Selective modification of the pyruvate dehydrogenase kinase isoform profile in skeletal muscle in hyperthyroidism: implications for the regulatory impact of glucose on fatty acid oxidation

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Abstract

The pyruvate dehydrogenase kinases (PDK1–4) regulate glucose oxidation through inhibitory phosphorylation of the pyruvate dehydrogenase complex (PDC). Immunoblot analysis with antibodies raised against recombinant PDK isoforms demonstrated changes in PDK isoform expression in response to experimental hyperthyroidism (100 μg/100 g body weight; 3 days) that was selective for fast-twitch vs slow-twitch skeletal muscle in that PDK2 expression was increased in the fast-twitch skeletal muscle (the anterior tibialis) (by 1·6-fold; $P<0·05$) but not in the slow-twitch muscle (the soleus). PDK4 protein expression was increased by experimental hyperthyroidism in both muscle types, there being a greater response in the anterior tibialis (4·2-fold increase; $P<0·05$) than in the soleus (3·2-fold increase; $P<0·05$). The hyperthyroidism-associated up-regulation of PDK4 expression was observed in conjunction with suppression of skeletal-muscle PDC activity, but not suppression of glucose uptake/phosphorylation, as measured *in vivo* in conscious unrestrained rats (using the 2-[^{3}H]deoxyglucose technique). We propose that increased PDK isoform expression contributes to the pathology of hyperthyroidism and to PDC inactivation by facilitating the operation of the glucose → lactate → glucose (Cori) and glucose → alanine → glucose cycles. We also propose that enhanced relative expression of the pyruvate-insensitive PDK isoform (PDK4) in skeletal muscle in hyperthyroidism uncouples glycolytic flux from pyruvate oxidation, sparing pyruvate for non-oxidative entry into the tricarboxylic acid (TCA) cycle, and thereby supporting entry of acetyl-CoA (derived from fatty acid oxidation) into the TCA cycle.

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Introduction

The skeletal muscle mass is quantitatively of major importance in glucose disposal (DeFronzo *et al.* 1981), but can also utilise other substrates including fatty acids (FAs) and exogenous lactate (Bertocci *et al.* 1997, Bertocci & Lujan 1999). In resting skeletal muscle, approx. 40% of oxygen uptake is normally used for carbohydrate oxidation, and the remainder, approx. 60%, is used for fat oxidation (Brooks & Mercier 1994). The relative rates of glucose and lipid utilisation vary according to muscle-fibre composition and nutritional and hormonal status (see, e.g., Storlien *et al.* 1986, Holness & Sugden 1990). Peripheral glucose metabolism is accelerated in hyperthyroidism (Okajima & Ui 1979*a*,*b*, Huang & Lardy 1981); lipid mobilisation and oxidation is also increased (Moller *et al.* 1996). The mechanisms that permit the concomitant use of both glucose and lipid at high rates in hyperthyroidism are incompletely understood, but may be linked to changes in energy metabolism introduced through modulation of, for example, myosin heavy-chain isoform composition (Canepari *et al.* 1998, Jakubiec-Puka *et al.* 1999), ATPase activity (Canepari *et al.* 1998) and uncoupling protein expression (Nagase *et al.* 1999). In addition, the expression of the insulin-regulatable glucose transporter GLUT4 is increased in hyperthyroidism (Casla *et al.* 1990). This latter effect is associated with increased glucose uptake and metabolism to lactate both in the absence and presence of insulin (Dimitriadis *et al.* 1997).

The pyruvate dehydrogenase complex (PDC) is a major target for the modulation of fuel selection through substrate competition (reviewed in Sugden & Holness 1994). Suppression of PDC activity in skeletal muscle during prolonged starvation is particularly important for sparing three carbon compounds (including lactate and pyruvate) that are required for gluconeogenesis to maintain glycaemia. PDC is rendered inactive by phosphorylation of the α-subunit of its pyruvate dehydrogenase component by pyruvate dehydrogenase kinase (PDK) (reviewed in

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Sugden & Holness 1994, Harris *et al.* 1995). NADH and acetyl-CoA, generated by increased rates of FA β-oxidation, activate PDK (reviewed in Sugden & Holness 1994, Harris *et al.* 1995). The activation of PDK by the products of FA oxidation, together with end-product inhibition, underlies the operation of the glucose–FA cycle in starvation.

Four PDK isoforms have been identified in mammalian tissues (Bowker-Kinley *et al.* 1998): two of these (PDK2 and PDK4) have been detected in both slow-twitch and fast-twitch skeletal muscle (Holness *et al.* 2000, Sugden *et al.* 2000). The suppression of PDC activity in oxidative skeletal muscle during prolonged starvation is observed in association with specific up-regulation of the expression of the PDK4 protein (Sugden *et al.* 2000). PDK4 protein expression is also up-regulated in fast-twitch muscle; however, PDK4 up-regulation in this muscle type is also associated with a modest increase in PDK2 expression (Sugden *et al.* 2000). *In vitro* studies with the recombinant proteins have shown that recombinant PDK4 (rPDK4) is a high-specific-activity isoform. Recombinant PDK2 (rPDK2) is stimulated only weakly by NADH (although stimulation is enhanced by further addition of acetyl-CoA), whereas rPDK4 is activated twofold by NADH alone, showing no further effects of the addition of acetyl-CoA (Bowker-Kinley *et al.* 1998). However, the activity of rPDK4 (relative to that of rPDK2) is insensitive to suppression by dichloroacetate, a pharmacological inhibitor of PDK that mimics the effect of the physiological inhibitor pyruvate (Bowker-Kinley *et al.* 1998). *In vivo*, up-regulation of PDK4 in skeletal muscle after prolonged starvation and high-fat feeding is associated with reduced susceptibility of PDK in isolated mitochondria to inhibition by pyruvate (Holness *et al.* 2000, Sugden *et al.* 2000).

The present study investigated the effect of experimental hyperthyroidism on PDK-isoform expression in both slow- and fast-twitch skeletal muscles in relation to changes in skeletal-muscle glucose uptake/phosphorylation and PDC activity *in vivo*. One of our aims was to test the hypothesis that modulation of PDK-isoform expression in skeletal muscle is a component of the metabolic response to hyperthyroidism. In addition, we wanted to establish whether a PDK-isoform shift in skeletal muscle in favour of the pyruvate-insensitive, lipid-responsive PDK isoform (PDK4) permits high rates of glycolysis and therefore the accelerated glucose turnover that is characteristic of the hyperthyroid state, but prevents activation of the PDC complex, thereby limiting the capacity for glucose oxidation and potentially favouring lipid oxidation.

Materials and Methods

Materials

Arylamine acetyltransferase was purified from pigeon-liver acetone powder (purchased from Europa Bioproducts Ltd, Ely, Cambridgeshire, UK). ECL reagents and 2-deoxy-D-[1-^{3}H]glucose were purchased from Amersham Radiochemicals (Amersham, Buckinghamshire, UK). Other chemicals and biochemicals were purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, Hertfordshire, UK), Boehringer Mannheim (Lewes, East Sussex, UK) or the Sigma Chemical Corporation (Poole, Dorset, UK). Female Wistar rats were purchased from Charles River Ltd (Margate, Kent, UK).

Rats

All studies were conducted in adherence to the regulations of the UK Animal Scientific Procedures Act (1986). Adult, female Wistar rats (initial weights 200–250 g) were provided with free access to food and water and were housed in individual cages in a temperature- (21 ± 2 °C) and light-controlled room (a ratio of 12 h light:12 h darkness). Age-matched rats were randomly allocated to one of two groups. Both groups were maintained with the standard high-carbohydrate/low-fat rodent laboratory diet (8% fat, 72% carbohydrate, 20% protein, by calories; supplied by Special Diets Services, Witham, Essex, UK) and water and were allowed to feed and drink *ad libitum*. Rats were sampled in the absorptive state or after 48 h of starvation. Rats were rendered hyperthyroid by injection of tri-iodothyronine (1 mg/kg body weight per day; 3 days). Control rats were injected with equivalent amounts of hormone solvent (10 mM NaOH/0·03% BSA). In some studies rats were, for 48 h, denied free access to food, but were allowed to drink water *ad libitum*. In all of the experimental protocols utilised, the two individual muscles (the anterior tibialis (AT) and the soleus (SOL)) were sampled from the same animal and analysed in parallel. The fibre profiles (% fast oxidative glycolytic: fast glycolytic:slow oxidative) of the AT and the SOL muscles are 66:32:2 and 0:0:100 respectively (Ariano *et al.* 1973).

Immunoblots

Mitochondria were prepared from the AT and SOL muscles 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 preparations (3 μg protein) were subjected to SDS-PAGE as described previously (Holness *et al.* 2000, Sugden *et al.* 2000).

Enzyme assay

Active pyruvate dehydrogenase (PDHa) activity was assayed both in freeze-clamped muscle extracts and in mitochondria, prepared as described in Fuller & Randle (1984). PDHa was assayed spectrophotometrically by

coupling to arylamine acetyltransferase (Caterson *et al.* 1982, Holness *et al.* 1989). Total PDC activities were measured after incubation of tissue extracts with PDH phosphate phosphatase and expressed relative to citrate synthase to correct for possible differences in mitochondrial extraction.

In vivo *glucose utilisation in individual muscles*

Estimations of glucose utilisation by the individual muscles *in vivo* 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 ([^{3}H]2DG) via a chronic indwelling cannula (Ferré *et al.* 1985, Holness & Sugden 1990). Blood samples (100 μl) for the determination of blood glucose concentrations and plasma tracer concentrations were obtained via the indwelling cannula at 1, 3, 5, 10, 20, 40 and 60 min after [^{3}H]2DG bolus administration. Throughout the study, the rats were awake and moving freely, with the connecting tubing suspended overhead. Rats were killed by the intravenous injection of pentobarbitone (60 mg/kg) via the indwelling cannula. Individual skeletal muscles (AT and SOL) were freeze-clamped when locomotor activity had ceased (within 5 s) and were stored in liquid nitrogen until analysis. No correction has been made for possible discrimination against 2-deoxyglucose versus glucose with respect to glucose transport and phosphorylation; hence, rates of tissue accumulation of 2-deoxy-D-[^{3}H] glucose 6-phosphate are referred to as glucose-utilisation indices (GUIs).

Statistical analysis

Experimental data are expressed as means ± S.E.M. The statistical significance of differences between groups was assessed using the Student's *t*-test.

Results

Experimental hyperthyroidism modifies the protein expression of individual PDK isoforms in skeletal-muscle mitochondria of fed rats

Western blot analysis was used to determine whether experimental hyperthyroidism is associated with targeted or muscle-fibre-type specific changes in PDK2 and/or PDK4 protein expression (Figs 1 and 2). Hyperthyroidism led to a 1·6-fold increase ($P<0·05$) in the amount of PDK2 protein present in the AT, the representative fast-twitch muscle, but did not affect PDK2 protein expression in the SOL (Fig. 1). In contrast, hyperthyroidism led to increases in PDK4 protein expression of an approximately similar magnitude (4·2- and 3·2-fold increases; $P<0·01$ in each case) in the AT and SOL mitochondria (Fig. 2). Thus, the expression of PDK4 relative to PDK2 increases in both the AT and the SOL muscles in response to experimental hyperthyroidism, but there is also specific up-regulation of PDK2 in the AT muscle.

Hyperthyroidism reduces PDHa activities in slow-twitch and fast-twitch skeletal muscles

We measured the percentage of active PDC (PDHa) in each of the skeletal muscles to determine whether increased PDK protein expression was associated with altered PDC phosphorylation (≡activity) status. Muscles from control and hyperthyroid animals in the absorptive state were freeze-clamped. In control rats, PDHa activities were considerably higher in the fast-twitch than in the slow-twitch muscle (Fig. 3). Nevertheless, hyperthyroidism was associated with significant suppression of PDHa activity in both the AT and the SOL. Hyperthyroidism produced no significant change in total PDC activity in either the fast-twitch (control (C), 38 ± 1 mU per U citrate synthase; tri-iodothyronine (T_3), 39 ± 1 mU per U citrate synthase) or the slow-twitch skeletal muscle (C, 24 ± 2 mU per U citrate synthase; T_3, 22 ± 1 mU per U citrate synthase). Since total PDC activities were unchanged by hyperthyroidism (see the Materials and Methods), the hyperthyroidism-associated lowering of the concentration of active PDC is a consequence of increased net phosphorylation of PDC, i.e. an increased PDK/PDC phosphate phosphatase ratio.

Hyperthyroidism does not suppress skeletal-muscle glucose utilisation in vivo

As the patterns of PDK-isoform expression evoked in fast- and slow-twitch muscle in response to hyperthyroidism are reminiscent of those induced by prolonged (48 h) starvation (see Sugden *et al.* 2000), and as both starvation (Sugden *et al.* 2000) and hyperthyroidism (present study) are associated with reduced skeletal-muscle PDHa activities, we investigated whether suppression of skeletal-muscle glucose utilisation (transport/phosphorylation) was a common feature. Rates of glucose utilisation (transport/phosphorylation) in the AT and the SOL muscles *in vivo* were measured in conscious, unrestrained euthyroid and hyperthyroid rats in the fed and starved (48 h) states (Fig. 4). Experimental hyperthyroidism significantly increased the GUIs in AT in the fed state (2·2-fold; $P<0·01$) and after 48 h starvation (3·3-fold; $P<0·001$). GUIs in the SOL of rats in the fed state were unaffected by experimental hyperthyroidism, but hyperthyroidism greatly attenuated the response to 48 h of starvation (a 78% decline ($P<0·01$) in euthyroid rats compared with a 58% decline ($P<0·001$) in hyperthyroid rats).

Discussion

Suppression of PDC activity by phosphorylation is achieved by the structurally related PDK isoforms

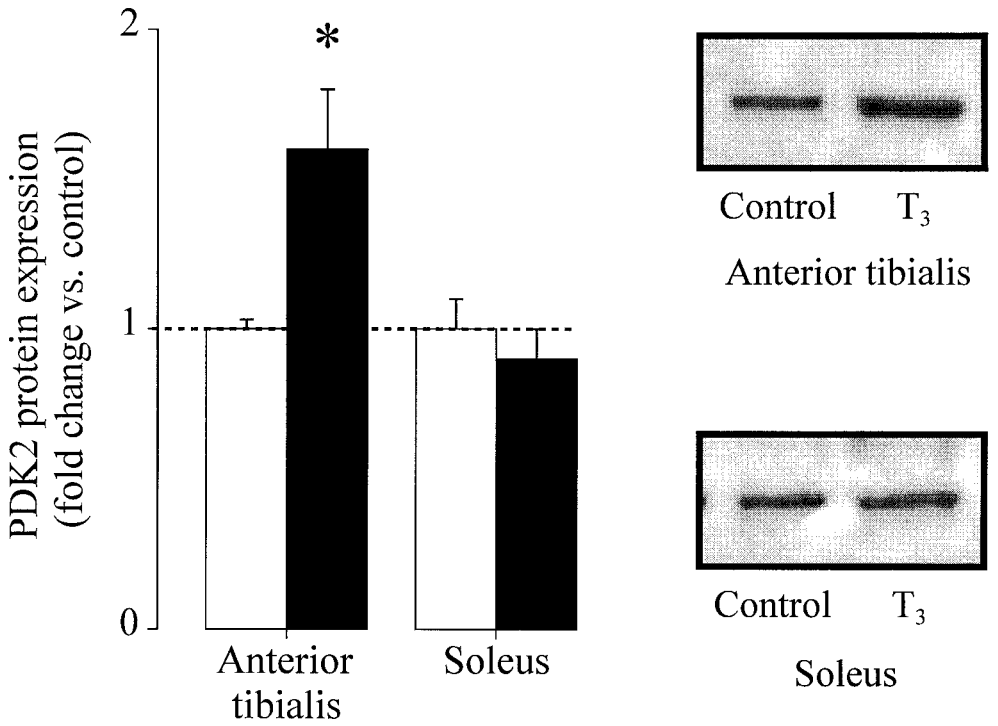

Figure 1 Effects of hyperthyroidism on PDK2 protein expression in mitochondria prepared from the anterior tibialis and soleus muscles of fed rats. Mitochondria were prepared from muscles of euthyroid (open bars) and hyperthyroid (filled bars) rats that were allowed to feed *ad libitum*. Rabbit polyclonal antisera raised against recombinant PDK2 were used to detect these proteins by means of Western blot analysis. Muscle mitochondrial extracts were denatured and subjected to SDS-PAGE and immunoblotting with these isoenzyme-specific antibodies, as described in the Materials and Methods. Western blots were analysed by scanning densitometry with MOLECULAR ANALYST Version 1·5 software. Values are means ± S.E.M. for 5–9 individual internally controlled experiments. *P<0·05 vs euthyroid. Representative immunoblots are shown.

(PDK1–4). PDK2 and PDK4 are expressed in rat skeletal muscle (Wu *et al.* 1999, Holness *et al.* 2000, Sugden *et al.* 2000), and up-regulation of PDK4 in starvation and high-fat feeding is associated with reduced sensitivity of PDK activity to suppression by pyruvate (Holness *et al.* 2000, Sugden *et al.* 2000). We demonstrate, here, a clear and novel effect of thyroid hormone on a key regulatory enzyme (PDK) of skeletal-muscle fuel metabolism. Experimental hyperthyroidism leads to stable up-regulation of the protein expression of both PDK2 and PDK4 in fast-twitch muscle, with a more marked up-regulation of PDK4 and up-regulation only of PDK4 in slow-twitch muscle. These effects are concomitant with suppression of skeletal-muscle PDC activity, but not of skeletal-muscle glucose uptake and phosphorylation. The study has important physiological implications for glucose homeostasis as our results suggest that relative up-regulation of PDK4 in hyperthyroidism uncouples the mechanism by which skeletal muscle increases the capacity for glucose oxidation in parallel with accelerated glycolytic flux. We further propose that the impairment in the ability of skeletal muscle to oxidise pyruvate facilitates the increased rates of Cori (glucose → pyruvate/lactate → glucose) cycling and glucose → alanine → glucose cycling that are characteristic of the hyperthyroid state.

Pyruvate is an allosteric inhibitor of PDK: increasing pyruvate concentrations activate PDC in respiring skeletal-muscle mitochondria (Holness *et al.* 2000, Sugden *et al.* 2000) and administration of the pyruvate analogue dichloroacetate increases the percentage of active PDC in skeletal muscles in the fed state *in vivo* (Holness *et al.* 1989). Studies with the recombinant proteins have shown that PDK2 is a low-specific-activity PDK isoform whose activity, compared with that of PDK4, is relatively sensitive to suppression by dichloroacetate (Bowker-Kinley *et al.* 1998). By analogy with the role of glucokinase as a 'glucose sensor' in the liver, we propose that PDK2 in skeletal muscle may act as a 'pyruvate sensor', coupling increases in pyruvate supply (generated via increased glycolytic flux or circulating lactate supply) with acetyl-CoA generation from pyruvate. Hyperthyroidism, which promotes glucose uptake and utilisation by skeletal muscle, would be expected to facilitate PDC activation and pyruvate oxidation in muscle via the suppression of PDK

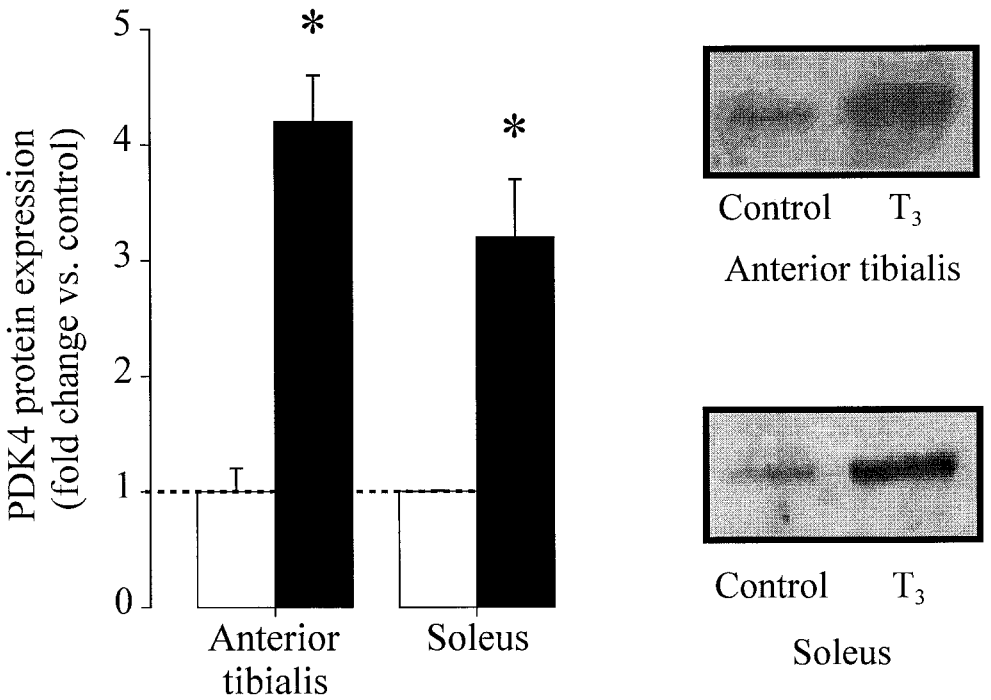

Figure 2 Effects of hyperthyroidism on PDK4 protein expression in mitochondria prepared from the anterior tibialis and soleus muscles of fed rats. Mitochondria were prepared from muscles of fed euthyroid (open bars) and hyperthyroid (filled bars) rats that were allowed to feed *ad libitum*. Rabbit polyclonal antisera raised against recombinant PDK4 were used to detect these proteins by means of Western blot analysis. Muscle mitochondrial extracts were denatured and subjected to SDS-PAGE and immunoblotting with these isoenzyme-specific antibodies, as described in the Materials and Methods. Western blots were analysed by scanning densitometry with MOLECULAR ANALYST Version 1·5 software. Values are means ± S.E.M. for 5–9 individual internally controlled experiments. *P<0·01 vs euthyroid. Representative immunoblots are shown.

activity by pyruvate. However, skeletal muscle responds to hyperthyroidism with PDC inactivation. We propose that the increased expression of PDK4 protein relative to PDK2 protein in skeletal muscle in hyperthyroidism desensitises the *in vivo* response of muscle glucose oxidation to pyruvate supply. The conversion of pyruvate to acetyl-CoA via PDC links glycolysis with the citric acid cycle and the synthesis of malonyl-CoA (Sugden & Holness 1994). Current research suggests that an increased glucose supply reduces FA oxidation in skeletal muscle through an increase in intracellular malonyl-CoA concentrations (the 'reverse' glucose–fatty acid cycle) (reviewed in McGarry & Brown 1997, Ruderman *et al.* 1999; see also Sidossis & Wolfe 1996, Sidossis *et al.* 1996, 1999). Maintenance of PDC activity at a low level relative to glycolytic flux means that the use of available pyruvate for malonyl-CoA synthesis is impaired and thus PDK4 up-regulation in the fed state in hyperthyroidism may attenuate regulatory links between an increased glucose supply and the suppression of FA oxidation.

In addition, we propose that the increased relative expression of PDK4 protein relative to PDK2 protein in skeletal muscle in hyperthyroidism sensitises the *in vivo* response of muscle glucose oxidation via PDC to increased rates of β-oxidation. Recent data obtained using a rat-hindlimb preparation perfused with [3-^{13}C]lactate has provided evidence for the non-oxidative entry of carbohydrate, mainly as oxaloacetate, into the TCA cycle (Bertocci & Lujan 1999). It was proposed that entry and exit of pyruvate from the TCA cycle via non-oxidative pathways are components of normal muscle metabolism and that carbohydrate depletion could impair aerobic energy metabolism by reducing the level of TCA cycle intermediates (Bertocci & Lujan 1999). Maintenance of PDC activity at a relatively low level – despite relatively high rates of glucose uptake and utilisation – in the hyperthyroid state ensures that a significant proportion of available pyruvate is 'spared' for oxaloacetate formation rather than being oxidised via PDC. We therefore propose that PDK4 up-regulation in rats in the fed state in hyperthyroidism facilitates the maintenance of TCA-cycle intermediates that would be predicted to support the complete oxidation (via the TCA cycle) of acetyl-CoA derived from FA β-oxidation.

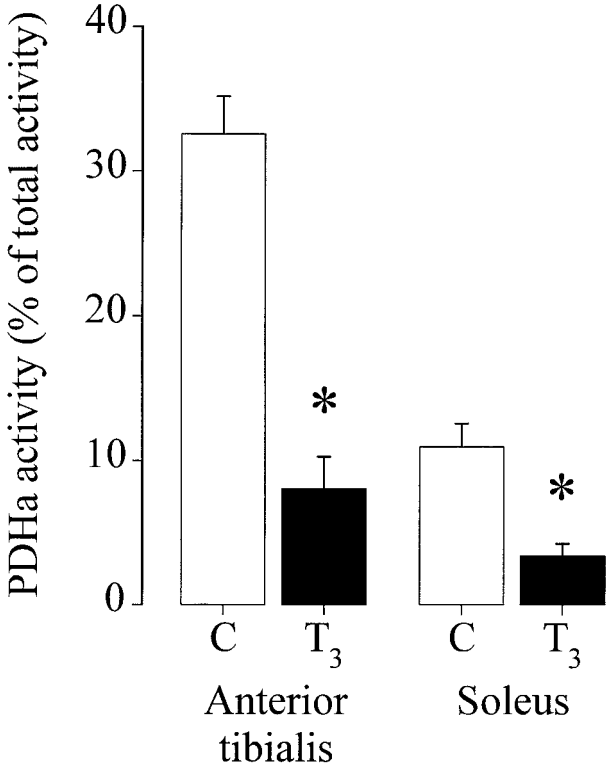

Figure 3 Effects of hyperthyroidism on PDHa activity in mitochondria prepared from the anterior tibialis and soleus muscles of fed rats. PDHa activities were measured in freeze-clamped muscle extracts of euthyroid (C, open bars) and hyperthyroid (T_3, filled bars) rats that were allowed to feed *ad libitum*. Values are means ± S.E.M. for 5–9 individual internally controlled experiments. *P<0·01 vs euthyroid.

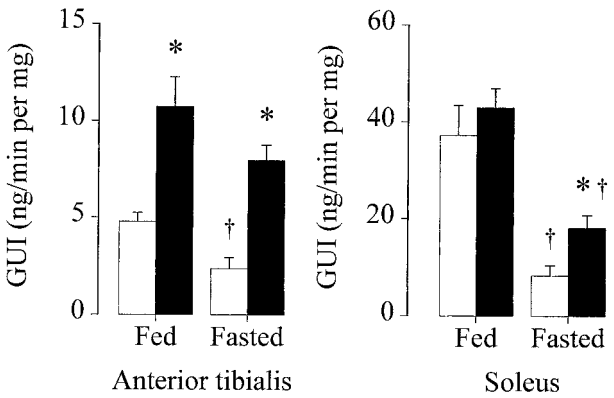

Figure 4 GUIs in the anterior tibialis and soleus muscles in euthyroid and hyperthyroid rats in the fed and fasted states. GUIs were measured in muscles of fed or fasted (48 h) euthyroid (open bars) and hyperthyroid (filled bars) rats that were allowed to feed *ad libitum*. Further details are given in the Materials and Methods. Values are means ± S.E.M. for 5–9 individual experiments. *P<0·01 vs euthyroid; †P<0·01 vs fed.

The physiological implications of concomitant up-regulation of both PDK isoforms in fast-twitch skeletal muscle remain to be determined. Fast-twitch muscle is more reliant on glucose than on FA as the energy substrate. Thus, up-regulation of both PDK2 and PDK4 in the fast-twitch muscle in response to hyperthyroidism may imply a requirement for PDC activity in this muscle type to retain limited sensitivity to changes in pyruvate supply, irrespective of whether acetyl-CoA/CoA and NADH/NAD^+ ratios are concomitantly increased through β-oxidation. Thus, PDC activity status can be matched to energy demand irrespective of the substrate that is being oxidised.

Skeletal muscle protein, particularly that of the non-working fast-twitch muscles, is relatively expendable (Preedy *et al.* 1990). In starvation, skeletal-muscle amino acids are transaminated with pyruvate to produce alanine, which is then released into the circulation and removed by the liver. Moderate hyperthyroidism increases muscle proteolysis (Tauveron *et al.* 1995, Grofte *et al.* 1997*a*,*b*) and reduces muscle nitrogen content and the overall nitrogen balance in the rat (Grofte *et al.* 1997*b*). A consequence of the breakdown of muscle protein is the need to dispose of the amine nitrogen generated by the conversion of the amino acids to their corresponding keto acids. In a previous study, we found that the specific increases in the expression of the PDK4 protein induced by starvation in both fast-twitch and slow-twitch skeletal muscle were associated with impaired activation of PDC by increased pyruvate concentrations (Sugden *et al.* 2000). By analogy, therefore, the relatively enhanced expression of the less pyruvate-sensitive PDK isoenzyme PDK4 in fast-twitch muscle in rats in the fed hyperthyroid state may be a mechanism by which PDC can remain relatively inactive, even though glucose uptake is increased, thereby facilitating the disposal of amine nitrogen generated by the conversion of amino acids (derived from protein breakdown) to their corresponding keto acids. However, the preferential elevation of PDK4 in skeletal muscle in response to hyperthyroidism is potentially detrimental to glucose homeostasis in that increased direction of glycolytically derived pyruvate away from oxidation towards lactate output (and hepatic glucose production) may form an important component of the elevated rates of glucose production characteristic of this condition.

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