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Rapid upregulation of pyruvate dehydrogenase kinase activity in human skeletal muscle during prolonged exercise

Matthew J. Watt,[1] George J. F. Heigenhauser,[2] Paul J. LeBlanc,[3] J. Greig Inglis,[3] Lawrence L. Spriet,[1] and Sandra J. Peters[3]

[1]*Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1;* [2]*Department of Medicine, McMaster University, Hamilton, Ontario L8N 3Z5; and* [3]*Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada L2S 3A1*

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Watt, Matthew J., George J. F. Heigenhauser, Paul J. LeBlanc, J. Greig Inglis, Lawrence L. Spriet, and Sandra J. Peters. Rapid upregulation of pyruvate dehydrogenase kinase activity in human skeletal muscle during prolonged exercise. *J Appl Physiol* 97: 1261–1267, 2004. First published May 28, 2004; 10.1152/japplphysiol.00132.2004.—Prolonged moderate-intensity exercise is characterized by a progressive reduction in carbohydrate oxidation and concomitant increase in fat oxidation. Pyruvate dehydrogenase (PDH) controls the entry of pyruvate into oxidative pathways and is a rate-limiting enzyme for carbohydrate metabolism. PDH is controlled by the activities of a kinase (PDK, inhibitory) and phosphatase (stimulatory). To test the hypothesis that increased PDK activity was associated with decreased PDH activity and carbohydrate oxidation during an acute exercise bout, seven recreationally active men completed 4 h of cycle exercise at 55% peak oxygen consumption. Muscle samples were obtained before and at 10 min and 4 h of exercise for the measurement of PDH activity and the extraction of intact mitochondria for the measurements of PDK activity and PDK-2 and PDK-4 protein expression. Carbohydrate oxidation was reduced ($P < 0.05$) with exercise duration. Muscle glycogen content was lower ($P \leq 0.05$) at 4 h compared with rest and there was no change in muscle pyruvate content from 10 to 240 min during exercise (10 min: 0.28 ± 0.05; 240 min: 0.35 ± 0.09 mmol/kg dry muscle). PDH activity increased ($P < 0.05$) above resting values at 10 min (2.86 ± 0.26 mmol·min^{-1}·kg wet muscle^{-1}), but was lower than 10 min after 4 h (2.23 ± 0.24 mmol·min^{-1}·kg wet muscle^{-1}) of exercise. PDK-2 and PDK-4 protein expression was not different from rest at 10 min and 4 h of exercise. PDK activity at rest averaged 0.081 ± 0.016 min^{-1}, was similar at 10 min, and increased ($P < 0.05$) to 0.189 ± 0.013 min^{-1} at 4 h. Although reduced glycolytic flux may have played a role in decreasing carbohydrate oxidation, the results suggest that increased PDK activity contributed to the reduction in PDH activity and carbohydrate oxidation late in prolonged exercise. The increased PDK activity was independent of changes in intra-mitochondrial effectors, and PDK-2 and PDK-4 protein content, suggesting that it was caused by a change in the specific activity of the existing kinases.

active form of pyruvate dehydrogenase; carbohydrate oxidation

PROLONGED MODERATE-INTENSITY exercise is characterized by a shift from carbohydrate to fat as the predominant fuel source (2, 41). Although there may be an upregulation of the enzymes and processes responsible for fat oxidation throughout the duration of the exercise, there may also be a limitation on the contribution of carbohydrate oxidation through pyruvate dehydrogenase (PDH). PDH is an important regulatory mitochondrial enzyme that is responsible for the irreversible oxidative decarboxylation of pyruvate to acetyl CoA. Previous work demonstrated decreased transformation to the active form of PDH (PDHa) late in prolonged exercise, which coincided with reduced carbohydrate oxidation (41). However, alterations in the modulators normally associated with short-term (acute) regulation of the PDH complex did not explain the decrease in PDHa transformation or activity (catalytic rate) at the end of exercise. Therefore, the mechanism(s) behind this downregulation remains obscure.

PDHa activity may be reduced late in exercise secondary to increased PDH kinase (PDK) activity, which phosphorylates and inactivates the PDH complex. Increased maximal skeletal muscle PDK activity, which was independent of the concentrations of intramitochondrial effectors, has been observed in response to as little as 24 h of a diet-induced increased reliance on fat oxidation (24, 25). There are four known PDK isoforms (PDK-1–4), with PDK-2 and PDK-4 being most abundant in human skeletal muscle. Changes in PDK-4 mRNA and protein concentration appear to be the most responsive to metabolic stressors such as starvation and high fat consumption (13, 23, 24). Although such changes in PDK gene expression were generally thought to require days for measurable changes to occur, recent studies have demonstrated rapid transcriptional activity of PDK-4 in response to exercise, with three- to sevenfold increases occurring immediately after 60–90 min of exhaustive knee extensor or 4 h of moderate intensity cycle exercise (28). These authors expected that this exercise-induced transcriptional regulation of metabolic genes would not have an immediate effect during the acute bout of exercise but may instead be important for eventual changes in PDK protein content during recovery or after repetitive bouts of exercise training (12, 28). However, to date there have been no studies that have investigated the changes in PDK activity or isoform protein during a single prolonged exercise bout.

The purpose of this study was to examine whether there were changes in PDK activity that might contribute to the observed reduction in PDHa activity and carbohydrate oxidation late in prolonged exercise. To determine whether changes in activity were due to increased gene expression of the isoforms, changes in PDK-2 and PDK-4 protein concentrations were examined. We hypothesized that PDK-4 protein and activity would increase late in exercise contributing to decreased PDHa activity and carbohydrate oxidation.

Address for reprint requests and other correspondence: M. J. Watt, School of Medical Sciences, RMIT Univ., Bundoora, Victoria 3083, Australia (E-mail: matthew.watt@rmit.edu.au).

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METHODS

Subjects

Seven recreationally active male subjects (25 ± 4 yr, 73 ± 3 kg; mean ± SD) volunteered to participate in the study. Subjects completed at least three endurance training sessions (>60 min) per week, and their peak oxygen consumption ($\dot{V}O_{2\,peak}$) averaged 53 ± 2 ml·min^{-1}·kg body mass^{-1}. Subjects provided written, informed consent after a detailed explanation of the experimental procedures was given. The ethics committees of all institutions approved the study, and experimental procedures conformed to The Declaration of Helsinki.

Preexperimental Protocol

Subjects completed an incremental test to volitional exhaustion on an electromagnetically braked cycle ergometer (Lode Excalibur, Quinton Instruments, Seattle, WA) to determine their $\dot{V}O_{2\,peak}$. At least 2 days later, subjects returned to the laboratory to perform a practice ride. Subjects were instructed to consume a light mixed meal (~150 g carbohydrate) 2 h before arriving at the laboratory. Subjects cycled for 3 h at 55% $\dot{V}O_{2\,peak}$ on the same cycle ergometer as used previously. The practice ride was completed to familiarize the subjects with the laboratory environment and to ensure that they were able to complete the prolonged exercise without premature fatigue. Respiratory gas samples were collected and analyzed online (Quinton Q-Plex 1, Quinton Instruments) at 30-min intervals throughout the practice ride.

Experimental Protocol

Subjects arrived at the laboratory having consumed a light mixed meal 2 h previously. Subjects were asked to consume a meal (≃150 g carbohydrate) that they would normally consume before a long training bout and refrain from alcohol and caffeine consumption and exercise for the 24 h before testing. On arrival at the laboratory, subjects voided and rested quietly on a bed. A Teflon catheter was inserted into a forearm vein, and a resting blood sample (~6 ml) was obtained. The catheter was kept patent by flushing with 0.9% saline. The subject's legs were then prepared for needle biopsy under local anesthesia (2% lidocaine without epinephrine).

Six muscle samples were obtained from the vastus lateralis throughout the experiment, four from one leg and two from the other leg. Two samples were obtained from *leg 1* immediately before exercise. Subjects commenced cycling at 55% $\dot{V}O_{2\,peak}$ and were instructed to maintain their pedal frequency between 75 and 100 rpm. After 10 min, two muscle samples were obtained from *leg 2* while the subject remained on the cycle ergometer. At 4 h, two muscle samples were obtained from *leg 1* while the subject remained on the cycle ergometer. The duplicate muscle samples were obtained from different incisions on the same leg, with the first sample being taken from a site ~3 cm proximal to the second. The first muscle sample obtained at each time point was immediately frozen in liquid N_2, removed from the needle while frozen, and stored in liquid N_2 until analysis. The time taken to freeze the first biopsy was always <20 s from exercise cessation and was used for PDHa and muscle metabolite measurements. The second sample was obtained within 60 s of exercise cessation and was used for PDK protein and total activity measures. Venous blood samples (~6 ml) were obtained at 10, 30, and 60 min of exercise and at 60-min intervals thereafter. Before blood sampling, expired gases were collected for 3 min and analyzed online. The last minute of data was recorded. Subjects were permitted to drink water ad libitum throughout exercise, and a fan was directed on subjects to facilitate heat loss.

Analysis

Blood analysis. One portion of heparinized whole blood was immediately deproteinized 1:2 with 0.6% (wt/vol) perchloric acid (PCA). The PCA extract was stored at −20°C and subsequently analyzed for blood glucose (3). A second portion of whole blood was centrifuged, and 400 μl of plasma were added to 100 μl NaCl and incubated at 56°C for 30 min to inactivate lipoprotein lipase. The plasma was subsequently analyzed for free fatty acids (FFA) via a colormetric method (Wako NEFA C test kit, Wako Chemicals, Richmond, VA). Another portion was centrifuged, and the supernatant was removed for the determination of insulin by radioimmunoassay (Coat-a-Count insulin test kit, Diagnostics Products, Los Angeles, CA). A final portion of blood (1.5 ml) was added to 30 μl of EGTA and GSH, mixed thoroughly, and centrifuged. The supernatant was analyzed for plasma epinephrine by radioimmunoassay (Adrenaline RIA, LDN Laboratory Diagnostiks, Nordhorn, Germany).

PDHa. A piece of frozen wet muscle (10–20 mg) was removed and analyzed for PDHa as previously described (4, 26). PDHa was normalized to the highest total creatine content from the three samples obtained for each subject to correct for nonmuscle contamination.

Mitochondrial preparation for PDK activity. Intact mitochondria were extracted by differential centrifugation as previously described (18, 25). Briefly, minced muscle was homogenized using a glass-on-glass Potter homogenizer in 20 volumes of a buffer containing (in mM) 100 KCl, 40 Tris·HCl, 10 Tris base, 5 magnesium sulfate, 1 EDTA, and 1 ATP (pH 7.5). The supernatant was retained after centrifugation at 700 *g* for 10 min, and a crude mitochondrial pellet was extracted with centrifugation at 14,000 *g* (10 min). The pellet was washed, resuspended, and pelleted twice (7,000 *g*, 10 min) in 10 volumes of (in mM) 100 KCl, 40 Tris·HCl, 10 Tris base, 1 magnesium sulfate, 0.1 EDTA, and 0.25 ATP (pH 7.5). The first wash buffer included 1% (wt/vol) bovine serum albumin, and the second was protein free. The final mitochondrial pellet was resuspended in a volume corresponding to 1 μl/mg fresh muscle extracted. The final buffer contained (in mM) 220 sucrose, 70 mannitol, 10 Tris·HCl, and 1 EDTA (pH 7.4). All procedures were carried out at 0–4°C. Mitochondrial recovery and quality was calculated from citrate synthase recovery in total muscle homogenate and mitochondrial suspensions (25). The final preparation was 90 ± 3% intact mitochondria, with a 17 ± 3% recovery.

The final mitochondrial suspension (50 μl) was diluted with 250 μl of buffer containing 10 μM carbonyl cyanide *m*-chlorophenylhydrazone, 20 mM Tris·HCl, 120 mM KCl, 2 mM EGTA, and 5 mM potassium (pH 7.4), and incubated for 20 min at 30°C, driving ATP concentration to zero and causing complete conversion of PDH to the active form as previously described (6). Mitochondria were pelleted at 7,000 *g* for 10 min and stored in liquid N_2 for later analysis of PDK.

PDK activity assay. PDK activity was determined as previously described (25). Briefly, the mitochondrial pellet was resuspended in ~300 μl of a buffer containing 30 mM KH_2PO_4, 5 mM EGTA, 5 mM dithiothreitol, 25 μg/ml oligomycin B, 1.0 mM tosyl-lysyl chloromethyl ketone, 0.1% (wt/vol) Triton X-100, and 1% (wt/vol) bovine serum albumin (pH 7.0), and it was freeze thawed twice to ensure that all mitochondria were broken. The suspension was warmed to 30°C, and two aliquots of the suspension were diluted 1:1 in a buffer containing (in mM) 200 sucrose, 50 potassium chloride, 5 magnesium chloride, 5 EGTA, 50 Tris·HCl, 50 sodium fluoride, and 5 dichloroacetate and 0.1% (wt/vol) Triton X-100 (pH 7.8) for later analysis of PDH activity. This point represents "zero time" or "total PDH." Magnesium ATP was added to the remaining suspension to bring the concentration to 0.3 mM, and timed samples were taken every 30 s for 3–5 min (depending on PDK activity) as previously described (6, 25). For our method, however, the samples were diluted 1:1 in sodium fluoride-dichloroacetate buffer to maintain the PDHa activity at that instant through inhibition of the phosphatase and kinase, respectively. The samples were stored on ice for analysis of PDHa activity by radioisotopic measurement as described previously. PDK activity is reported as the apparent first-order rate constant of the inactivation of PDH (min^{-1}) or as the slope of ln {%[PDHa activity (with ATP addition)]/total PDH (without ATP addition)} vs. time (6). There was

no appreciable loss of activity in the absence of ATP over the 3- to 5-min experiment.

Immunoblots. Mitochondria were diluted to a final protein concentration of 1 μg/μl in 50 mM Tris·HCl, pH 6.8, containing 2% (wt/vol) SDS, 0.1 M dithiothreitol, 0.1% (wt/vol) bromophenol blue, 10% (vol/vol) glycerol, 1 mM benzamidine, 0.1 mg/ml trypsin inhibitor, 1 μg/ml aprotinin, 0.1 mM tosyl-lysyl phenylmethyl ketone, 1 μM leupeptin, and 1 μM pepstatin A. Samples were solubilized by being boiled for 5 min and then cooled on ice for 5 min. Samples of mitochondrial preparations (18–19 μg protein/lane) were subjected to SDS-PAGE (12% running and 4% stacking), electroblotting, and immunodetection by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Primary polyclonal antibodies for PDK-2 and PDK-4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used as recommended by the supplier (1:200 dilution). Blots were blocked using 5% skim milk in TBST [20 mM Tris base, 137 mM NaCl, 0.1% (vol/vol) Tween 20, pH 7.5] for 1 h, and then they were incubated overnight with primary antibody at +4°C. Secondary antibody was peroxidase-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology) at 1:20,000 in 5% skim milk/TBST. Blots revealed a single band just above the 41-kDa standard.

Muscle Metabolites

Muscle from the rapidly frozen biopsy was freeze-dried and extracted in a volume of 0.5 M PCA (1 mM EDTA) and neutralized with 2.2 M $KHCO_3$. This extract was used for the determination of ATP, phosphocreatine, creatine, and lactate by spectrophotometric assays (3, 9). Pyruvate was determined on the extract fluorometrically (21), and acetyl-CoA and acetylcarnitine were determined by a radiometric assay (4). Glycogen content was determined in a second aliquot of freeze-dried muscle according to the methods of Harris et al. (9).

Calculations and Statistics

Whole body carbohydrate and fat oxidation rates were estimated using the following equations: carbohydrate oxidation = 4.585 ·carbon dioxide production − 3.226·oxygen consumption, and fat oxidation = 1.695·oxygen consumption − 1.701·carbon dioxide production (22). All data are expressed as means ± SE. Statistical significance was assessed by a one-way analysis of variance with repeated measures. Specific differences were located by using a Student-Newman-Keuls post hoc test. Statistical significance was set at $P \leq 0.05$.

RESULTS

Performance and Respiratory Measures

Of the seven subjects, six completed the 240 min of exercise and one reached volitional exhaustion at 200 min. The average exercise time was 234 ± 6 min. $\dot{V}O_2$ averaged 2.01 ± 0.14 l/min (52 ± 2% $\dot{V}O_{2\,peak}$) at 10 min and increased progressively throughout exercise reaching significance ($P < 0.05$) at 120 min (Table 1). Respiratory exchange ratio was decreased ($P < 0.05$) from 10 min by 120 min of exercise and continued to decrease until exercise cessation (Table 1). Accordingly, carbohydrate oxidation progressively decreased ($P < 0.05$, Fig. 1) and fat oxidation increased ($P < 0.05$) after 120 min of exercise.

Table 1. *Respiratory responses during 240 min of exercise at 55% $\dot{V}O_{2\,peak}$*

	10 min	60 min	120 min	180 min	240 min
$\dot{V}O_2$, l/min	2.01±0.14	2.05±0.16	2.15±0.14	2.15±0.10	2.15±0.06*†
RER	0.94±0.01	0.92±0.01	0.86±0.01*†	0.82±0.02*†‡	0.79±0.01*†‡§

Values are means ± SE for 7 subjects. $\dot{V}O_2$, oxygen consumption; $\dot{V}O_{2\,peak}$, peak $\dot{V}O_2$; RER, respiratory exchange ratio. *Significant difference from 10 min, $P < 0.05$. †Significant difference from 60 min, $P < 0.05$. ‡Significant difference from 120 min, $P < 0.05$. §Significant difference from 180 min, $P < 0.05$.

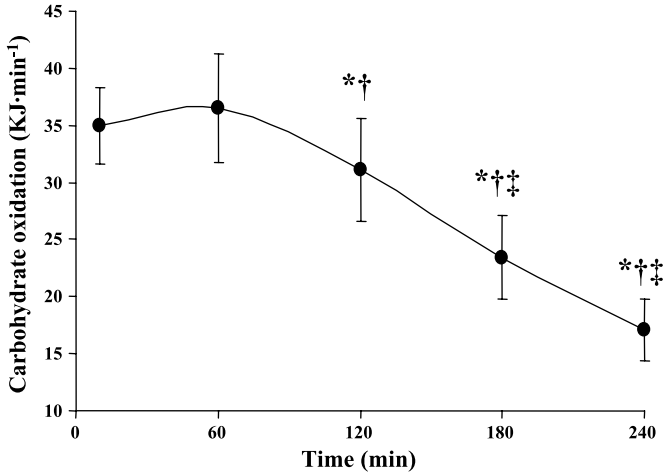

Fig. 1. Whole body carbohydrate oxidation during 240 min at 55% peak oxygen consumption. Values are means ± SE for 7 subjects. *Significant difference from 10 min, $P < 0.05$. †Significant difference from 60 min, $P < 0.05$. ‡Significant difference from 120 min, $P < 0.05$.

Blood Metabolites and Hormones

Blood glucose averaged 4.9 ± 0.2 mM at rest and was decreased ($P < 0.05$) at 180 min until exercise cessation (Table 2). Plasma FFA was increased ($P < 0.05$) from rest by 60 min and continued to increase, reaching a peak value of 1.76 ± 0.05 mM at 240 min (Table 2). Exercise induced a decrease ($P < 0.05$) in plasma insulin after 60 min, which continued to decline with exercise duration (Table 2). Plasma epinephrine was increased ($P < 0.05$) from rest at all exercise time points. After 60 min, plasma epinephrine increased ($P < 0.05$) each hour, reaching 5.90 ± 1.36 nM at 240 min (Table 2).

Muscle Metabolites

Muscle metabolite data are presented in Table 3. Muscle glycogen content was decreased ($P \leq 0.05$) during 4 h of exercise. Muscle pyruvate was unchanged from rest at 10 and 240 min of exercise, whereas acetyl-CoA, acetylcarnitine, and

Table 2. *Blood metabolite and plasma hormone responses during 240 min of exercise at 55% $\dot{V}O_{2\,peak}$*

Time, min	Glucose, mM	FFA, mM	Insulin, pM	Epinephrine, nM
0	4.9±0.2	0.21±0.04	68.6±13.0	0.47±0.11
10	4.6±0.1	0.24±0.03	41.8±13.3	0.91±0.23*
60	4.7±0.2	0.53±0.10*	23.1±8.7*	1.14±0.08*
120	4.5±0.2*	0.98±0.07*†	11.9±2.3*†	2.00±0.33*†
180	4.4±0.2*	1.45±0.11*†‡	5.5±0.6*†	2.87±0.43*†
240	4.0±0.2*†	1.76±0.05*†‡§	2.4±0.5*†‡	5.90±1.36*†‡§

Values are means ± SE for 7 subjects. FFA, free fatty acids. *Significant difference from 0 min, $P < 0.05$. †Significant difference from 10 and 60 min, $P < 0.05$. ‡Significant difference from 120 min, $P < 0.05$. §Significant difference from 180 min, $P < 0.05$.

Table 3. *Muscle metabolite responses during 240 min of exercise at 55% $\dot{V}O_{2\ peak}$*

Muscle Metabolite	0 min	10 min	240 min
ATP, mmol/kg dry muscle	26.5±1.0	25.5±1.0	27.5±0.7
PCr, mmol/kg dry muscle	74.1±2.5	59.3±3.0*	48.7±5.9*
Creatine, mmol/kg dry muscle	47.7±2.2	62.4±4.1*	73.6±4.6*
Lactate, mmol/kg dry muscle	5.4±1.1	14.4±3.4*	9.8±3.1
Pyruvate, mmol/kg dry muscle	0.16±0.02	0.28±0.05	0.35±0.09
Glycogen, mmol/kg dry muscle	486±21		125±31*
Acetyl-CoA, μmol/kg dry muscle	9.0±1.4	17.2±2.5*	10.3±1.8
Acetyl-carnitine, μmol/kg dry muscle	5.4±1.6	12.5±1.8*	6.1±1.3†

Values are means ± SE for 7 subjects. PCr, phosphocreatine, *Significant difference from 0 min, $P < 0.05$. †Significant difference from 10 min, $P < 0.05$.

lactate were increased ($P \leq 0.05$) at 10 min and returned to resting levels by 4 h. ATP was unchanged from rest throughout exercise. Muscle phosphocreatine was lower ($P \leq 0.05$) than resting values at 10 min of exercise and remained lower at 4 h. Muscle creatine content was elevated ($P \leq 0.05$) at all exercise time points compared with rest.

PDH Activity

PDHa activity averaged 0.79 ± 0.08 mmol acetyl CoA·min^{-1}·kg wet mass^{-1} at rest (Fig. 2). PDHa activity was elevated ($P < 0.05$) during exercise at 10 min. PDHa activity remained higher than rest ($P < 0.05$) at 240 min but was lower ($P < 0.05$) than 10 min.

PDK Protein Expression and Activity

PDK-2 and PDK-4 protein did not change in response to exercise (Fig. 3). PDK activity averaged 0.081 ± 0.016 min^{-1} at rest and was unchanged from resting values at 10 min (0.119 ± 0.16 min^{-1}). PDK activity increased by ~2.5-fold to 0.189 ± 0.013 min^{-1} by 240 min (Fig. 4).

Relationship Between PDK, PDH, and Carbohydrate Oxidation

Carbohydrate oxidation and PDHa activity decreased by 51 ± 4 and 22 ± 5%, respectively, whereas PDK increased by 59 ± 25% from 10 min to 4 h of exercise. The relationships between *1*) PDK activity and PDHa activity and *2*) PDHa activity and carbohydrate oxidation are shown in Fig. 5. These data demonstrate a significant correlation between the change in PDHa activity and carbohydrate oxidation ($P = 0.006$) and a tendency ($P = 0.09$) for PDK activity to increase as PDHa activity decreases.

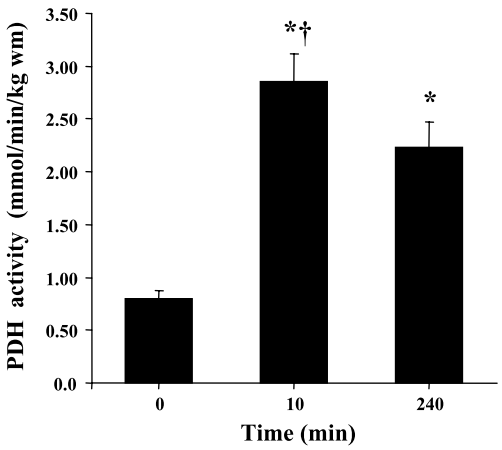

Fig. 2. Active form of pyruvate dehydrogenase (PDHa) activity at rest and during 240 min of exercise at 55% peak oxygen consumption. Values are means ± SE for 7 subjects. wm, Wet mass. *Significant difference from rest, $P < 0.05$. †Significant difference from 240 min, $P < 0.05$.

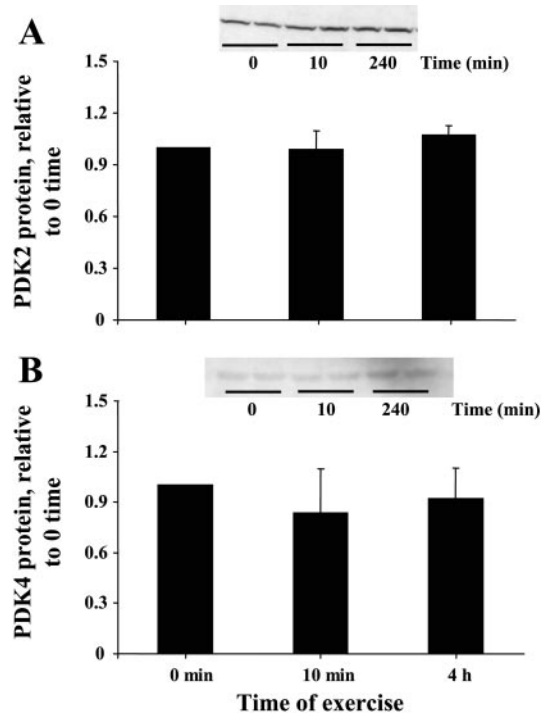

Fig. 3. Mitochondrial pyruvate dehydrogenase kinase (PDK)-2 (*A*) and -4 (*B*) protein concentration activity at rest and during 240 min of exercise at 55% peak oxygen consumption. Values were normalized to resting intensities. Representative immunoblots are shown above the corresponding graphs. Values are means ± SE for 7 subjects.

DISCUSSION

We have demonstrated that the reduced carbobohydrate oxidation observed late in prolonged exercise is at least partly due to an increase in PDK activity. The increased PDK activity

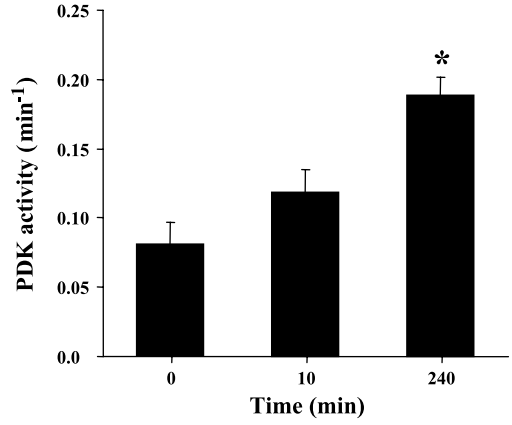

Fig. 4. PDK activity at rest and during 240 min of exercise at 55% peak oxygen consumption. Values are means ± SE for 7 subjects. *Significant difference from rest and 10 min, $P < 0.05$.

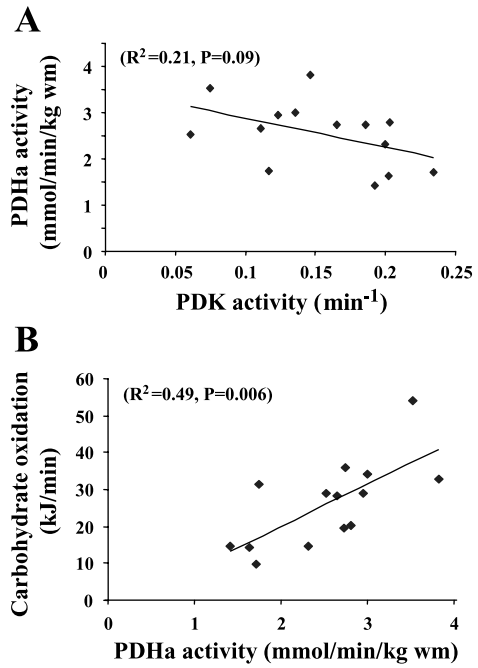

Fig. 5. Relationships between PDHa activity and PDK activity (*A*) and carbohydrate oxidation and PDHa activity (*B*) during 240 min of exercise at 55% peak oxygen consumption. Data points represent individual responses.

was accompanied by a decrease in transformation to PDHa and reduced carbohydrate oxidation. Contrary to our hypothesis, we observed no change in PDK-2 and PDK-4 protein expression, suggesting that the increased PDK activity resulted from an enhanced specific activity of existing kinases and that it was not due to increases in the absolute amounts of mitochondrial PDK protein. Although previous work has demonstrated an increase in PDK4 mRNA and transcriptional rate in response to prolonged exercise, it would appear that this is not translated into active protein in the short term and that it likely plays a role in adaptation to exercise training or during recovery from exercise (12, 27, 28).

The present data are the first measurements of PDK activity during exercise in human skeletal muscle. PDK activity remained at resting levels after 10 min of exercise and increased by ~2.5-fold by 4 h. The degree of increase in PDK activity late in exercise was similar in magnitude to that observed after 24 h of high-fat feeding (24). The regulatory mechanisms underlying the rapid increase in PDK activity are not readily apparent but must relate to stable upregulation of the enzyme-specific activity because PDK-2 and -4 protein expression was unchanged during exercise. The increased PDK activity must be "stable," and not due to short-term regulation, because the effect was maintained despite purification and washing of the intact mitochondria before measuring kinase activity. Therefore, increased PDK activity must be due to either upregulation of another PDK isoform or a persistent upregulation of specific activity that is "effector independent." Previous work has also documented a dissociation between PDK protein expression and activity (24). In this study, although PDK-4 protein was elevated after only 1 day on a high-fat diet, it increased no further on *days 2* and *3* and could not account for the continuing increase in PDK activity. It is unlikely that PDK-1 and PDK-3 are increased during exercise, and it is currently difficult to determine whether they are contributing because they are in such low concentrations that it is impossible to detect protein by standard immunoblotting or Northern blotting techniques (S. J. Peters, unpublished observations). Another possible mechanism for increased kinase activity could relate to the binding of PDK protein to the dihydrolipoamide acetyltransferase core. Both PDK-2 and PDK-3 are potently activated through enhanced binding to the dihydrolipoamide acetyltransferase core, and binding is enhanced by acetylation and reduction of the dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase enzymes (30). Changes in the maximal activity of another mitochondrial enzyme, citrate synthase, was recently reported in response to a single prolonged exercise bout (8, 40). In response to 75-min exercise, maximal citrate synthase activity increased by ~45% through mechanism(s) that appear to be independent of protein synthesis and are as yet unexplained (40). It is possible that there are exercise-induced changes or associations within the mitochondria that affect the specific activity of several mitochondrial enzymes.

Alterations in the modulators normally associated with acute regulation of PDH activity were unlikely to have influenced PDHa during prolonged exercise. The increased PDHa early in exercise was likely due to calcium-mediated stimulation of pyruvate dehydrogenase phosphatase 1 and reduced PDK activity secondary to increased pyruvate flux and increased free ADP (14, 34). Our laboratory previously hypothesized that, as exercise progresses, conditions that favor the inhibition of PDK are likely to prevail (41). However, we observed no significant change in muscle pyruvate content, despite a reduction in muscle glycogen content and carbohydrate oxidation. Although this demonstrates that total cellular pyruvate content was not reduced late in prolonged exercise, the subcellular localization of pyruvate may be important for the control of PDK. Acetyl-CoA was increased after 10 min of exercise but had returned to resting levels after 4 h of cycling (Table 3). An increased acetyl-CoA-to-reduced CoA ratio acutely increases PDK activity (26) and decreases PDH activity in vitro, but it is of questionable importance as a regulator during muscle contraction (34), and because the levels had returned to baseline at 4 h, it would not have contributed to decreased activation of the complex at the end of the exercise bout. It has been argued that mitochondrial NADH is increased during exercise (20) during instances of high FFA availability and increased rates of β-oxidation (37). Previous studies reported reduced PDH activity during exercise after a high-fat diet (29) or Intralipid and heparin infusion (19, 20). In the present study, FFA availability and fat oxidation (β-oxidation) were markedly elevated with exercise duration, supporting the possibility that NADH was increased late in exercise. We were unable to directly measure NADH in the present study given the uncertainty surrounding the methods used to estimate mitochondrial NADH.

In the present study, carbohydrate oxidation decreased to a larger extent than the decrease in PDHa during 4 h of exercise. Carbohydrate oxidation decreased by ~50% (Fig. 1), whereas transformation to active PDHa only decreased by ~25% (Fig. 2). There appears to be a mismatch between the proportion of the complex transformed to the active form (PDHa activity) and the flux through the enzyme (catalytic rate). This apparent mismatch may be explained by limited substrate as a consequence of reduced glycogenolytic flux late in exercise. Indeed,

in both the present study and our laboratory's previous study using endurance-trained cyclists (41), muscle glycogen was significantly depleted after 4 h of exercise. Putman et al. (29) also demonstrated reduced carbohydrate oxidation and maintenace of PDHa during exercise at 75% $\dot{V}_{O_2\,peak}$ when glycogen levels were ~30 mmol/kg dry muscle. Collectively, these data suggest that a severe reduction in substrate availability limits carbohydrate oxidation despite the potential for greater flux through PDH.

Situations that chronically decrease carbohydrate availability and increase the reliance of skeletal muscle on fat produce increases in the mRNA and protein of the PDK-4 isoform and the activity of PDK (24). This ultimately leads to a decreased fraction of PDH in the active form and decreased whole body carbohydrate oxidation at rest (24). Whereas these changes occur over hours and days, recent studies in exercising human subjects demonstrate rapid (hours) upregulation of PDK-4 mRNA immediately after and early in the recovery period after prolonged exercise (27, 28). In contrast to the increased mRNA content observed in previous studies, PDK-2 and PDK-4 protein contents were not increased during the 4-h exercise bout. Taken together, these data demonstrate that transcriptional, but not translational, responses occur during a single prolonged exercise bout and indicate that, although the molecular mechanisms that mediate the cellular adaptations to exercise occur both during and after exercise, there is no adaptive and therefore functional increase in PDK protein during prolonged exercise.

The rapid but stable increase in PDK activity suggests that the rapid PDK upregulation functions to preserve glucose and maintain glycemia both late in exercise and during recovery from exercise. It is tempting to speculate that the increased plasma FFA availability and flux occurring late in exercise targets PDK to prevent glucose metabolism for maintenance of glycemia. Indeed, fasting is characterized by elevated plasma FFA levels and elicits increased PDK activity in rat skeletal muscle (42). In the present study, plasma FFA was markedly elevated (~1.8 mM at 4 h) and coincided with increased PDK activity; however, given the multitude of cellular responses occurring during exercise, we cannot ascribe a causal relationship.

In summary, the results of the present study provide evidence that diminished glycolytic flux and chronic increases in PDK activity attenuate PDH activity and carbohydrate oxidation late in prolonged exercise. The increased PDK activity is independent of concentrations of intramitochondrial effectors and is not due to changes in PDK-2 or PDK-4 protein. Therefore, it appears to be related to an upregulation of the intrinsic activity of the existing kinases. Possibly, the increased PDK activity resulted from enhanced binding of the existing PDKs to the E2 core of the PDH complex, although it has yet to be determined whether this would persist in the face of a mitochondrial extraction.

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Present address of M. J. Watt: School of Medical Sciences, RMIT Univ., Bundoora, Victoria 3083, Australia.

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