

# Targeted Upregulation of Pyruvate Dehydrogenase Kinase (PDK)-4 in Slow-Twitch Skeletal Muscle Underlies the Stable Modification of the Regulatory Characteristics of PDK Induced by High-Fat Feeding

Mark J. Holness, Alexandra Kraus, Robert A. Harris, and Mary C. Sugden

**In using Western blot analysis with antibodies raised against recombinant pyruvate dehydrogenase kinase (PDK) isoforms PDK2 and PDK4, this study demonstrates selective PDK isoform switching in specific skeletal muscle types in response to high-fat feeding that is associated with altered regulation of PDK activity by pyruvate. The administration of a diet high in saturated fats led to stable (~2-fold) increases in PDK activities in both a typical slow-twitch (soleus [SOL]) muscle and a typical fast-twitch (anterior tibialis [AT]) muscle. Western blot analysis revealed that high-fat feeding significantly increased (~2-fold;  $P < 0.001$ ) PDK4 protein expression in SOL, with a modest (1.3-fold) increase in PDK2 protein expression. The relative increase in PDK4 protein expression in SOL was associated with a 7.6-fold increase in the pyruvate concentration that was required to elicit a 50% active pyruvate dehydrogenase complex, which indicates a marked decrease in the sensitivity of PDK to inhibition by pyruvate. In AT muscle, high-fat feeding elicited comparable (1.5- to 1.7-fold) increases ( $P < 0.05$ ) in PDK4 and PDK2 protein expression. Loss of sensitivity of PDK to inhibition by pyruvate was less marked. The data suggest that a positive correlation exists between increases in PDK4 expression and the propensity with which muscles use lipid-derived fuels as respiratory substrates rather than with the degree of insulin resistance induced in skeletal muscles by high-fat feeding. In conclusion, high-fat feeding leads to selective upregulation of PDK4 expression in slow-twitch muscle in response to high-fat feeding in vivo, which is associated with a pronounced loss of sensitivity of PDK activity to acute inhibition by pyruvate. Thus, increased PDK4**

**expression may underlie the stable modification of the regulatory characteristics of PDK observed in slow-twitch muscle in response to high-fat feeding. *Diabetes* 49:775-781, 2000**

**R**egulation 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 starvation when 3 carbon compounds (including pyruvate) are required for gluconeogenesis to maintain glycemia. PDC is rendered inactive by phosphorylation of the  $\alpha$ -subunit of its pyruvate dehydrogenase component by pyruvate dehydrogenase kinase (PDK) (1,2). PDK activity is itself regulated. Short-term mechanisms of PDK regulation include suppression of activity by pyruvate generated by glycolysis or from circulating lactate and activation resulting from high mitochondrial acetyl-CoA/CoA and NADH/NAD<sup>+</sup> concentration ratios that are generated by increased rates of fatty acid (FA)  $\beta$ -oxidation. In addition, PDK activity can be enhanced by a longer-term mechanism that is independent of the acute effects of small molecular weight effectors (3). A total of 4 PDK isoforms have been identified in mammalian tissues (4,5). Studies with the individual recombinant PDK isoforms expressed in *Escherichia coli* have demonstrated important differences in their specific activities and in their regulatory characteristics (4). In studies in the rat in vivo, stable effects of starvation to enhance PDK activity have been observed in association with the upregulation of the protein expression of at least 2 of these 4 PDK isoforms. PDK4 is upregulated in response to starvation in the heart (6). PDK4 is a high-specific activity PDK isoform whose activity is relatively insensitive to suppression by dichloroacetate (4), which is a highly specific synthetic inhibitor of PDK that is believed to mimic the effect of the physiological inhibitor pyruvate. This response to starvation appears to be specific for PDK4 with no change in protein expression of PDK2 (a lower-specific activity pyruvate-sensitive isoform) (6). By contrast, PDK2 protein expression is upregulated by starvation both in the liver (7) and in the kidneys (8). This suggests that the responses of individual PDK isoforms to insulin deficiency are dictated by tissue type and presumably function.

From the Department of Diabetes and Metabolic Medicine (M.J.H., A.K., M.C.S.), Division of General and Developmental Medicine, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Queen Mary and Westfield College, University of London, London, U.K.; and the Department of Biochemistry and Molecular Biology (R.A.H.), Indiana University School of Medicine, Indianapolis, Indiana.

Address correspondence and reprint requests to M.C. Sugden, MA, DPhil, DSc, Department of Diabetes and Metabolic Medicine, Medical Sciences Bldg., Queen Mary and Westfield College, Mile End Rd., London E1 4NS, U.K. E-mail: m.c.sugden@qmw.ac.uk.

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[<sup>3</sup>H]2DG, 2-deoxy-D-[1-<sup>3</sup>H]glucose; AT, anterior tibialis; ECL, enhanced chemiluminescence; FA, fatty acid; GUI, glucose utilization index; PDC, pyruvate dehydrogenase complex; PDHa, active pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PPAR- $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ;  $R_p$ , glucose disappearance rate; SOL, soleus; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween.

The skeletal muscle mass is quantitatively of major importance for glucose disposal (9). In nondiabetic Pima Indians (a population with a high prevalence of type 2 diabetes associated with obesity), the expression of PDK2 and PDK4 mRNA in muscle biopsies correlates positively with fasting plasma insulin levels (an indicator of insulin resistance) and negatively with insulin-mediated glucose uptake (10). This study suggested that insufficient downregulation of PDK by insulin in the skeletal muscles of insulin-resistant individuals may be a cause of increased PDK expression, thus leading to impaired glucose oxidation followed by increased FA oxidation (10). Recent findings have also implicated a direct role for FA acting via the peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) in signaling increased PDK4 expression in skeletal muscle (11). However, the skeletal muscle mass displays considerable heterogeneity regarding glucose utilization (both in the basal [postabsorptive] state and during insulin stimulation) and the capacity for FA utilization. Under resting conditions, the slow-twitch muscles (e.g., soleus [SOL] muscle) display higher rates of glucose utilization (12,13) and greater insulin sensitivity (13) than the fast-twitch muscles (e.g., anterior tibialis [AT] muscle). Furthermore, slow-twitch muscles (which contain higher proportions of oxidative fibers [14]) avidly oxidize FA, and glucose utilization is markedly reduced in these metabolically active muscles to a greater degree than the reduction in whole-body glucose disposal after high-fat feeding (15). In contrast, fast-twitch muscles containing mainly glycolytic fibers are little affected by high-fat feeding (15). PDK2 and PDK4 mRNA expression has been demonstrated in rat gastrocnemius muscle (4); in the rat, this muscle displays characteristics of fast-twitch muscle regarding glucose utilization in the fed and fasted states (12) and during insulin stimulation (13,16).

Researchers have demonstrated previously that skeletal muscle PDK activity increases in response to the administration of a diet high in saturated fat and low in carbohydrates (17). In the present study, we examined to what extent PDK2 and/or PDK4 protein expression in skeletal muscle is altered and whether individual muscle types respond differently regarding changes in expression of different PDK isoforms in response to high-saturated fat feeding, which elicits insulin resistance in both slow- and fast-twitch muscles. We evaluated whether changes in the expression of either of these PDK isoforms in slow- and fast-twitch skeletal muscle *in vivo* correlate positively with their capacity for FA utilization or negatively with changes in skeletal muscle insulin sensitivity assessed directly *in vivo* with the 2-deoxy[1- $^3$ H]glucose technique in combination with the euglycemic-hyperinsulinemic clamp. Finally, to assess whether PDK isoform switching, by virtue of the distinct regulatory properties identified *in vitro* with recombinant proteins (4), is of potential physiological significance for the control of glucose oxidation *in vivo*, we investigated whether changes in the PDK isoform profile in individual skeletal muscles are reflected in altered sensitivity of PDK activity to suppression by pyruvate.

## RESEARCH DESIGN AND METHODS

**Materials.** Human Actrapid insulin was purchased from Novo Nordisk (Bagsvaerd, Denmark). Kits for determination of plasma insulin concentrations were purchased from Phadeseoph Pharmacia (Uppsala, Sweden). Radiochemicals and enhanced chemiluminescence (ECL) reagents were purchased from Amersham International (Buckinghamshire, U.K.). Arylamine acetyltransferase was purified from pigeon liver acetone powder (Europa Bio-

products, Ely, Cambridgeshire, U.K.). Other chemicals and biochemicals were purchased from Bio-Rad (Hemel Hempstead, Hertfordshire, U.K.), Boehringer Mannheim (Lewes, East Sussex, U.K.), or Sigma (Poole, Dorset, U.K.). Female Wistar rats were purchased from Charles River (Margate, Kent, U.K.). Diets and individual components of diets were purchased from Special Diet Services (Witham, Essex, U.K.).

**Rats and diets.** Adult female Wistar rats (initial weights 230–250 g) with free access to food and water were housed in individual cages in a temperature- $(21 \pm 2^\circ\text{C})$  and light-controlled room (a 12-h light-dark cycle). Age-matched rats were randomly allocated to 2 groups. The first group was maintained on standard high-carbohydrate/low-fat rodent laboratory diet (72% digestible carbohydrate, 20% protein, 8% lipid by energy), whereas the second group was maintained on a semisynthetic low-carbohydrate high-saturated fat diet (33% carbohydrate, 20% protein, and 47% lipid by energy) (17) for 28 days. In all of the experimental protocols, the 2 individual muscles were sampled from the same animal and were analyzed in parallel.

**Euglycemic-hyperinsulinemic clamps.** For the euglycemic-hyperinsulinemic clamp studies, each rat was fitted with 2 chronic indwelling cannulas. One cannula was placed in the right jugular vein, and the other cannula was placed in the left jugular vein (for infusion and sampling, respectively) under Hypnorm (Janssen Pharmaceuticals, Oxford, U.K.) (fentanyl citrate [0.315 mg/ml]/fluanisone [10 mg/ml], 1 ml/kg body wt via intraperitoneal injection) and Diazepam (Phoenix Pharmaceuticals, Gloucester, U.K.) (5 mg/ml, 1 ml/kg body wt via intraperitoneal injection) anesthesia at 5–7 days before study (18). On the day of the experiment, food was withdrawn at the end of the dark (feeding) phase, and the rats were studied in the postabsorptive state at 1400 (i.e., 6 h after food withdrawal). Euglycemic-hyperinsulinemic clamps were performed in conscious unstressed freely moving rats (18). In brief, a primed continuous intravenous infusion of insulin was administered at a fixed rate ( $4.2 \text{ mU} \cdot \text{kg body wt}^{-1} \cdot \text{min}^{-1}$ ) for 2.5 h. This insulin dose was selected on the basis of previous studies demonstrating that it produces plasma insulin concentrations comparable to those observed after ingesting a carbohydrate-rich meal (16). A variable rate of glucose infusion was initiated at 1 min after the start of insulin infusion. Blood was sampled from the right jugular vein at 5- to 10-min intervals. A steady state was reached after 60–90 min. Coefficients of variation of blood glucose concentrations during the hyperinsulinemic clamp were  $<12\%$  in all studies. Whole-body glucose kinetics were estimated in awake unstressed freely moving rats in the basal (postabsorptive) state and during euglycemia-hyperinsulinemia with a primed (0.5  $\mu\text{Ci}$ ) continuous (0.2  $\mu\text{Ci} \cdot \text{min}^{-1} \cdot \text{rat}^{-1}$ ) intravenous infusion of [ $^3\text{H}$ ]-labeled glucose as previously described (18,19). Whole-body glucose disappearance rates ( $R_d$ ) were calculated as previously described (18).

**In vivo glucose utilization in individual muscles.** Estimations of glucose utilization by individual skeletal muscles *in vivo* were obtained by measuring the accumulation of 2-deoxy-D-[1- $^3\text{H}$ ] glucose-6-phosphate in the tissue after the bolus intravenous injection of tracer amounts (30  $\mu\text{Ci}$ ) of 2-deoxy-D-[1- $^3\text{H}$ ]glucose ([ $^3\text{H}$ ]2DG) in the basal state or during hyperinsulinemia (at 90 min after the start of the clamp) (12). Blood samples (100  $\mu\text{l}$ ) for determination of blood glucose concentrations and plasma tracer concentrations were obtained at 1, 3, 5, 10, 20, 40, and 60 min after [ $^3\text{H}$ ]2DG 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  $\mu\text{l}$ ) was added to a heparinized tube and was immediately centrifuged at  $4^\circ\text{C}$ , and plasma was frozen at  $-20^\circ\text{C}$  for subsequent insulin determinations. Rats were killed by the intravenous injection of pentobarbitone (60 mg/kg body wt) via the right jugular cannula. Individual skeletal muscles (SOL and AT) were freeze-clamped when locomotor activity had ceased (within 5 s). The fiber profiles (fast oxidative glycolytic:fast glycolytic:slow oxidative) of SOL and AT in the rat are 0:0:100 and 66:32:2, respectively (14). The freeze-clamped muscles were stored in liquid nitrogen until analysis as previously described (12,20). No correction was made for possible discrimination against 2-deoxyglucose versus glucose regarding glucose transport and phosphorylation, and hence rates of tissue accumulation of 2-deoxy-D-[ $^3\text{H}$ ]glucose-6-phosphate are referred to as glucose utilization index (GUI) values.

**Enzyme assays.** Active PDC (PDHa) activity was assayed spectrophotometrically by coupling the generation of acetyl-CoA to the acetylation of *p*-(*p*-aminophenylazo)benzene sulfonic acid by arylamine acetyltransferase (21). PDHa activities are expressed relative to citrate synthase to correct for possible differences in mitochondrial extraction (21). Total PDC activity was assayed as active PDC after complete activation through the action of endogenous PDC phosphate phosphatase in mitochondria incubated for 15 min in the absence of respiratory substrate and in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (22). PDK activities were determined at  $30^\circ\text{C}$  in mitochondrial extracts at a pH of 7.0 by the rate of ATP-dependent inactivation of PDHa (17,23). PDK activities are expressed as first-order rate con-

stands for ATP-dependent PDHa inactivation. To test the effects of pyruvate, mitochondria were incubated at 30°C in KCl medium (100 mmol/l KCl, 20 mmol/l Tris, 5 mmol/l  $\text{KH}_2\text{PO}_4$ , 2 mmol/l EGTA, pH 7.4) in the presence of respiratory substrate (5 mmol/l 2-oxoglutarate/0.5 mmol/l L-malate) together with the concentrations of pyruvate indicated. Incubations were terminated by centrifugation after 5 min, and mitochondrial extracts were assayed for PDHa activity (24).

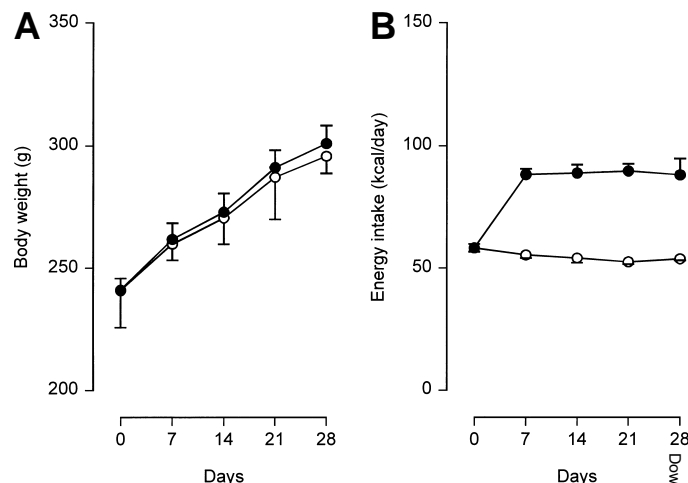
**Western blotting analysis.** Mitochondria were prepared from SOL and AT and were stored at -70°C until analysis (within 1 week). Mitochondria were extracted in 50 mmol/l  $\text{KH}_2\text{PO}_4$ , 50 mmol/l  $\text{K}_2\text{HPO}_4$ , 10 mmol/l EGTA, 1 mmol/l benzamide, 50  $\mu\text{mol/l}$  aprotinin, 50  $\mu\text{mol/l}$  pepstatin, and 10  $\mu\text{mol/l}$  leupeptin. Samples of mitochondrial extracts were denatured by heating to 60°C with a 1:2 dilution of Laemmli electrophoresis buffer (0.25 mol/l Tris, pH 6.8, 10% glycerol, 0.01% bromophenol blue, 2%  $\beta$ -mercaptoethanol, 2% SDS, 0.01 mol/l dithiothreitol). A total of 3  $\mu\text{g}$  mitochondrial protein was then separated by discontinuous SDS-PAGE electrophoresis and was subsequently transferred electrophoretically to nitrocellulose membranes with the electrophoresis semi-dry apparatus. Nitrocellulose filters were then blocked overnight at 4°C with Tris-buffered saline (TBS) (150 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.6) supplemented with 0.05% Tween 20 and 5% (wt/vol) nonfat powdered milk, incubated for 3 h at room temperature with polyclonal antisera raised against specific recombinant PDK isoforms, washed with TBS with Tween (TBST; 0.05% Tween 20 in TBS) ( $3 \times 5$  min), and incubated with horseradish peroxidase-linked secondary antibody IgG anti-rabbit (1:2,000 in 1% [wt/vol] nonfat milk in TBST) for 2 h at room temperature. The blots were then extensively washed in TBST, and bound antibody was visualized with ECL. The blots were then exposed to Hyperfilm (Amersham International, Buckinghamshire, U.K.), and the signals were quantified by scanning densitometry and were analyzed with Molecular Analyst 1.5 software (Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.).

**Statistical analysis.** Experimental data are means  $\pm$  SE. The statistical significance of differences between groups was assessed with Student's unpaired *t* test. Statistical comparisons were made with StatView (Abacus Concepts, Berkeley, CA). Curve fitting was carried out with Fig P software (Biosoft, Cambridge, U.K.).

## RESULTS

**Food intake and body weight.** Food intake and body weight during the 4-week study period are shown in Fig. 1. Caloric intake was ~65% higher in the high-fat-fed group compared with the control group. Similar increases in caloric intake in response to high-fat feeding have been observed in previous studies by other researchers (25). The increase in caloric intake was not associated with an increase in body weight during the 4-week period of high-fat feeding. Although we did not systematically measure physical activity, no obvious differences were evident in physical activity between the 2 dietary groups.

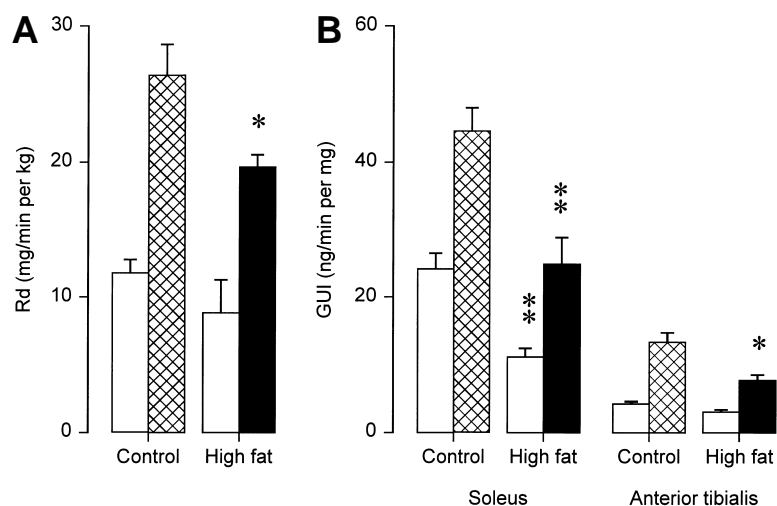
**Effect of high-fat feeding on insulin action in vivo.** Insulin infusion increased plasma insulin concentrations from  $12 \pm 2$  ( $n = 13$ ) to  $82 \pm 5$   $\mu\text{U/ml}$  ( $n = 16$ ) in the control rats and from  $13 \pm 1$  ( $n = 11$ ) to  $73 \pm 4$   $\mu\text{U/ml}$  ( $n = 12$ ) in the high-fat-fed rats. Neither the basal nor the clamped insulin concentrations differed significantly between the control and high-fat-fed groups. Basal glucose concentrations were  $4.1 \pm 0.1$  ( $n = 13$ ) and  $3.8 \pm 0.1$  mmol/l ( $n = 11$ ), respectively, in the control and high-fat-fed groups. During hyperinsulinemia, glucose was infused to maintain blood glucose concentrations, and steady-state glucose concentrations were  $4.2 \pm 0.2$  ( $n = 16$ ) and  $4.0 \pm 0.1$  mmol/l ( $n = 12$ ), respectively, in the control and high-fat-fed groups. The  $R_d$  was significantly reduced (by 26%,  $P < 0.05$ ) by high-fat feeding (Fig. 2A), which indicates the existence of peripheral insulin resistance. In control rats, glucose utilization (transport + phosphorylation) rates (GUI values) were significantly higher in SOL muscle than in AT muscle both in the basal state and during hyperinsulinemia (by 5.9- and 3.4-fold, respectively;  $P < 0.001$ ). In addition, the response to insulin infusion was 2.2-fold greater in SOL muscle than during hyperinsulinemia in AT muscle (Fig. 2B). This pattern is typical of slow-twitch versus fast-twitch muscle (11). GUI values



**FIG. 1.** Body weight and daily energy intake of rats maintained on control or high-fat diets. Rats were maintained on either a standard diet (○) or a high-fat diet (●). Data for body weight (A) and daily energy intake (B) are means  $\pm$  SE for 8–10 rats. No statistically significant effects of high-fat feeding on body weights were evident. Effects of high-fat feeding on daily energy intake were significant at all of the time points studied ( $P < 0.05$ ).

were significantly reduced by high-fat feeding in both the basal state (by 54%,  $P < 0.01$ ) and during hyperinsulinemia in SOL muscle (by 44%,  $P < 0.01$ ) and during hyperinsulinemia in AT muscle (by 42%,  $P < 0.05$ ). The mean increment in glucose utilization induced by hyperinsulinemia was reduced by 33% in SOL muscle and by 49% in AT muscle after high-fat feeding. We can conclude that the high-fat feeding protocol induced insulin resistance regarding glucose utilization in both muscle types in vivo.

**Interconversion of active and inactive forms of PDC in slow-twitch and fast-twitch skeletal muscle mitochondria.** Skeletal muscle PDC is converted from the inactive phosphorylated form to the active dephosphorylated form (PDHa) by incubation of skeletal muscle mitochondria in the absence of respiratory substrates and with respiratory inhibitors (26). Conversely, incubation of mitochondria with respiratory substrates increases the ATP concentration, thus allowing phosphorylation of PDC by endogenous PDK. Skeletal muscle mitochondria were prepared from control rats in the fed state. Total PDC activities measured in the absence of substrate and in the presence of respiratory inhibitors and expressed relative to the mitochondrial marker citrate synthase (see RESEARCH DESIGN AND METHODS) were similar in mitochondria prepared from SOL and AT muscles in control rats that were fed a standard diet ad libitum (Table 1). The administration of the high-fat diet produced no significant change in total PDC activity in either type of skeletal muscle. In contrast, high-fat feeding reduced the percentage of active PDC in mitochondria from SOL muscle incubated with respiratory substrate (2-oxoglutarate/L-malate) from  $37.6 \pm 4.6\%$  of total PDC in mitochondria from control rats to  $14.7 \pm 1.5\%$  of total PDC in mitochondria from high-fat-fed rats ( $P < 0.05$ ). Because total PDC activity in SOL mitochondria was unchanged by high-fat feeding, the effect of high-fat feeding to lower the percentage of active PDC is a consequence of increased net phosphorylation of PDC. By contrast, the percentage of active PDC in mitochondria from



**FIG. 2.** Whole-body  $R_d$  and GUI values in SOL and AT muscles in the basal state and during euglycemia-hyperinsulinemia in control and high fat-fed rats. Whole-body  $R_d$  (A) and GUI (B) values were measured in the basal (postabsorptive) state (□, both control and high fat-fed rats) or after 2.5 h of euglycemia-hyperinsulinemia in control (▨) or high fat-fed (■) rats. Data are means  $\pm$  SE for 5–12 rats. \* $P < 0.05$  vs. control; \*\* $P < 0.01$  vs. control.

the AT muscle of high fat-fed rats incubated with respiratory substrate remained similar to that found with mitochondria from control rats (control rats  $29.3 \pm 4.2\%$  of total PDC, high fat-fed rats  $24.2 \pm 3.2\%$  of total PDC).

**Effect of high-fat feeding on PDK activities in slow-twitch and fast-twitch skeletal muscle.** In control rats, no significant differences in PDK activity were evident between SOL and AT muscles (Table 1). PDK activity in SOL mitochondria was increased 2.2-fold ( $P < 0.01$ ) by high-fat feeding (Table 1). PDK activity in AT mitochondria was also significantly increased ( $P < 0.001$ ) in response to high-fat feeding (Table 1). The fold increase in PDK activity observed in AT muscle (2.1-fold) was approximately similar to that observed in SOL muscle (Table 1).

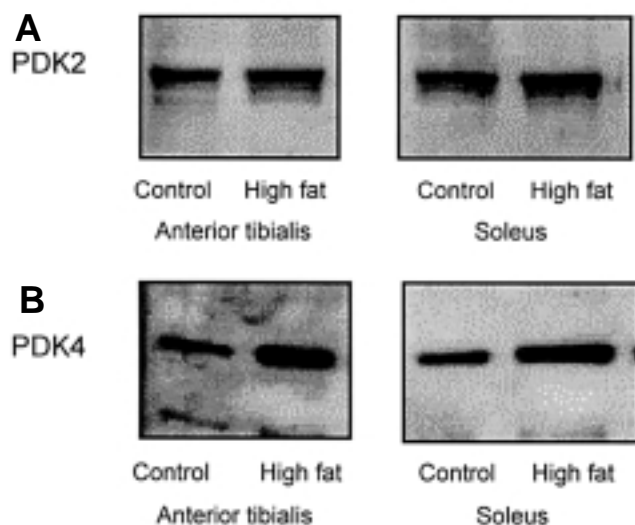
**Protein expression of individual PDK isoforms in skeletal muscle mitochondria.** Western blot analysis was used to determine whether the increased skeletal muscle PDK activities elicited by high-fat feeding were because of increased expression of PDK2 and PDK4 (the 2 PDK isoforms of which mRNA is expressed in significant amounts in rat gastrocnemius muscle) (4). High-fat feeding caused a significant 1.96-fold ( $P < 0.001$ ) increase in the amount of PDK4 protein expressed in SOL mitochondria (Figs. 3 and 4). In contrast, high-fat feeding elicited only a modest (1.29-fold,  $P < 0.01$ ) increase in the amount of PDK2 protein present in SOL muscle (Figs. 3 and 4). Thus, the relative expression of PDK4 to PDK2 increases in SOL muscle in response to high-fat feeding. High-fat feeding led to a 1.68-fold increase ( $P < 0.05$ ) in PDK4 protein expression and to a 1.50-fold increase

( $P < 0.05$ ) in PDK2 protein expression in AT muscle (Figs. 3 and 4). Thus, the relative expression of PDK4 and PDK2 protein in AT muscle is relatively unchanged by high-fat feeding. **Effects of pyruvate on the percentage of PDHa in skeletal muscle mitochondria from control and high fat-fed rats.** In mitochondria incubated with respiratory substrate, pyruvate addition increases the percentage of active PDC through suppression of PDK activity (assessed by incorporation of [ $^{32}$ P] from [ $^{32}$ P]-labeled inorganic phosphate in mitochondria incubated with 2-oxoglutarate/L-malate) (26). The percentage of PDHa in respiring mitochondria from SOL and AT muscles of control rats increased progressively when the pyruvate concentration was increased successively from 0.01 to 10 mmol/l (Fig. 5). Increasing the pyruvate concentration further to 100 mmol/l did not lead to further activation of PDC (data not shown). In control rats, the pyruvate concentrations giving 50% PDHa were  $\sim 0.25$  and  $\sim 0.51$  mmol/l for mitochondria prepared from SOL and AT muscles, respectively (Fig. 5). The pyruvate concentration giving 50% PDHa in SOL muscle was increased 7.6-fold (to  $\sim 1.9$  mmol/l) in response to high-fat feeding. As a consequence, the percentage of active PDC in SOL mitochondria incubated with physiological pyruvate concentrations (0.01, 0.1, and 1 mmol/l) was significantly reduced by high-fat feeding (by 61, 66, and 37%, respectively;  $P < 0.05$ ) (Fig. 5). In AT muscle, the effect of high-fat feeding in decreasing the sensitivity of PDK to inhibition by pyruvate was more modest compared with that in SOL muscle, with only an  $\sim 3.9$ -fold increase in the pyruvate concentration giving 50% PDHa (to  $\sim 2.0$  mmol/l) (Fig. 5). Thus,

**TABLE 1**  
Total PDC and PDK activities in SOL and AT muscle from control and high fat-fed rats

	Total PDC activity (mU/U citrate synthase)		PDK activity ( $\text{min}^{-1}$ )	
	Control	High fat	Control	High fat
SOL	$182.0 \pm 15.8$ (5)	$175.3 \pm 5.7$ (4)	$0.348 \pm 0.023$ (5)	$0.761 \pm 0.094$ (8)*
AT	$178.1 \pm 7.4$ (5)	$162.1 \pm 29.0$ (4)	$0.385 \pm 0.029$ (6)	$0.789 \pm 0.083$ (14)†

Data are means  $\pm$  SE for the numbers of preparations from individual rats shown in parentheses. Each assay was run in duplicate. PDHa activities were measured in freeze-clamped muscle extracts. Total PDC activities were measured in extracts of freshly isolated skeletal muscle mitochondria incubated for 15 min in the absence of respiratory substrate. PDK activities were measured in mitochondrial extracts. Rate constants for PDK activity were calculated by least-squares linear regression analysis of  $\ln[\% \text{ of } 0 \text{ time activity}]$  against time. \* $P < 0.01$  vs. control; † $P < 0.001$  vs. control.



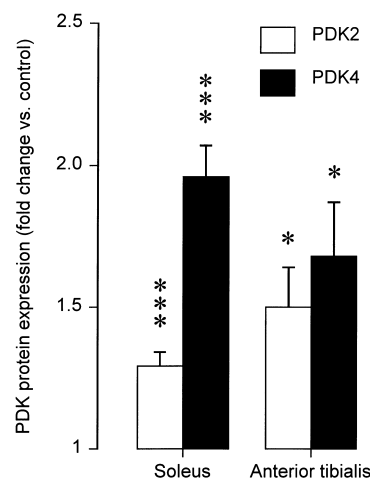
**FIG. 3.** Effects of high-fat feeding on PDK2 and PDK4 protein expression in mitochondria prepared from SOL and AT muscles of control and high fat-fed rats. Rabbit polyclonal antisera raised against PDK2 and PDK4 were used to detect these proteins with Western blot analysis. Typical immunoblots of PDK2 (A) and PDK4 (B) protein expression are shown for SOL and AT muscles of control and high fat-fed rats. Muscle mitochondrial extracts were denatured and subjected to SDS-PAGE and immunoblotting with these isoform-specific antibodies as described in RESEARCH DESIGN AND METHODS. Each lane corresponds to 3  $\mu$ g of mitochondrial protein. A total of 5–9 preparations of mitochondria were analyzed. Representative results are shown.

as shown graphically in Fig. 6, high-fat feeding was generally less effective in impairing the pyruvate sensitivity of PDK in mitochondria prepared from fast-twitch AT muscle than that from slow-twitch SOL muscle.

## DISCUSSION

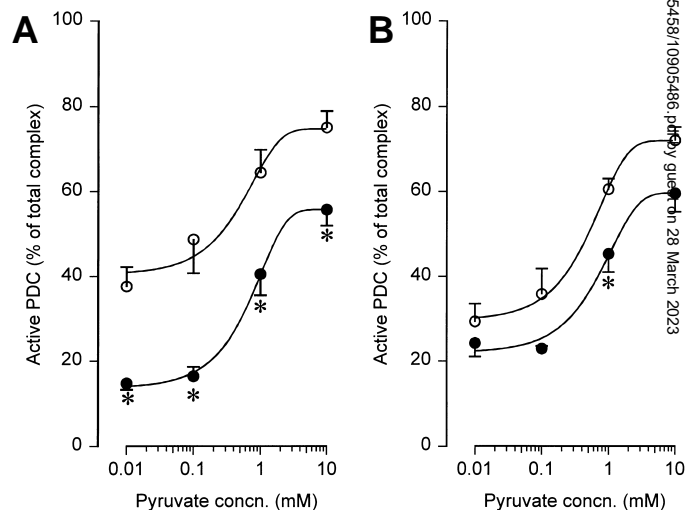
In the present study, Western blot analysis with antibodies raised against recombinant PDK2 and PDK4 conclusively demonstrates that PDK2 and PDK4 proteins are expressed in both slow-twitch (SOL) and fast-twitch (AT) skeletal muscle in the fed state. The sensitivity of PDK to suppression by pyruvate was found to be ~2-fold greater in SOL than in AT muscle, which mirrors the increased insulin sensitivity that is characteristic of slow-twitch versus fast-twitch muscle (13). Furthermore, stable increases in PDK activities evoked in response to high-fat feeding in both skeletal muscles are associated with increased protein expression of 1 or both PDK isoforms. The increase in PDK activity observed in response to high-fat feeding in SOL muscle (the representative slow-twitch muscle) is mainly because of targeted upregulation of PDK4. However, the enhanced PDK activity in the fast-twitch muscle observed after high-fat feeding can be attributed to increased protein expression of both PDK2 and PDK4.

To assess the physiological implications of muscle fiber-specific changes in PDK isoform protein expression, we examined the regulation of PDK activity by pyruvate. In vivo, pyruvate may be derived from the glycolytic pathway or from circulating lactate and alanine. Studies with recombinant PDK isoforms have shown that PDK2 and PDK4 are differentially sensitive to inhibition by dichloroacetate, a pyruvate analog (4). In vivo, the effectiveness of dichloroacetate to activate PDK differs between slow-twitch and fast-twitch mus-

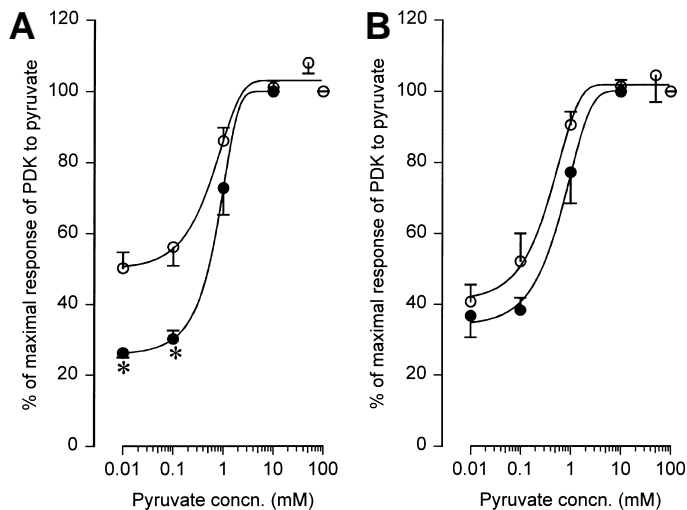


**FIG. 4.** Quantification of Western analysis of PDK isoform expression in extracts of mitochondria from the SOL and AT muscles of control and high fat-fed rats. Western blots were analyzed by scanning densitometry using Molecular Analyst 1.5 software. Data are means  $\pm$  SE for 5–9 individual internally controlled experiments. \* $P$  < 0.05 vs. control; \*\*\* $P$  < 0.001 vs. control.

cles, with a more marked response in the slow-twitch muscles (27). Mitochondria prepared from SOL and AT muscles were incubated with respiratory substrate and varying pyruvate concentrations to determine whether differences in the sensitivity of PDK to inhibition by pyruvate were evident between slow-twitch and fast-twitch skeletal muscles and whether the selective changes in PDK isoform expression patterns evoked in these distinct skeletal muscle types in response to high-fat feeding are associated with differential



**FIG. 5.** Effect of high-fat feeding on inhibition of PDK activity by pyruvate in freshly isolated mitochondria from SOL and AT muscles of control and high fat-fed rats. Freshly isolated mitochondria (0.5–1.0 mg mitochondrial protein) from SOL (A) and AT (B) muscles of rats maintained on a control diet (○) or a high-fat diet (●) were incubated at 30°C with respiratory substrate (2-oxoglutarate/L-malate) and the concentrations of pyruvate indicated as described in RESEARCH DESIGN AND METHODS. Mitochondria were precipitated by centrifugation, and steady-state PDC activity was measured in mitochondrial extracts. Data for 18 (control) and 6 (high fat-fed) mitochondrial preparations from individual rats are means  $\pm$  SE for each pyruvate concentration (concn.). \* $P$  < 0.05 vs. control.



**FIG. 6. A comparison of the sensitivity of PDK activity to suppression by pyruvate in freshly isolated mitochondria from the SOL and AT muscles of control and high fat-fed rats. The results of the effects of pyruvate on PDK activity in freshly isolated mitochondria from the SOL (A) and AT (B) muscles of rats maintained on a control diet (○) or a high-fat diet (●) are expressed as percentages of the maximal response to pyruvate observed with each individual mitochondrial preparation. Further details are given in the legend for Fig. 5. Data for 18 (control) and 6 (high fat-fed) mitochondrial preparations from individual rats are means  $\pm$  SE for each pyruvate concentration (concn.). \* $P < 0.05$  vs. control.**

alterations in the sensitivity of PDK to inhibition by pyruvate in vivo. In control rats, inhibition of PDK activity by pyruvate was greater in mitochondria from slow-twitch muscle than in mitochondria from fast-twitch skeletal muscle. Furthermore, a more pronounced effect of high-fat feeding to reduce the sensitivity of PDK to inhibition by pyruvate was observed in slow-twitch skeletal muscle, whereas the effect of high-fat feeding in fast-twitch skeletal muscle was relatively modest. We therefore conclude that the pyruvate sensitivity of PDK in skeletal muscle varies both with muscle fiber type (slow-twitch vs. fast-twitch muscle) and with nutritional status (control vs. high fat-fed rats). In addition, important differences in PDK responses between skeletal muscles differing in fiber composition can be revealed by the administration of a diet high in saturated fat, which induces insulin resistance at the level of glucose uptake and phosphorylation in skeletal muscle. In SOL muscle (the representative slow-twitch muscle), the findings of concomitant changes in PDK activity and PDK4 expression together with decreased pyruvate sensitivity are compatible with a functional switch to the higher-specific activity less-pyruvate-sensitive PDK isoform (PDK4) after high-fat feeding.

A previous study in vitro has shown that the presence of an FA and dibutyl cAMP in culture is necessary to maintain differences in PDK activities between freshly prepared SOL strips from fed and starved rats (28). The data are consistent with the concept that relatively high PDK activities are maintained in slow-twitch oxidative skeletal muscle when FA oxidation rates are high. The response of PDK4 expression to increased dietary lipids is higher in SOL than in AT muscle; this response parallels the propensity with which these muscles use lipid-derived fuels as respiratory substrates. In addition, given the specificity of the PDK isoform response to high-

fat feeding in SOL muscle (selective upregulation of PDK4), our data suggest that FAs (or their metabolites) or metabolic changes secondary to increased FA oxidation (e.g., decreased glucose utilization) are necessary for or facilitate increased expression of PDK4 in slow-twitch oxidative skeletal muscle. In the present study, the high fat-fed rats exhibited a marked increase in caloric intake, but no increase in body weight was observed (Fig. 1). A recent study by Mollica et al. (29) demonstrated that a significant increase in energy intake but no increase in body weight in high fat-fed rats resulted from a significant increase in energy expenditure. Skeletal muscle homogenates from these animals showed a marked increase in FA-stimulated oxygen consumption, which led to the suggestion that increased skeletal muscle FA oxidation rates prevented excess fat deposition (29). Support for a direct role of FA in signaling increased PDK4 expression in skeletal muscle has recently been obtained. Feeding rats WY14643, which is a very potent and selective agonist for PPAR- $\alpha$  (30,31), mimics the effects of starvation and diabetes to increase PDK4 expression in gastrocnemius muscle (11). Increasing evidence exists that long-chain FAs or their metabolites function as naturally occurring activators for PPAR- $\alpha$  (31–35). Thus, conceivably, the increased rates of FA oxidation observed in skeletal muscle together with possible direct effects of FAs on PPAR- $\alpha$  underlie the increase in PDK4 expression in skeletal muscles of high fat-fed rats. However, we cannot infer whether FAs (or their metabolites) directly upregulate PDK4 expression in skeletal muscle or whether high-fat feeding prevents downregulation of PDK4 expression by inducing insulin resistance in skeletal muscle. However, the degree of insulin resistance at the level of glucose uptake and phosphorylation evoked by high-fat feeding was similar in both SOL (slow-twitch) and AT (fast-twitch) skeletal muscle (15). Thus, our data imply that a positive correlation exists between increases in PDK4 expression and the propensity with which different skeletal muscle types use lipid-derived fuels as respiratory substrates but not with the degree of insulin resistance induced by high-fat feeding in individual skeletal muscles.

Exposure of fed rats maintained on a standard (high-carbohydrate/low-fat) diet to a high concentration of dichloroacetate leads to almost complete PDK activation in a range of skeletal muscles within 2 h (27). This finding indicates that PDK is active even in the fed state. Furthermore, it suggests that regulation of skeletal muscle PDK by changes in the pyruvate supply is likely to be important in the regulation of PDK phosphorylation status. Physiological conditions that increase glycolytic flux in slow-twitch skeletal muscle (e.g., refeeding or exercise) would therefore be expected to increase skeletal muscle pyruvate levels and suppress PDK, thereby facilitating PDK activation and pyruvate oxidation. Our data indicate that the specific increase in the protein expression of PDK4 induced by high-fat feeding in SOL muscle is associated with a stable modification in the characteristics of regulation of PDK by pyruvate such that activation of PDK secondary to inhibition of PDK by pyruvate is greatly impaired. This finding suggests that differences in the regulatory characteristics of individual PDK isoforms demonstrated with recombinant proteins in vitro (4) are relevant in vivo. The loss of sensitivity of skeletal muscle PDK to suppression by pyruvate observed after high-fat feeding may facilitate the direction of glycolytically derived pyruvate toward lactate

output rather than oxidation. During starvation, such an adaptation would be considered to be beneficial because pyruvate (and related 3C intermediates) could be released into the blood and used for glucose synthesis by the liver for use by the brain (36). However, this adaptation is potentially detrimental to glucose homeostasis after high-fat feeding because redirection of glycolytically derived pyruvate from oxidation toward lactate output by skeletal muscle would facilitate hepatic glucose production from lactate and may contribute to the overproduction of glucose and ultimately the development of hyperglycemia. Evidence that an increase in the supply of gluconeogenic precursors from skeletal muscle may contribute to overproduction of glucose by the liver has been obtained in an animal model of type 2 diabetes (low-dose streptozotocin treatment) (37). Lactate production by perfused hindlimbs is significantly (3- to 4-fold) greater in diabetic rats than in control rats (either perfused at normal glucose levels or at diabetic glucose levels) (37). Targeted pharmacological inhibition of the expression and/or activity of PDK4 in slow-twitch muscle may therefore prove to be of considerable importance as a strategy to prevent or ameliorate hyperglycemia associated with insulin resistance.

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