

Human skeletal muscle pyruvate dehydrogenase kinase activity increases after a low-carbohydrate diet

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Peters, Sandra J., Timothy A. St. Amand, Richard A. Howlett, George J. F. Heigenhauser, and Lawrence L. Spriet. Human skeletal muscle pyruvate dehydrogenase kinase activity increases after a low-carbohydrate diet. *Am. J. Physiol.* 275 (Endocrinol. Metab. 38): E980–E986, 1998.—To characterize human skeletal muscle enzymatic adaptation to a low-carbohydrate, high-fat, and high-protein diet (LCD), subjects consumed a eucaloric diet consisting of 5% of the total energy intake from carbohydrate, 63% from fat, and 33% from protein for 6 days compared with their normal diet (52% carbohydrate, 33% fat, and 14% protein). Biopsies were taken from the vastus lateralis before and after 3 and 6 days on a LCD. Intact mitochondria were extracted from fresh muscle and analyzed for pyruvate dehydrogenase (PDH) kinase, total PDH, and carnitine palmitoyltransferase I activities and mitochondrial ATP production rate (using carbohydrate and fat substrates). β -Hydroxyacyl CoA dehydrogenase, active PDH (PDH_a), and citrate synthase activities were also measured on whole muscle homogenates. PDH kinase (PDHK) was calculated as the absolute value of the apparent first-order rate constant of the inactivation of PDH in the presence of 0.3 mM Mg²⁺-ATP. PDHK increased dramatically from $0.10 \pm 0.02 \text{ min}^{-1}$ to $0.35 \pm 0.09 \text{ min}^{-1}$ at 3 days and $0.49 \pm 0.06 \text{ min}^{-1}$ after 6 days. Resting PDH_a activity decreased from 0.63 ± 0.17 to $0.17 \pm 0.04 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ after 6 days on the diet, whereas total PDH activity did not change. Activities for all other enzymes were unaltered by the LCD. In summary, severe deficiency of dietary carbohydrate combined with a twofold increase in dietary fat and protein caused a rapid three- to fivefold increase in PDHK activity in human skeletal muscle. The increased PDHK activity down-regulated the amount of PDH in its active form at rest and decreased carbohydrate metabolism. However, an increase in the activities of enzymes involved in fatty acid oxidation did not occur.

carbohydrate metabolism; β -hydroxyacyl coenzyme A dehydrogenase; carnitine palmitoyltransferase I; citrate synthase; mitochondrial ATP production

PYRUVATE DEHYDROGENASE (PDH) is a multienzyme complex enzyme that catalyzes the conversion of pyruvate to acetyl-CoA. The reaction is the first irreversible step in the oxidation of carbohydrate-derived carbon and regulates the entry of carbohydrate into the tricarboxylic acid cycle. PDH activity is tightly regulated by reversible phosphorylation and dephosphorylation reactions catalyzed by an intrinsic kinase and phosphatase

(18, 19, 36, 39). Phosphorylation of the E1 catalytic subunit of PDH by PDH kinase (PDHK) causes inactivation of the enzyme, whereas PDH phosphatase removes phosphate and returns the enzyme to its active form (PDH_a) (18, 19). The relative activities of the phosphatase and PDHK determine the proportion of the complex that is in the PDH_a form.

A previous study from this laboratory demonstrated that a 3-day low-carbohydrate diet (LCD) attenuated the activation of PDH in the rest-to-exercise transition compared with a control mixed diet (29). Acutely, PDHK is regulated by intramitochondrial effectors. High ATP-to-ADP, NADH-to-NAD⁺, and acetyl-CoA-to-free CoA ratios and a low pyruvate concentration favor PDHK activation (6, 8, 27, 36, 39), whereas the PDH phosphatase requires Mg²⁺ and is accelerated by increasing Ca²⁺ concentration (32). At rest, Ca²⁺ and pyruvate are low and the other mitochondrial ratio effectors are high, so the activity of the phosphatase is limited, and PDHK is active, leaving a small proportion of PDH in the active form.

With the onset of exercise, the phosphatase is activated by increasing intracellular Ca²⁺ levels. As well, decreasing ATP-ADP and increasing pyruvate favor PDHK inhibition. The net result is increased dephosphorylation of the catalytic PDH enzyme and activation. However, despite the fact that the exercise and phosphatase activation would be similar in both trials of their study, Putman et al. (29) observed significantly less PDH_a formation both at rest and during exercise after 3 days on the LCD. The authors suggested that a partial explanation could be found in the decreased pyruvate concentration during exercise after the LCD condition that would allow less PDHK inhibition.

However, there was a possibility that with LCD the complex was more resistant to activation because of a stable increase in PDHK activity. Animal models have demonstrated an adaptive increase in PDHK activity in rat skeletal and cardiac muscle after periods of carbohydrate restriction (e.g., starvation, diabetes) (13, 15, 35) or increased dietary fat (26). The increase in PDHK persists even after a rigorous mitochondrial preparation, suggesting that the modification was not simply the result of effector accumulation (because of an increased reliance on fat fuel), but is instead a stable adaptation or covalent modification. A similar stable adaptation has not been observed in PDH phosphatase (8).

The purpose of this study was to investigate whether a stable increase in PDHK occurred in human skeletal muscle after 3 and 6 days on the LCD compared with a

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normal mixed diet. An existing PDHK assay from rat muscle was modified to accommodate the smaller mass available with human skeletal muscle biopsies. A secondary purpose was to examine the effect of the LCD on the maximal activities of other enzymes that represent important steps in the pathways of fat metabolism. We chose carnitine palmitoyltransferase I (CPT I, fatty acid entry into the mitochondrion), β -hydroxyacyl CoA dehydrogenase (β -HAD, β -oxidation of fatty acids), and a representative of the tricarboxylic acid cycle (citrate synthase, CS). In addition, we measured the mitochondrial ATP production rate (MAPR), which monitors the rate of ATP production through oxidative phosphorylation from both carbohydrate and fat substrates.

Our hypothesis was that PDHK activity would increase on the LCD, and this would be manifested as a decrease in the amount of PDH in the active form. We also expected to see increased rates in enzymes that are responsible for fat metabolism.

MATERIALS AND METHODS

Eleven university students (9 male, 2 female) volunteered for this study. They were in good health, with a mean age of 21.8 ± 0.5 yr (range 20–24 yr) and weight of 62 ± 11 kg (range 57–91 kg). The subjects were diverse in their regular exercise frequency. Nine of eleven subjects were not very active and engaged only in activity associated with daily living. The mean relative maximum O_2 consumption ($\dot{V}O_{2\max}$) was 47 ± 3 ml $\cdot$ min $^{-1}$ $\cdot$ kg $^{-1}$ (range 33–69 ml $\cdot$ min $^{-1}$ $\cdot$ kg $^{-1}$). Subjects were informed of the risks of the procedures before giving informed consent, and the study was approved by the ethics committees of both universities.

Study design. Subjects reported to the laboratory on three or four occasions. On the first visit, they provided a 3-day record of their normal diet before the study. They were asked to eliminate simple and complex carbohydrates from their normal diet, and the kilojoules were replaced with fat-containing foods. An outline of foods to avoid (i.e., potatoes, pasta, bread, rice, fruits), foods to eat only once per day (i.e., broccoli, cauliflower, tomatoes), and foods to eat freely (i.e., oils, butter, mayonnaise, cheese, meats) was provided. The LCD was tailored on an individual basis for each subject, and they were allowed to choose from the choices provided in the outline. They were instructed to record everything they ingested throughout the experiment. On the second day, all subjects reported after a normal breakfast for a resting biopsy (Pre) taken from the vastus lateralis muscle as previously described (2). Subjects immediately began the LCD. They were instructed to continue with their normal daily activities but to refrain from formal exercise sessions during the diet intervention. All subjects then returned to the laboratory for a second muscle biopsy after 6 days on the diet. A group of subjects ($n = 5$) also had an intermediate time-point biopsy at 3 days. Biopsies (3 and 6 days) were taken at the same time of day as the Pre biopsy after a low-carbohydrate, high-fat breakfast. Because of biopsy size, not all measurements could be made on all subjects, and therefore n values of <11 are reported.

In addition, on *day 0* and *day 6*, a 3- to 4-ml blood sample was drawn from the antecubital vein into heparinized tubes. One portion of whole blood was deproteinized 1:2 with 6% perchloric acid for analysis of β -hydroxybutyrate, glucose, lactate, and glycerol (1). A second portion of whole blood was centrifuged, and a 400- μ l aliquot of plasma was removed, added to 100 μ l of 6 M sodium chloride, and incubated for 30

min at 56°C to inactivate lipoprotein lipase. This plasma supernatant was analyzed for free fatty acids with Wako NEFA C test kit (Wako Chemicals, Richmond, VA). Insulin was measured on an aliquot of the remaining plasma with a Coat-a-Count Insulin test kit (Diagnostics Products, Los Angeles, CA).

Processing of muscle samples. Intact mitochondria were extracted from fresh muscle (~50–80 mg) for the analysis of PDHK, total PDH, CPT I, CS activities, and MAPR. A second portion of the biopsy (~20–25 mg) was frozen immediately in liquid nitrogen for later analysis of total homogenate CS (to calculate mitochondrial recovery), PDH_a, and β -HAD activities.

Mitochondrial preparation. Intact mitochondria were extracted by differential centrifugation as previously described (14, 21). Briefly, minced muscle was homogenized by a few turns in a glass-on-glass Potter homogenizer in 20 vol of a buffer containing (in mM) 100 KCl, 40 Tris $\cdot$ 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 centrifuged twice (7,000 g , 10 min) in 10 vol of (in mM) 100 KCl, 40 Tris $\cdot$ HCl, 10 Tris base, 1 magnesium sulfate, 0.1 EDTA, and 0.25 ATP (pH 7.5). The first wash buffer included 1% bovine serum albumin, and the second was protein free. The final mitochondrial pellet was resuspended in a volume corresponding to 1 μ l/1 mg fresh muscle extracted. The final buffer contained 220 mM sucrose, 70 mM mannitol, 10 mM Tris $\cdot$ HCl, and 1 mM EDTA (pH 7.4). All procedures were carried out at 0–4°C. Unless specifically stated, all chemicals were obtained from Sigma (St. Louis, MO).

Incubation of mitochondria for total PDH and PDHK activities. The final mitochondrial suspension (50 μ l) was diluted with 250 μ l of buffer containing 10 μ M carbonyl cyanide *m*-chlorophenyl-hydrazone, 20 mM Tris $\cdot$ HCl, 120 mM KCl, 2 mM EGTA, and 5 mM potassium phosphate (monobasic) (pH 7.4) and was incubated for 20 min at 30°C. This procedure decreased ATP concentration to zero and caused complete conversion of PDH to the active form as previously described (7). Mitochondria were pelleted at 7,000 g for 10 min and stored in liquid nitrogen for later analysis of total PDH and PDHK.

Total PDH and PDHK activity. The mitochondrial pellet was resuspended in ~300 μ l of a buffer containing 30 mM KH_2PO_4 , 5 mM EGTA, 5 mM dithiothreitol, 25 μ g/ml oligomycin B, 1.0 mM tosyl-lysyl-phenylmethylketone, 0.1% Triton, and 1% bovine serum albumin (pH 7.0), and 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 $\cdot$ HCl, 50 sodium fluoride, 5 dichloroacetate, and 0.1% Triton (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 20–30 s for 4–5 min (depending on PDHK activity) as previously described (7, 37).

In the present study, however, the samples were diluted 1:1 in the sodium fluoride, dichloroacetate buffer described above to lock the PDH_a activity through inhibition of the phosphatase and kinase, respectively. The samples were stored on ice for analysis of PDH_a activity by radioisotopic measurement as described previously (5, 29). PDHK activity is reported as the apparent first-order rate constant of the inactivation of PDH

(min^{-1}), or the slope of $\ln [\%(\text{PDH}_a \text{ activity with ATP addition}) / (\text{total PDH without ATP addition})]$ vs. time (7, 37). The slope was determined by regression analysis of the line. Intercept values averaged 4.57 ± 0.01 (which corresponds to 96.5% total PDH), with mean r value of 0.92 ± 0.02 . A typical analysis is outlined in Fig. 1. There was no appreciable loss of activity in the absence of ATP over the 4- to 5-min experiment.

MAPR. MAPR was determined as the luminescent release of light from the reaction of ATP and luciferin with firefly luciferase (BioOrbit ATP monitoring kit, Turku, Finland) as previously described (38). Reactions were monitored with a BioOrbit 1253 Luminometer. Substrate combinations used were 1) 1 mM malate, 1 mM pyruvate, 5 μM palmitoylcarnitine, and 10 mM α -ketoglutarate (P + PC + α -KG + M); 2) 5 μM palmitoylcarnitine and 1 mM malate (PC + M); 3) 1 mM pyruvate and 1 mM malate (P + M); and 4) 1 mM pyruvate, 5 μM palmitoylcarnitine, and 1 mM malate (P + PC + M).

CPT I activity. Maximal CPT I activity was measured with the intact mitochondrial suspension as the production of [^3H]palmitoylcarnitine from [^3H]carnitine (400 μM) as adapted from McGarry et al. (23) as previously described (3).

Homogenization of whole muscle, β -HAD, and PDH_a activities. Frozen muscle was homogenized in 80 vol of 0.1 M phosphate buffer (pH 7.3, 0.05% bovine serum albumin) with a polytron (Brinkman Instruments, Mississauga, ON) for 10 s. Total muscle homogenates were used to determine total CS (see *Mitochondrial and total homogenate CS activity*), and β -HAD activities (20). The substrate used for determination

of β -HAD was aceto-acetyl CoA (a 4-carbon β -hydroxyacyl CoA). A separate piece was homogenized with a Teflon pestle in a glass tube in a buffer containing (in mM) 200 sucrose, 50 potassium chloride, 5 magnesium chloride, 5 EGTA, 50 Tris·HCl, 50 sodium fluoride, 5 dichloroacetate, and 0.1% Triton (pH 7.8) for measurement of homogenate PDH_a as previously described (5).

Mitochondrial and total homogenate CS activity. CS activities on the total muscle homogenate (CS_{homog}) and mitochondrial suspensions were measured as previously described (34). A small volume of the mitochondrial suspension was diluted 20-fold with the final sucrose and mannitol buffer and divided into two fractions. Extramitochondrial CS (CS_{em}) was measured in the intact mitochondrial preparation, and CS activity in the total suspension (CS_{ts}) was measured after the preparation was frozen and thawed twice to break mitochondria. Triton (0.1%) was included in the cuvette for measurement of CS_{ts} and CS_{homog} .

Calculation of recovery and quality of mitochondrial preparation. Recovery of intact mitochondria was calculated as

$$\text{fractional recovery} = (\text{CS}_{\text{ts}} - \text{CS}_{\text{em}}) / \text{CS}_{\text{homog}}$$

This calculation was used to convert the mitochondrial activities of CPT I, MAPR, and total PDH ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ mitochondrial suspension) to micromole per minute per kilogram wet muscle

activity ($\text{mmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)

$$= \text{activity} (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}) / (\text{fractional recovery})$$

As with previous work (3), the quality of the preparation ($88 \pm 1\%$ for this study) was checked with each extraction and was calculated as the percentage of intact mitochondria

$$\% \text{intact mitochondria} = 100 \times (\text{CS}_{\text{ts}} - \text{CS}_{\text{em}}) / \text{CS}_{\text{ts}}$$

Diet analysis and statistics. Prediets and LCDs were analyzed with Nutripro Diet Analysis Software (West Publishing, Salem, OR) and then reported as the mean analysis for 3 days (Pre) and 6 days (LCD).

Enzyme results for Pre, 3, and 6 days were analyzed with a one-way ANOVA, with a Fisher's protected least significant difference post hoc test. For data with Pre and 6-day results, a paired Student's t -test was used. Significance was accepted at $P < 0.05$.

RESULTS

Diet analysis. Analysis of the Pre (or normal diet) and LCD consumed by the subjects revealed that the total kilojoules consumed did not differ between the two diets (Table 1). Compliance was good during the LCD, and carbohydrate content for the subjects ranged from 3 to 7% (fat 59–65%, protein 30–35%). The average carbohydrate intake as a percentage of the total kilojoules consumed with LCD was extremely restricted, whereas total fat increased approximately twofold. As a percentage of total fat in the diet, saturated, mono-, and polyunsaturated fat were ~ 36 , 37, and 18%, respectively, and this was unaltered with LCD. The protein content also increased approximately twofold with the LCD.

Blood glucose, lactate, glycerol, β -hydroxybutyrate, and plasma insulin and free fatty acid concentrations. Plasma free fatty acid, blood glycerol, and β -hydroxybutyrate concentrations were significantly elevated after

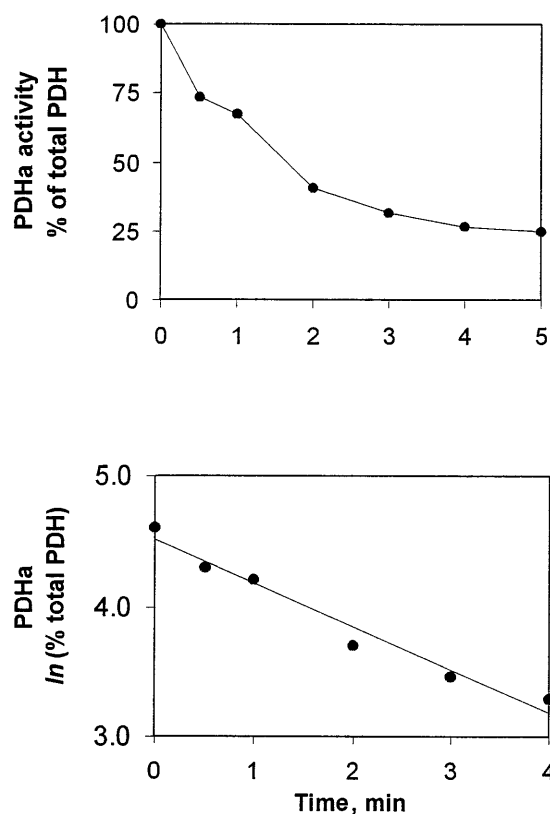


Fig. 1. Determination of PDH kinase (PDHK) in human skeletal muscle. *Top*: percentage of PDH in active form (PDH_a) vs. time after addition of 0.3 mM ATP. *Bottom*: plot of natural log ($\ln$) of percentage of PDH_a vs. time after addition of 0.3 mM ATP. Slope of plot was analyzed with linear regression, and absolute value of slope was recorded as apparent first-order rate constant of PDHK activity in units of min^{-1} . Linear regression of plot yields the equation $y = -0.332 + 4.508x$, and, therefore, PDHK activity = 0.33 min^{-1} .

Table 1. Diet analysis for prediet and low-carbohydrate diet

	Pre	LCD
Total intake, kJ	9,655 ± 660	9,673 ± 861
%Carbohydrate	52 ± 1	4.8 ± 0.6
%Fat	33 ± 2	62.7 ± 0.7
%Protein	14 ± 1	32 ± 1
Protein, g	81 ± 12	167 ± 14
Fat, g		
Total	87 ± 6	171 ± 16
Saturated	33 ± 4	61 ± 5
Monounsaturated	30 ± 2	67 ± 7
Polyunsaturated	17 ± 4	31 ± 4

Values are means ± SE in amount per day and represent the average of 3 days [prediet (Pre)] or 6 days [low-carbohydrate diet (LCD)] of diet for each subject. See text for details of protocol. Data were analyzed by Nutripro Diet Analysis Software (West Publishing, Salem, OR).

6 days on the LCD (Table 2). Blood lactate concentrations were significantly depressed, as were the plasma insulin levels. Blood glucose was unchanged by LCD.

PDHK, PDH_a, and total PDH activities. PDHK activity was significantly increased by the LCD as early as 3 days, with no significant further increase by 6 days (Fig. 2, top). PDH_a decreased significantly in 6 days (Fig. 2, bottom), but total PDH activity did not change and was 4.6 ± 0.9 and 4.8 ± 0.5 mmol·min⁻¹·kg⁻¹ wet muscle for Pre and 6 days on LCD, respectively.

CPT I, β-HAD, CS, and MAPR activities. No significant differences were found in the activities of CPT I, β-HAD, and CS after 6 days on a LCD (Table 3). MAPR was unaffected by diet intervention, regardless of the substrate combination (Fig. 3). Palmitoyl-carnitine and malate (PC + M) represented ATP generated through β-oxidation of fatty acids, whereas pyruvate and malate (P + M) represented ATP production from carbohydrate sources after glycolysis. The other two substrate mixtures incorporated both palmitoyl-carnitine and pyruvate (fat and carbohydrate sources) in combination with one (malate) or two (malate and α-ketoglutarate) tricarboxylic acid intermediates.

DISCUSSION

The most important finding of this study was the dramatic increase in PDHK activity in human skeletal

Table 2. Venous concentrations of glucose, β-hydroxybutyrate, lactate, free fatty acids, glycerol, and insulin before and after 6 days on LCD

	Pre	LCD
Glucose, mmol/l	4.84 ± 0.12	4.61 ± 0.17
Lactate, mmol/l	1.18 ± 0.13	0.60 ± 0.06*
FFA, mmol/l	0.20 ± 0.04	0.73 ± 0.09*
Glycerol, μmol/l	7.7 ± 2.5	23.6 ± 6.7*
β-Hydroxybutyrate, mmol/l	0.17 ± 0.02	1.83 ± 0.49*
Insulin, mIU/l	21.0 ± 6.7	7.0 ± 1.7*

Values are means ± SE. Glucose, β-hydroxybutyrate, lactate, and glycerol were measured on perchloric acid extracts of whole blood. Insulin and free fatty acids (FFA) were measured using plasma. Data reported before (Pre) and after 6 days on LCD. *Significantly different from control condition, $P < 0.05$.

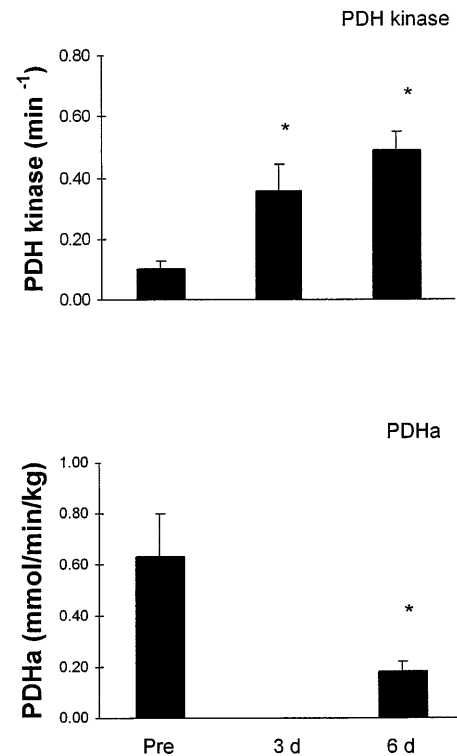


Fig. 2. Activities of PDHK and PDH_a in human skeletal muscle in response to 3 and 6 days on low-carbohydrate diet. PDHK activity (top) was measured on isolated intact mitochondria and is reported as apparent first-order rate constant for ATP-dependent inhibition of PDH_a (units are min⁻¹). PDH_a activity (bottom) was measured on whole muscle homogenates and is reported in units of mmol·min⁻¹·kg wet muscle⁻¹. PDH_a was not measured at 3 days. For prediet (Pre) and 6-day data, $n = 8$; for 3-day data, $n = 5$. *Significantly different from the prediet value.

muscle after a low-carbohydrate dietary intervention that shifts reliance to fat and ketone metabolism. Human skeletal muscle PDHK increased three- to fivefold after only 3 days on a LCD (Fig. 2). This increase was stable and persists despite a rigorous mitochondrial preparation, and it was not simply the result of an increase in the intramitochondrial effectors that are known to upregulate PDHK activity. With the greater supply of fatty acids and ketone bodies available for oxidation during the LCD, there are many possible sites of regulation for the shift in substrate

Table 3. Maximal enzyme activities before and after 6 days on LCD

	Pre	LCD
CPT I	262 ± 21 (7)	242 ± 34 (7)
β-HAD	2.9 ± 0.3 (11)	3.0 ± 0.4 (11)
CS	17 ± 2 (11)	16 ± 2 (11)

Values are means ± SE; nos. in parentheses are no. of observations. Citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) were measured on whole muscle homogenate; units are mmol·min⁻¹·kg wet muscle⁻¹. Carnitine palmitoyltransferase I (CPT I) activity was measured on isolated intact mitochondria and corrected to whole muscle value (see METHODS for details); units are μmol·min⁻¹·kg wet muscle⁻¹. No significant differences were observed with 6 days of LCD intervention. Data reported before (Pre) and after 6 days on LCD.

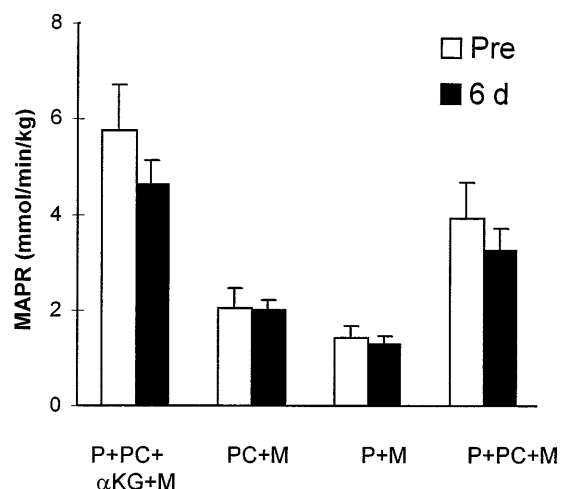


Fig. 3. Mitochondrial ATP production (MAPR) from carbohydrate and fat substrates. Maximal production of ATP in intact mitochondria through PDH (from pyruvate) or β -oxidation (palmitoylcarnitine) as well as tricarboxylic acid cycle and oxidative phosphorylation. Substrates: P, 1 mM pyruvate; PC, 5 μ M palmitoylcarnitine; α -KG, 10 mM α -ketoglutarate; M, 1 mM malate. Activity is measured on intact mitochondria and corrected to a whole muscle value (see METHODS for details). Activity is reported as $\text{mmol} \cdot \text{min}^{-1} \cdot \text{kg wet muscle}^{-1}$. No significant differences occurred between Pre and 6 days for any substrate combination ($n = 6$).

oxidation. These include decreased glucose uptake (insulin receptor binding, insulin signaling cascade events, and GLUT-4 translocation), inhibition of glycolytic enzymes (phosphofructokinase, hexokinase), in addition to the inhibition of PDH through an increase in PDHK. This study demonstrated a rapid and dramatic adaptive increase in PDHK activity, indicating that this is one important mechanism involved in the shift. The present data appear to be the first reported human skeletal muscle PDHK data. In contrast, the maximal activity of enzymes responsible for fat metabolism measured in this study did not increase during the 6-day time course of the LCD.

PDHK activity. A comparison of the PDHK values obtained in this study with activity levels in rat skeletal and cardiac muscle in the literature reveals that qualitatively the PDHK activities from this study match well with dietary manipulations in rodent models. PDHK increased twofold in both rat heart muscle (26) and rat soleus muscle (35) after 2 days of starvation. A rapid and profound drop in circulating insulin concentrations, accompanied by markedly elevated serum free fatty acid levels, is observed with starvation in rats. The present study also demonstrated a significant increase in availability of fat fuel as evidenced by the large increases in circulating free fatty acids, glycerol, and β -hydroxybutyrate. Decreased insulin (from 21 to 7 mIU/l) and increased free fatty acid concentrations (from 0.2 to 0.7 mmol/l) such as those observed in this study have been implicated as potential regulators of the stable PDHK increase. Orfali et al. (26) demonstrated in rat hearts that the increase in PDHK was much slower with a diet that was high in fat but not severely restricted in carbohydrates. PDHK activity was unaltered after 10 days on this diet and required a

minimum of 28 days for measurable PDHK increases. During this manipulation, insulin was unchanged during the first 10 days of the high-fat diet, and this was accompanied by a delay in PDHK activity upregulation and a slow increase in serum free fatty acid concentrations. This suggests that the rapid decrease in insulin and increase in plasma free fatty acids that accompany severe carbohydrate restriction were major factors in determining the acute PDHK increase in this study.

Fryer et al. (9) studied rat hearts and found that the fatty acid composition of the high-fat diet was an important determining factor for PDHK increases. A diet enriched in n-3 fatty acids does not increase PDHK activity in rat hearts compared with a normal high-fat diet. As little as 1 day with n-3 fatty acid supplementation after a standard 28-day high-fat diet will return PDHK activity to rat heart control values. Fish oils and flax seed contain high levels of these n-3 fatty acids, but the diet records of our subjects indicate that they did not consume large quantities of these foods. Therefore, the n-3 fatty-acid content of their LCD was low (12). Saturated and monounsaturated fats made up the greatest proportion of the consumed fatty acids. Because saturated fatty acids are implicated as a regulator in stable increases of PDHK (9, 26), the LCD used in this study would effectively promote an increase in maximal PDHK activity.

The stable increase in PDHK in this study is not only important in decreasing PDH_a activity during rest but also during exercise. There are three possible phosphorylation sites on the E1 catalytic subunit of the PDH complex. Only site 1 is necessary for inactivation. However, with prolonged upregulation of PDHK, sites 2 and 3 are eventually phosphorylated, rendering the subunit more resistant to the phosphatase (31). The marked increase in PDHK after 3 days of LCD in the present study offers another explanation for the attenuated increase in PDH_a observed in our previous study with the onset of exercise (29). The 3-day LCD intervention and adaptive increase in PDHK likely caused multisite phosphorylation of the catalytic enzyme at rest. With an increasing number of sites 2 and 3 occupied with phosphates, the E1 subunit would be more resistant to PDH phosphatase. Intracellular Ca^{2+} , which increased during exercise, should have been as effective in activating phosphatase during exercise. However, the increased occupation of the PDH E1 regulatory sites made it more difficult to remove the site 1 phosphate and activate this enzyme.

It is not clear from the present study whether the increase in PDHK activity is because of a stable upregulation of the enzyme specific activity (which would persist despite rigorous extraction procedures) or because of an increase in protein synthesis. Earlier work in rat heart and hepatocytes suggested that although protein synthesis played a role in PDHK activation, it did not appear to account for the magnitude of the PDHK activity increase (15, 28). However, more recent work revealed that there are currently four known isoenzymes of PDHK (10, 30) that differ significantly in their tissue distribution (4, 10) as well as in their

specific activity and sensitivity to effectors (4). It is interesting to note that the tissue distribution of the four isoforms is also different between rats and humans (4, 10). For instance, PDK3 is found exclusively in human heart and skeletal muscle but in the rat is most abundant in testes (with only trace amounts in heart and skeletal muscle). Wu et al. (41) recently demonstrated a dramatic increase in rat heart PDK4 in response to starvation and diabetes that could adequately explain the associated increase in PDHK activity in rats. It is possible that PDK4 could also be responsible for the dramatic increase in PDHK activity in humans. However, because PDK3 is found exclusively in human heart and skeletal muscle, it is possible that there is a species difference in PDK expression. Further work to elucidate the mechanism in human skeletal muscle is necessary.

CPT I. CPT I is a key enzyme in free fatty acid oxidation, catalyzing the conversion of fatty acyl-CoA to fatty acylcarnitine on the outer membrane of the mitochondria. In conjunction with carnitine acylcarnitine translocase and CPT II, the fatty acid moiety is transported and reconverted to fatty acyl-CoA in the inner matrix of the mitochondrion, ready for β -oxidation (22). The CPT I reaction is rate limiting and is a likely candidate as a regulator of fatty acid oxidation. However, there were no changes in the maximal activity of this enzyme with dietary adaptation after 6 days. Either the maximal activity of this enzyme is not responsive to alterations in fat reliance, or 6 days is not an adequate length of time to cause an adaptive change. However, the *in vitro* assay utilized in this study would not account for the *in vivo* activation of CPT I because of increased long-chain fatty acyl-CoA esters (36). As well, CPT I is acutely inhibited by malonyl-CoA concentrations *in vitro* and in rodent skeletal muscle (3, 40); however, the role that it plays in regulating human skeletal muscle CPT I is unclear (25). Malonyl-CoA is synthesized in skeletal muscle by acetyl-CoA carboxylase. We did not examine the effect of LCD on maximal acetyl-CoA carboxylase activity or malonyl-CoA and long-chain fatty acyl-CoA concentrations. Therefore, it is possible that *in vivo* CPT I activity at rest could be increased after LCD through increased long-chain fatty acyl-CoA esters or decreased maximal acetyl-CoA carboxylase activity.

CS and β -HAD. Few human studies have addressed the question of adaptive change in the maximal activities of skeletal muscle CS and β -HAD because of dietary intervention. Kiens et al. (16) did not observe increases in the maximal activities of CS or β -HAD after 4 wk on a modestly increased fat diet in humans (control fat intake, 43% of total energy intake vs. 54% in fat diet). However, β -HAD activity increased by 120% after 7 wk when the dietary fat was increased to ~60% compared with 37% control (11). No change in CS activity occurred. This β -HAD upregulation agrees with rat studies that used more severe increases in dietary fat content (60–70% fat). However, many of the rat studies also observed increases in CS activity of ~20% (17, 24, 33). Although the severe carbohydrate restriction and high-fat content of our diet manipula-

tion forced an immediate reliance on fat fuel, no increase was seen in either β -HAD or CS after 6 days. In the short term, it would appear that the maximal activity of the fat-oxidizing enzymes is adequate in combination with carbohydrate sparing. Possibly, an adaptation to an excess of fuel is slower than to a deficiency.

MAPR. The determination of MAPR allows for a direct assessment of ATP production in intact mitochondria. It is a maximal activity determination that incorporates PDH or β -oxidation (for carbohydrate and fat sources, respectively) through to the tricarboxylic acid cycle and oxidative phosphorylation to produce ATP. MAPR rates did not differ in our study after 6 days on the LCD with any of the substrate combinations (Fig. 3). Because the maximal activities of β -HAD or CS did not change, any change observed with the fat substrate (palmitoyl-carnitine) in a combination would be because of an alteration in the electron transport chain. We would conclude from this that the enzymes responsible for oxidative phosphorylation did not change in response to the diet, and there was no increase in oxidative potential with the LCD for 6 days. With the increased PDHK and decreased PDH_a observed in this study, we might have expected a downregulation in MAPR activity with pyruvate as a substrate. However, this preparation measures the maximal PDH_a activity, because the high pyruvate concentration in the assay buffer would inhibit PDHK activity *in vitro*, allowing full activation of PDH. As no difference was observed with pyruvate as a substrate, this reinforces the conclusion that the total maximal PDH activity was unaffected by LCD.

Summary. This study investigated the adaptation of human skeletal muscle enzymes to 6 days of severe dietary carbohydrate restriction. A PDHK assay was adapted to accommodate small human muscle biopsies. Human skeletal muscle PDHK activity dramatically increased three- to fivefold after 3 and 6 days on the LCD diet and was paralleled by a decrease in resting PDH_a activity. Total PDH activity did not change, and maximal activities of CPT I, β -HAD, CS, and MAPR were unaffected by the diet. It appears that the downregulation of PDH through a stable increase in PDHK is one important mechanism in the first line of defense to spare carbohydrate and shift fuel reliance to fat. Further work is necessary to determine the mechanism of PDHK activation in human skeletal muscle.

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