch skeletal-muscle mitochondria

No significant differences in PDK activity existed between soleus and anterior tibialis muscles in rats fed *ad libitum* (Table 1). PDK activity in soleus mitochondria was increased 2.3-fold ($P < 0.01$) by 48 h-starvation (Table 1). PDK activity in anterior tibialis mitochondria was also significantly increased ($P < 0.001$) in response to 48 h-starvation, but the fold increase in PDK activity observed in anterior tibialis (3.1-fold) was greater than that observed in soleus (Table 1). Western blot analysis was used to determine whether the increased PDK activities elicited by 48 h-starvation in the representative slow-twitch and fast-twitch skeletal-muscles were achieved by targeted or muscle-type specific changes in PDK2 and/or PDK4 expression. Representative

Table 1 Glucose utilization indices and pyruvate dehydrogenase kinase (PDK) activities in soleus and anterior tibialis muscles in the fed and starved states and at 4 h after refeeding

Glucose utilization indices were measured in muscles of fed *ad libitum* rats, 48 h-starved rats and 48 h-starved rats at 4 h after refeeding. PDK activities were measured in mitochondrial extracts of muscles of fed *ad libitum* rats, 48 h-starved rats, and 48 h-starved rats at 4 h after refeeding. Each PDK assay was run in duplicate. Further details are given in the Materials and Methods section. Rate constants for PDK activity were calculated by least-squares linear regression analysis of ln [% of zero time activity] against time. Values are means ± S.E.M. for 5–12 rats. Statistically significant effects of starvation are indicated by: ** $P < 0.01$; *** $P < 0.001$. Statistically significant effects of refeeding after 48 h starvation are indicated by: † $P < 0.05$; ††† $P < 0.001$.

	GUI (ng/min per mg)		PDK activity (min^{-1})	
	Soleus	Anterior tibialis	Soleus	Anterior tibialis
Fed	57.8 ± 4.0	17.8 ± 1.9	0.366 ± 0.060	0.349 ± 0.076
Starved (48 h)	8.3 ± 1.6***	2.4 ± 0.4***	0.851 ± 0.172**	1.095 ± 0.160***
Refed (4 h)	51.5 ± 10.3†††	19.8 ± 2.1†††	0.499 ± 0.075†	0.737 ± 0.123†

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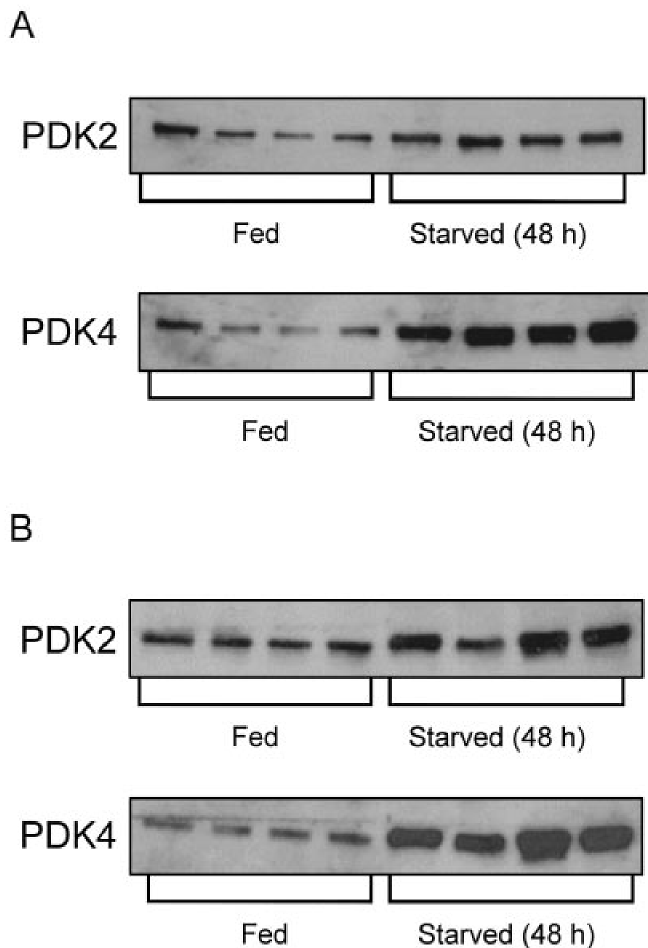

Figure 1 Effects of starvation on PDK2 and PDK4 protein expression in mitochondria prepared from soleus and anterior tibialis muscles

Mitochondria were prepared from muscles of fed *ad libitum* rats and 48 h-starved rats. Rabbit polyclonal antisera raised against recombinant PDK2 and PDK4 were used to detect these proteins by Western blot analysis. Typical immunoblots of PDK2 and PDK4 protein expression are shown for soleus (panel **A**) and anterior tibialis (panel **B**) muscles of fed and starved rats. Muscle mitochondrial extracts were denatured and subjected to SDS/PAGE and immunoblotting with these isoenzyme-specific antibodies, as described in the Materials and Methods section. Each lane corresponds to 3 μg of mitochondrial protein. Five to nine preparations of mitochondria were analysed. Representative results are shown.

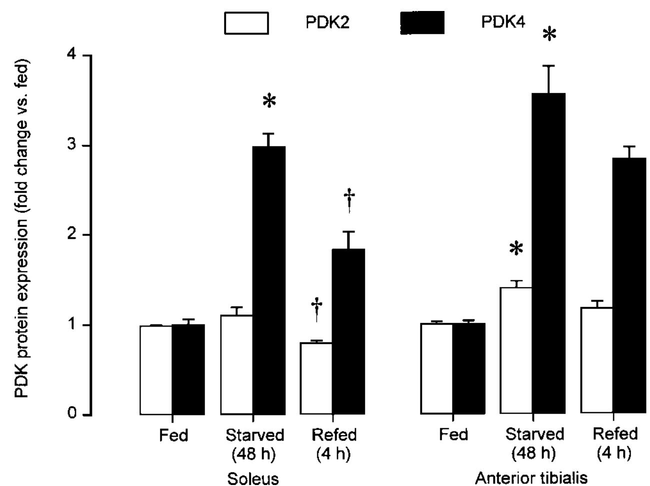

Figure 2 Quantification of Western analysis of effects of starvation and refeeding after starvation on PDK2 and PDK4 protein expression in mitochondria prepared from soleus and anterior tibialis muscles

Details are provided in the legend to Figure 1. Western blots were analysed by scanning densitometry using Molecular Analyst 1.5 software. Values are means ± S.E.M. for 5–9 individual internally controlled experiments. *$P < 0.001$ versus fed. †$P < 0.05$ versus 48 h-starved.

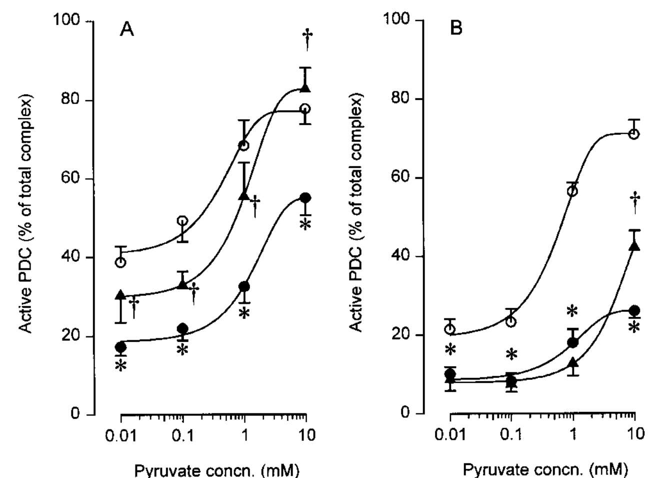

Figure 3 Effects of starvation and refeeding after starvation on suppression of PDK activity by pyruvate in freshly isolated mitochondria prepared from soleus and anterior tibialis muscles

Freshly isolated mitochondria (0.5–1 mg of mitochondrial protein) from soleus (panel **A**) and anterior tibialis (panel **B**) muscles of fed *ad libitum* rats (open circles), 48 h-starved rats (closed circles) and 48 h-starved rats at 4 h after refeeding (closed triangles) were incubated at 30 °C with respiratory substrate (2-oxoglutarate/malate) and the concentrations of pyruvate indicated as described in the Materials and Methods section. Mitochondria were precipitated by centrifugation, and steady-state PDC activity measured in mitochondrial extracts. Values for 16 (fed), 9 (48 h-starved) and 8 (4 h-refed) mitochondrial preparations from individual rats are presented as means ± S.E.M. for each pyruvate concentration. *$P < 0.001$ versus fed. †$P < 0.01$ versus 48 h-starved.

immunoblots are shown in Figure 1, and results of 5–9 independent experiments are quantified in Figure 2. Starvation for 48 h significantly ($P < 0.001$) increased (2.98-fold) the amount of PDK4 protein expressed in soleus mitochondria. In contrast, the amount of PDK2 protein present in soleus was unaffected by 48 h-starvation. Thus, the relative expression of PDK4:PDK2 greatly increases in soleus in response to prolonged starvation. Starvation for 48 h also led to a marked 3.56-fold increase ($P < 0.001$) in PDK4 protein expression in anterior tibialis. In this case, this was accompanied by a modest, yet nonetheless significant, 1.40-fold increase ($P < 0.001$) in the PDK2 protein expression in anterior tibialis.

Interconversion of active and inactive forms of PDC in slow-twitch and fast-twitch skeletal-muscle mitochondria

Incubation of mitochondria with respiratory substrates increases the ATP concentration, thus allowing phosphorylation of PDC by endogenous PDK [20]. Thus the percentage of active PDC gives a functional index of PDK activity. Slow-twitch and fast-twitch skeletal-muscle mitochondria prepared from fed and 48 h-starved rats were incubated with respiratory substrate (2-oxoglutarate/malate) for a fixed 5 min period. Starvation reduced the percentage of active PDC in mitochondria from soleus

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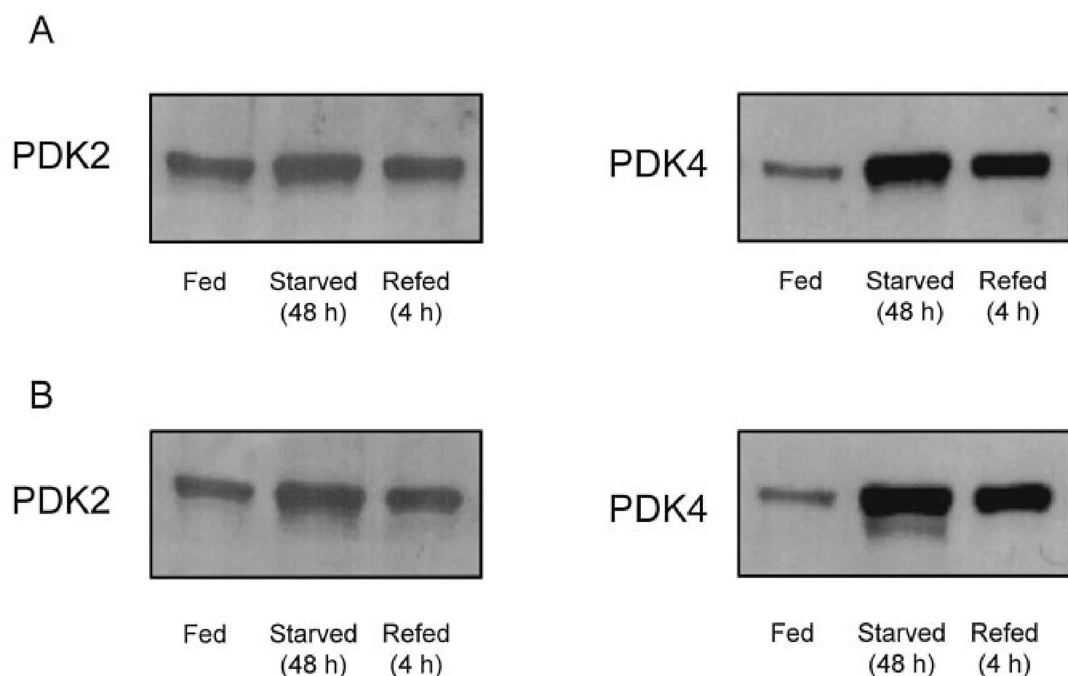

Figure 4 Effects of refeeding after starvation on PDK2 and PDK4 protein expression in mitochondria prepared from soleus and anterior tibialis muscles

Mitochondria were prepared from muscles of fed *ad libitum* rats, 48 h-starved rats and 48 h-starved rats at 4 h after refeeding. Typical immunoblots of PDK2 and PDK4 protein expression are shown for soleus (panel **A**) and anterior tibialis (panel **B**) muscles of starved and refed rats. Further details are given in the legend to Figure 1. Representative results are shown. Results are quantified in Figure 2.

incubated with respiratory substrate from $38.6 \pm 4.1\,\%$ of total PDC activity to $17.3 \pm 2.3\,\%$ of total PDC activity ($P < 0.001$). The percentage of active PDC in mitochondria from anterior tibialis muscle incubated with respiratory substrate was also decreased by prolonged starvation (fed, $21.5 \pm 2.6\,\%$ of total PDC activity; starved, $10.1 \pm 1.8\,\%$ of total PDC activity; $P < 0.01$). Total PDC activities (measured in the absence of substrate and in the presence of respiratory inhibitors) expressed relative to the mitochondrial marker citrate synthase were similar in mitochondria prepared from soleus and anterior tibialis muscles and unaffected by prolonged (48 h) starvation (soleus: fed, 219 ± 27 m-units/unit of citrate synthase, 48-h starved, 194 ± 28 m-units/unit of citrate synthase; anterior tibialis: fed, 297 ± 22 m-units/unit of citrate synthase, 48-h starved, 268 ± 33 m-units/unit of citrate synthase; $n \geqslant 9$ mitochondrial preparations in each case).

Effects of pyruvate on the percentage of active PDC in skeletal-muscle mitochondria from fed and 48 h-starved rats

In mitochondria incubated with respiratory substrate, pyruvate addition increases the percentage of active PDC through suppression of PDK activity [20]. The percentage of active PDC in respiring mitochondria from soleus and anterior tibialis muscles of fed rats increased progressively when the pyruvate concentration was raised successively from 0.01–10 mM (Figure 3). Raising the pyruvate concentration further to 100 mM did not lead to further PDC activation (results not shown). In fed rats, the pyruvate concentrations giving 50 % active PDC were approx. 0.25 mM and approx. 0.51 mM for mitochondria prepared from soleus and anterior tibialis muscles respectively (Figure 3). Starvation for 48 h led to rightward shifts in the pyruvate-sensitivity curves for PDK in both muscles. Furthermore, the magnitude of the response of PDK to pyruvate was greatly attenuated by starvation in both muscles. The percentages of active PDC in mitochondria incubated with physiological pyruvate concentrations (0.01, 0.1 and 1 mM) were therefore significantly reduced by starvation (by 55 %, 55 % and 52 % respectively; $P < 0.05$) in soleus. In anterior tibialis, the effect of starvation to decrease the sensitivity and response of PDK to inhibition by pyruvate was similar or even more marked than that of soleus (Figure 3). As a consequence, the percentages of active PDC in mitochondria prepared from anterior tibialis and incubated with 0.01, 0.1 and 1 mM pyruvate were reduced by 53 %, 65 % and 68 % respectively ($P < 0.05$).

Persistence of the effects of prolonged starvation after 4 h of refeeding

Refeeding for 4 h after 48 h-starvation decreased PDK activity by 41 % ($P < 0.05$) in soleus and by 33 % ($P < 0.05$) in anterior tibialis (Table 1). Refeeding for 4 h after 48 h-starvation also reduced PDK4 protein expression in both muscles (Figure 2; representative blots are shown in Figure 4). However, the magnitude of the response of PDK4 protein expression to 4 h-refeeding differed between the two skeletal muscles, with a more marked effect in soleus (39 % decline in PDK4 protein expression) than in anterior tibialis (20 % decline in PDK4 protein expression) (Figure 2). Refeeding for 4 h partially reversed the effect of prolonged starvation to reduce the percentage of active PDC in mitochondria from soleus incubated with respiratory substrate (2-oxoglutarate/malate). Thus, the percentage of active PDC increased from $17.3 \pm 2.3\,\%$ of total PDC to $30.3 \pm 6.8\,\%$ of total PDC after 4 h of refeeding. Refeeding also partially reversed the effect of starvation to suppress PDK pyruvate sensitivity and response in soleus (Figure 3). The

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percentage of active PDC in soleus mitochondria incubated at pyruvate concentrations of 0.01, 0.1 and 1 mM was increased by 75%, 49% and 70% respectively. By contrast, 4 h refeeding failed to increase the percentage of active PDC in mitochondria prepared from anterior tibialis and incubated with respiratory substrate (starved, $10.1 \pm 1.8\%$ of total PDC; refed, $8.7 \pm 2.8\%$ of total PDC) and had little effect on PDK pyruvate sensitivity and response in anterior tibialis, except at the highest pyruvate concentration (10 mM; Figure 3).

DISCUSSION

Suppression of PDC activity is not the property of a unique protein, but is a feature of a family of structurally-related proteins [3,31]. Four PDK isoenzymes (PDK1–4) have been expressed in bacteria, and all are capable of PDC inactivation *in vitro* [3]. The relative specific activity of the recombinant isoenzymes varies [3]. PDK2, the lowest specific activity isoenzyme, is ubiquitously expressed in the fed state [3]. PDK1, PDK2 and PDK4 are expressed in rat heart and PDK2 and PDK4 in rat skeletal muscle (gastrocnemius) [3]. PDK3 has a limited tissue distribution (testis, kidney and brain) [3]. Using Western blot analysis, the present study demonstrates that PDK2 and PDK4 proteins are expressed in both slow-twitch (soleus) and fast-twitch (anterior tibialis) skeletal muscles in the fed state. It also demonstrates, for the first time, that stable increases in PDK activities evoked by prolonged starvation in both skeletal-muscle types are associated with markedly increased protein expression of PDK4. The starvation-induced increase in PDK activity in slow-twitch muscle can be specifically attributed to up-regulation of PDK4 (PDK2 protein expression is unchanged). Both PDK2 and PDK4 protein expression increase in fast-twitch muscle after prolonged starvation. However, given the relative magnitude of response, and differences in specific activity, it is concluded that the starvation-induced enhancement of PDK activity in fast-twitch muscle also predominantly reflects up-regulation of PDK4.

The presence of a FA and dibutyryl cAMP is necessary to maintain differences in PDK activities between freshly-prepared soleus strips from fed and starved rats in culture [21]. Furthermore, PDC inactivation in both slow- and fast-twitch skeletal muscle in starvation is accelerated by elevation of circulating FA levels, and reversed by pharmacological inhibition of adipose-tissue lipolysis [25]. The present study provides evidence that starvation-induced increases in PDK activity are linked to upregulation of PDK4. PDK4 is relatively responsive to an increased $NADH/NAD^+$ concentration ratio compared with PDK2, and this study therefore provides the first evidence for a mechanism linking increased lipid-fuel availability and oxidation to PDC inactivation in response to starvation in both muscle types [25].

The exaggerated response of PDK4 protein expression to starvation in anterior tibialis compared with soleus does not correlate with the relative propensity with which these muscles oxidize lipid-derived fuels. Furthermore, a relatively high level of PDK4 protein expression is retained in fast-twitch muscle after refeeding at a time when PDK4 protein expression in soleus is significantly suppressed. These observations suggest the existence of mechanism(s) for regulation of PDK4 protein expression in fast-twitch muscle in addition to any mechanism responsive to lipid-fuel oxidation. There is a positive correlation between PDK2 and PDK4 mRNA expression in skeletal muscle and fasting plasma insulin levels (an index of insulin resistance) in Pima Indians, suggesting that PDK expression is down-regulated by insulin [32]. As insulin levels fall during starvation, greater upregulation of PDK4 expression in fast- compared with slow-twitch muscle could reflect the lesser sensitivity of fast-twitch muscle to insulin [14]. We therefore propose that insulin imposes a constraint on PDK4 expression in skeletal muscle, particularly in fast-twitch muscle.

Dichloroacetate is a pharmacological inhibitor of PDK that mimics the effect of pyruvate to inhibit PDK activity [7]. Dichloroacetate administration to fed rats increases the percentage of active (dephosphorylated) PDC in a range of skeletal muscles [25], implying that PDK activity in muscle is significant even in the fed state. Furthermore, it suggests that regulation of skeletal-muscle PDK by pyruvate is important in the regulation of PDC phosphorylation status (and therefore of pyruvate oxidation). *In vivo*, pyruvate may be derived from glycolysis *in situ*. In addition, since skeletal muscle readily oxidizes exogenous lactate, which enters the myocyte via one or more fibre-type specific MCTs [16], lactate can be derived from adjacent muscle cells or from circulating lactate. Physiological conditions that promote muscle glycolysis or increase exogenous lactate levels would, therefore, be predicted to increase pyruvate levels and suppress PDK, thereby facilitating PDC activation and pyruvate oxidation. Conversely, suppression of muscle glycolysis or lactate uptake would be predicted to de-suppress PDK and suppress pyruvate oxidation.

PDK2 not only has the lowest specific activity, but is also the most sensitive to inhibition by dichloroacetate [3]. PDK activity in respiring skeletal muscle mitochondria (rat hindlimb) can be inhibited directly by pyruvate [20]. Activation of PDC by dichloroacetate in the fed state *in vivo* is observed in slow-twitch and fast-twitch muscles, with more marked activation in the former [25]. In the present study, mitochondria prepared from soleus and anterior tibialis were incubated with respiratory substrate and pyruvate to determine whether these distinct muscle types differ with respect to sensitivity of PDK to pyruvate inhibition. We demonstrate that, in the fed state, sensitivity of PDK to suppression by pyruvate is approx. 2-fold greater in soleus than in anterior tibialis, suggesting that the functional contribution of PDK2 to total PDK activity in the fed state is greater in soleus than in anterior tibialis. A preponderance of relatively pyruvate-sensitive PDK2 in slow-twitch muscle in the fed state is predicted to render PDK activity relatively responsive to acute changes in pyruvate supply, facilitating the oxidation of lactate entering via MCT4. Conversely, higher relative expression of PDK4 in fast-twitch muscle in the fed state may confer a degree of pyruvate insensitivity to pyruvate oxidation, permitting partial uncoupling of glycolysis from glucose oxidation. Under anaerobic conditions, reduction of pyruvate thus generated to actate, regenerates the cytosolic NAD^+ required for maintenance of high glycolytic rates. This may be physiologically important since fast-twitch (white) muscle has a relatively poor blood supply and obtains much of the ATP required for contraction during exercise via anaerobic glycolysis. In addition, since the MCTs are proton-linked, exit of excess lactate from the cell will remove H^+, increasing intracellular pH and reducing fatigue.

We assessed the physiological implications of upregulation of PDK4 expression in starvation by examining the regulation of PDK activity by pyruvate. Our results suggest that the specific increase in PDK4 protein expression induced by starvation in both muscle types is likely to underlie the stable modification of the characteristics of regulation of native PDK by pyruvate. Thus differences in the regulatory characteristics of individual recombinant PDK isoenzymes demonstrated *in vitro* [3] are immediately relevant to native PDK *in vivo*. We also demonstrate that the more pronounced effect of starvation to impair PDK sensitivity to inhibition by pyruvate in fast-twitch compared with slow-twitch muscle is paralleled by relatively greater enhancement

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of PDK4 protein expression. Thus our data demonstrate that altered PDK pyruvate sensitivity in skeletal muscle evoked by starvation correlates with changes in PDK4 protein expression. This correlation is also apparent during refeeding, where partial suppression of PDK activity in soleus is associated with decreased PDK4 protein expression together with partial restoration of PDK pyruvate sensitivity. The more refractory response of PDK activity and PDK4 protein expression to refeeding in anterior tibialis is associated with a failure to reverse starvation-induced changes in PDK pyruvate sensitivity. Thus our results indicate that a functional switch to the higher specific activity, less pyruvate sensitive, PDK isoenzyme occurs after prolonged starvation, with relatively slow reversal after refeeding.

The changes in relative protein expression of PDK2 and PDK4, with selective upregulation of PDK4, may be fundamental to the metabolic response of skeletal muscle to starvation in several respects. Firstly, since skeletal muscle is the major peripheral site of glucose disposal, suppression of muscle glucose oxidation is vital for conservation of 3-carbon derivatives of glucose for use as gluconeogenic precursors. Since muscle glycogen stores are lost in starvation [30], a proportion of these gluconeogenic precursors may be derived from glycogenolysis and glycolysis *in situ*. In addition, PDC inactivation in skeletal muscle will minimize the use of blood-borne lactate as an oxidative substrate. Skeletal-muscle protein is relatively expendable in prolonged starvation. The carbon skeletons of constituent amino acids of skeletal-muscle protein may be an important source of gluconeogenic precursors. Breakdown of muscle protein requires the disposal of the amine nitrogen generated by conversion of amino acids into their keto acid derivatives. In starvation, skeletal-muscle amino acids are transaminated with pyruvate to produce alanine, which is released into the circulation. Thus increased expression of the less pyruvate sensitive isoform may be a mechanism for suppression of PDC activity [thereby limiting pyruvate oxidation to facilitate the Cori (glucose → lactate → glucose) cycle], whilst maintaining a sufficiently high pyruvate concentration to ensure unimpaired operation of the glucose → alanine → glucose cycle. Finally, we propose that upregulation of PDK4 protein in skeletal muscle is related to muscle ATP requirements under circumstances where carbohydrate is scarce. Anaplerotic entry of carbohydrates to maintain tricarboxylic acid cycle intermediates is required to support normal muscle oxidative capacity during exercise [11]. Data obtained using a rat hindlimb preparation perfused with [3-^{13}C]lactate have provided evidence for non-oxidative entry of carbohydrate into the tricarboxylic acid cycle [11]. It was proposed that entry and exit of pyruvate from the tricarboxylic acid cycle via non-oxidative pathways are components of normal muscle metabolism, that non-oxidative tricarboxylic acid entry of pyruvate during contraction may be required for normal muscle function, and that carbohydrate depletion during prolonged exercise can impair aerobic energy metabolism by reducing the level of tricarboxylic acid cycle intermediates [11]. A similar situation may exist after prolonged starvation, when carbohydrate is scarce. A requirement for the maintenance of tricarboxylic acid cycle intermediate concentrations in working muscle would be introduced if $V\text{O}_2$ and oxidative phosphorylation were limited not only by oxygen itself, but also by the tricarboxylic acid cycle intermediate pool size. Switching from a pyruvate-sensitive to a pyruvate-insensitive PDK isoenzyme would ensure that available pyruvate was not oxidized, but 'spared' for anaplerotic entry into the tricarboxylic acid cycle to maintain tricarboxylic acid cycle intermediates. In turn, this would support the oxidation (via the tricarboxylic acid cycle) of acetyl-CoA derived from FA oxidation. Thus we propose that PDK4 upregulation may both force and facilitate FA oxidation. In the event that the ATP requirement was met, anaplerotic entry of pyruvate would permit the accumulation of citrate, which is known to signal FA oxidation and to suppress anaerobic glycolysis in muscle [33]. Thus upregulation of PDK4 is perceived as an essential component of the operation of the glucose-FA cycle in skeletal muscle in starvation.

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