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Carbohydrate metabolism during prolonged exercise and recovery: interactions between pyruvate dehydrogenase, fatty acids, and amino acids

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Mourtzakis, Marina, Bengt Saltin, Terry Graham, and Henriette Pilegaard. Carbohydrate metabolism during prolonged exercise and recovery: interactions between pyruvate dehydrogenase, fatty acids, and amino acids. *J Appl Physiol* 100: 1822–1830, 2006. First published January 19, 2006; doi:10.1152/japplphysiol.00571.2005.—During prolonged exercise, carbohydrate oxidation may result from decreased pyruvate production and increased fatty acid supply and ultimately lead to reduced pyruvate dehydrogenase (PDH) activity. Pyruvate also interacts with the amino acids alanine, glutamine, and glutamate, whereby the decline in pyruvate production could affect tricarboxycylic acid cycle flux as well as gluconeogenesis. To enhance our understanding of these interactions, we studied the time course of changes in substrate utilization in six men who cycled at 44 ± 1% peak oxygen consumption (mean ± SE) until exhaustion (exhaustion at 3 h 23 min ± 11 min). Femoral arterial and venous blood, blood flow measurements, and muscle samples were obtained hourly during exercise and recovery (3 h). Carbohydrate oxidation peaked at 30 min of exercise and subsequently decreased for the remainder of the exercise bout ($P < 0.05$). PDH activity peaked at 2 h of exercise, whereas pyruvate production peaked at 1 h of exercise and was reduced (~30%) thereafter, suggesting that pyruvate availability primarily accounted for reduced carbohydrate oxidation. Increased free fatty acid uptake ($P < 0.05$) was also associated with decreasing PDH activity ($P < 0.05$) and increased PDH kinase 4 mRNA ($P < 0.05$) during exercise and recovery. At 1 h of exercise, pyruvate production was greatest and was closely linked to glutamate, which was the predominant amino acid taken up during exercise and recovery. Alanine and glutamine were also associated with pyruvate metabolism, and they comprised ~68% of total amino-acid release during exercise and recovery. Thus reduced pyruvate production was primarily associated with reduced carbohydrate oxidation, whereas the greatest production of pyruvate was related to glutamate, glutamine, and alanine metabolism in early exercise.

alanine; pyruvate dehydrogenase kinase 4 mMRNA; carbohydrate oxidation; nitrogen balance

THE REGULATION OF CARBOHYDRATE provision during prolonged exercise and recovery is a profound challenge for the human body. Muscle carbohydrate metabolism does not happen in isolation but requires integration across tissues as well as regulation with the other major substrates, such as fats and amino acids. Because carbohydrate stores are limited in the body (i.e., muscle, liver), the highly coordinated integration of liver, adipose tissue, pancreas, and muscle metabolism is important for constant energy provision during prolonged exercise (1, 30, 34–36) and in reestablishing homeostasis during recovery (2). Arterial glucose delivery is one key element in energy provision, and the body attempts to maintain glucose delivery during exercise by activating liver glucose production with decreased insulin, increased glucagon, and catecholamines, as well as enhanced availability of gluconeogenic precursors, glutamine, and alanine (1, 30, 36).

Although reduced carbohydrate oxidation is evident during prolonged exercise, the mechanisms underlying this reduction are unclear since much of our understanding is based on exercise studies lasting up to 90 min. Rarely have there been detailed, quantitative assessments for longer exercise bouts to comprehensively identify regulatory factors involved in substrate utilization. Decreased carbohydrate oxidation has been primarily associated with the decline in muscle glycogen stores (5, 30, 37) and more specifically a decrease in pyruvate dehydrogenase (PDH) activity (37, 38). Watt et al. (38) demonstrated that, at the end of a prolonged exercise bout, PDH kinase (PDK) activity was elevated and coupled with reduced PDH activity in absence of changes in the known modulators (i.e., muscle pyruvate and cellular status) of PDH activity. However, the time course of these alterations remains unknown, which ultimately makes it difficult to further study whether PDH activity specifically regulates carbohydrate oxidation. Because pyruvate is a potent regulator of PDH activity, it is important to examine the amount of pyruvate available for oxidation during exercise. Similarly, investigating the potential adaptive, transcriptional responses of PDK4 to enhanced free fatty acid (FFA) availability, as observed with dietary perturbations and/or exercise, may also provide insightful information on the role of PDH activity in substrate utilization following prolonged exercise (16, 23–26, 31).

Although protein and amino acid metabolism may contribute modestly to substrate utilization (20), amino acids such as glutamine and alanine are gluconeogenic precursors that interlink with pyruvate metabolism. Muscle glutamine and alanine are derived from glutamate through different reactions that are coupled with pyruvate metabolism and the tricarboxycylic acid (TCA) cycle. These three amino acids, which demonstrate the most dynamic changes compared with other amino acids during exercise (5, 12, 15, 21, 29), are essential not only to nitrogen balance but also to carbohydrate oxidation. Although glutamine, alanine, and glutamate are important in nitrogen balance and are intimately associated with carbohydrate regulation as well as energy metabolism, there is a limited understanding of amino acid metabolism during prolonged exercise (>60 min) (5, 12, 15, 21, 29).

Thus the present study examined the time course of potential alterations in net pyruvate production in relation to changes in

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PDH activity as well as carbohydrate oxidation during moderately low-intensity, prolonged exercise. At this intensity, a relevant combination of carbohydrate and fat would be utilized near the onset of exercise, and we anticipated that a decline in carbohydrate oxidation in early exercise would be associated with a decrease in pyruvate availability, which would ultimately reduce PDH activity. The effects of altered FFA metabolism were also examined in association with pyruvate supply and energy provision. Because pyruvate is closely linked to various amino acid reactions, this study also investigated the potential effects of altered pyruvate supply on amino acids, especially glutamine, glutamate, and alanine, that have important implications on gluconeogenesis and nitrogen balance during exercise and in recovery.

METHODS

Six healthy, recreationally active men were recruited to participate in this study. The mean ± SE age, body mass, and maximal oxygen uptake ($\dot{V}O_2$) of the subjects was 28 ± 1 yr, 80.0 ± 1.8 kg, and 49.8 ± 1.1 ml·kg^{-1}·min^{-1} (3.98 ± 0.10 l/min), respectively. Subjects were informed verbally and in writing of the purpose of the study as well as the procedures and risks involved with the experiment. The experimental protocol was approved by the Copenhagen and Frederiksberg Ethics Committee in Denmark. The study was conducted under the guidelines of the Declaration of Helsinki.

Experimental protocol. To minimize the variability of food intake, subjects were provided a standardized diet (65% carbohydrate, 15% protein, 20% fat) for the day before testing. After an overnight fast, subjects arrived at the laboratory and rested in the supine position. While resting and before catheterization, pulmonary and blood flow measurements were made. Arterial blood flow was measured at rest and recovery using ultrasound Doppler, as described by Rådegran et al. (28), and was estimated during exercise using measured femoral arteriovenous oxygen differences and rate of pulmonary $\dot{V}O_2$ at a given workload according to Fick's principle (33). Cannulation of the femoral artery and vein was performed under local anesthesia. The femoral arterial catheter was inserted ~2 cm below the inguinal ligament and set proximally ~10 cm, whereas the femoral venous catheter was placed ~2–5 cm below the inguinal ligament and forwarded ~10 cm in the distal direction. After the insertion of the catheters, subjects rested for 20 min before resting blood samples were drawn. Subsequently, a biopsy was obtained from the vastus lateralis muscle under local anesthesia using the needle biopsy technique as described by Bergström (4).

Following the preparatory procedures and the resting measurements, subjects cycled at 44 ± 1% maximal $\dot{V}O_2$ (mean ± SE) with a cadence of 70 rpm until exhaustion. This exercise intensity was chosen so that subjects could maintain steady-state exercise for a prolonged time period to allow for the evaluation of changes in substrate utilization. Pulmonary measurements were taken 30 min into the exercise protocol. Thereafter, pulmonary measurements as well as blood and muscle samples were obtained at hourly intervals. When subjects could not maintain 70 rpm for longer than 30 s, this was denoted as exhaustion, at which point they stopped exercising and a muscle biopsy was taken immediately. The subjects remained in the supine position and refrained from eating during the following 3 h of recovery. Pulmonary and blood flow measurements as well as blood samples were taken hourly, whereas muscle biopsies were obtained at 1 and 3 h postexercise.

Blood analysis. Heparinized syringes were used to collect blood samples for measuring blood P_{CO_2} and P_{O_2} (ABL5, Radiometer), hemoglobin, oxygen saturation (OSM3 hemoximeter, Radiometer), and hematocrit. Arterial and venous plasma samples from the heparinized syringes were used to analyze amino acids using ultraviolet detection on high-performance liquid chromotography (14) and ammonia using a fluorometric method (3). Arterial blood samples were collected in tubes containing EDTA and glutathione, which were centrifuged and plasma stored at −40°C for the analysis of insulin (DAKO), glucagon (Linco Research), and catecholamines. Catecholamines were analyzed using high-performance liquid chromotography with electrochemical detection as described by Hallman et al. (13). Arterial and venous samples were collected for the analysis of glucose, lactate, and FFAs, whereby blood glucose and lactate were measured on an automatic analyzer (EML105, Radiometer) and plasma lactate and glucose were analyzed with an enzymatic method on an automatic analyzer (Cobas Fara, Roche). Plasma FFAs were measured using a WAKO NEFA-C kit (Wako Chemical).

Muscle analysis. Biopsy samples were immediately frozen (<10 s) and stored in liquid nitrogen. A 10- to 20-mg piece of muscle sample was used to measure PDH activity (6, 8) as modified by Putman et al. (27). These data were corrected for differences in blood and connective tissue by adjusting, within a subject, to the highest total creatine concentration in the neutralized perchloric acid extracts of wet muscle homogenates used for the PDH activity analyses. Another part of the biopsy sample was freeze-dried, powdered, and stored at −80°C. The freeze-dried muscle was then extracted using 0.5 M perchloric acid (with 1 mM EDTA) and neutralized with 2.2 M $KHCO_3$. The extract was then used to measure muscle amino acids (14), glycogen (22), pyruvate (22), and acetyl-CoA (6). Adenine nucleotides, phosphocreatine, and creatine were also measured and are presented elsewhere (39).

RNA isolation, reverse transcription, and PCR. Total RNA was isolated from ~25 mg of tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method adapted from Chomczynski and Sacchi (7) and as described previously (26). RNA was resuspended overnight (4°C) in 2 μl/mg muscle tissue in DEPC-treated H_2O containing 0.1 mM EDTA. Reverse transcription of 11 μl of total RNA sample was performed using the Superscript II RNase H^- system (Invitrogen) as previously described (26). Reverse transcription products were diluted in nuclease-free H_2O to a total volume of 150 μl. The mRNA content of the PDK4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as endogenous control) genes was determined by real-time PCR (ABI PRISM 7700 sequence detection system, Applied Biosystems). The following oligonucleotides were designed from human-specific sequence database (Entrez-NIH) using computer software (Primer Express, Applied Biosystems) to amplify a fragment of the PDK4 gene: forward primer 5′-TCCACTGCACCAACGCCT-3′; reverse primer 5′-TGGCAAGCCGTAACCAAAA-3′ and TaqMan probe 5′-ATAATTCCCGGAATGCTCCTTTGGCTG-3′. The probe was 5′ 6-carboxyfluorescein and 3′ 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine labeled. Prior optimization was conducted determining optimal primer and probe concentrations and verifying the efficiency of the amplification. The expected size of the PCR product was initially confirmed by gel electrophoresis (2.5% agarose gel) as part of the pretesting. GAPDH was amplified using a predeveloped assay reagent (Applied Biosystems). PCR amplification was performed (in triplicate) in a total reaction volume of 25 μl. The reaction mixture consisted of 2.5 μl of diluted template, forward and reverse primers and probe as determined from the prior optimization, ×2 TaqMan Universal MasterMix optimized for TaqMan reactions (Applied Biosystems; containing AmpliTaq Gold DNA polymerase, AmpErase uracil NH_2-glycosylase, 2-deoxynucleotide 5′-triphosphate with dUTP, ROX as passive reference, and buffer components) and nuclease-free water. The following cycle profile was used for both genes: 50°C for 2 min + 95°C for 10 min + (95°C for 15 s + 60°C for 1 min) × 40 cycles. For each gene, the threshold cycle (the cycle number where the amplification curve crosses a defined fluorescence threshold) was determined for each sample and converted to a (relative) PDK4 mRNA amount and GAPDH amount, respectively, by using a standard curve constructed from the results of a serial dilution of a representative sample run together with the

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Table 1. *Pulmonary $\dot{V}O_2$, blood flow, leg $\dot{V}O_2$, and RER during prolonged exercise and recovery*

	Rest	30 min	1 h	2 h	3 h	EXH	Post 1 h	Post 2 h	Post 3 h
Pulmonary $\dot{V}O_2$, ml O_2/min	301±11		1,693±179[a]	1,855±101[a]	1,712±65[a]	1,926±81[a]	355±12[b–e]	349±17[b–e]	327±19[b–e]
Blood flow, ml·min^{-1}·leg^{-1}	391±47		3,649±415[a]	4,147±304[a]	4,042±267[a]	4,602±365[a,b]	798±102[b–e]	750±81[b–c]	767±97[b–e]
Leg $\dot{V}O_2$, ml O_2·min^{-1}·leg^{-1}	21±5		616±12[a]	615±12[a]	611±15[a]	609±9[a]	21±5[b–c]	20±4[b–c]	18±6[b–c]
RER	0.81±0.02	0.84±0.02[a]	0.82±0.02[a]	0.81±0.01[a]	0.80±0.01[a]	0.81±0.04[a]	0.73±0.03[b]	0.70±0.01[a–d]	0.70±0.02[a–d]

Values are means ± SE. EXH, exhaustion; RER, respiratory exchange ratio; $\dot{V}O_2$, oxygen uptake. Significance difference ($P < 0.05$): [a]from rest; [b]from 1 h of exercise; [c]from 2 h of exercise; [d]from 3 h of exercise; [e]from EXH.

unknown samples. The amount of PDK4 was normalized to the amount of GAPDH for each sample.

Statistics and calculations. Values are expressed as means ± SE. All data were analyzed using one-way repeated-measures ANOVA, and Tukey's post hoc analysis was used. Statistical significance was accepted at $P < 0.05$. Statistical analyses of mRNA expression was performed on logarithmic transformed ratios (PDK4/GAPDH), and these data are presented as fold change relative to preexercise values. All uptake calculations were based on the Fick principle, and hematocrit data were used when plasma flow data were required. The term "flux" is used interchangeable with "net flux," and they maintain the same meaning.

Energy (kcal) use from fat and carbohydrate sources, excluding protein oxidation, during prolonged exercise was calculated by assuming that only the exercising legs expended energy [i.e., respiratory exchange ratio (RER) = respiratory quotient] (9). The energy sources that were calculated included whole body carbohydrate oxidation, blood glucose uptake by the leg, estimated muscle glycogen use, whole body fat utilization, blood FFA uptake by the leg, as well as estimated intramuscular triglyceride utilization. Whole body carbohydrate and fat utilization rates were calculated using pulmonary RER and $\dot{V}O_2$, whereas muscle carbohydrate and fat utilization were calculated using leg $\dot{V}O_2$, assuming RER is the same as leg respiratory quotient (18). Blood glucose uptake (mmol·min^{-1}·leg^{-1}) was multiplied by the appropriate exercise time (for each subject) and then converted to grams per leg by multiplying the molecular weight of glucose (180 g/mmol) and then to calories per gram of carbohydrate (~4.2 kcal/g) for resulting calculations to be in kilocalories per leg. These results were multiplied by 2 to represent both legs. From this information, pyruvate oxidized was calculated. Thus the amount of pyruvate produced was calculated by adding net lactate efflux, net alanine production, and changes in muscle pyruvate concentrations to the amount of pyruvate oxidized. The same calculations were used for FFA uptake except that molecular mass of palmytoyl-stearoyl-oleoyl-glycerol was used (861 g), which accounts for a combination of fatty acids as suggested by Frayn (9). Subsequently, this was multiplied by calories per gram of fat (9.5 kcal/g). The use of intramuscular triacylglycerol (IMTG) and glycogen was derived by calculating total calories of fat and carbohydrates used, respectively, and subtracting blood glucose and FFAs from the total use, respectively. Finally, to present data in per kilogram muscle (dry weight), leg muscle mass was assumed to be 8 kg wet wt or ~2 kg dry wt.

Branched-chain amino acids (BCAA) were calculated as the sum of valine, leucine, and isoleucine. The sum of threonine, methionine, phenylalanine, lysine, and BCAA was referred to as total essential amino acids. Due to its importance in protein metabolism, tyrosine was also added to the list of amino acids that were presented. Total amino acids (TAA) were calculated as the sum of all amino acids measured, which included essential amino acids, tyrosine, aspartate, serine, asparagine, glycine, taurine, histidine, ariginine, proline, and ornithine. Net exchange of specific amino acids was calculated as the sum of delta muscle concentrations between two time points and the net total amount released or taken over the given period of time. Despite the fact that glutamine consists of two amino groups, net exchange of glutamine was assumed to represent one amino group because the source of the second amino group could not be determined, since it can arise from either glutamate (2 amino groups) or protein degradation (1 amino group).

RESULTS

Pulmonary $\dot{V}O_2$, RER, blood flow, and leg $\dot{V}O_2$. Subjects exercised at ~44% maximal $\dot{V}O_2$ for 3 h 23 min ± 11 min. As expected, pulmonary and leg $\dot{V}O_2$ as well as leg blood flow were elevated ($P < 0.05$) for the entire exercise duration (Table 1), and leg $\dot{V}O_2$ comprised ~80% of pulmonary $\dot{V}O_2$. RER peaked at 30 min and declined ~13% during exercise ($P < 0.05$; Table 1), reflecting a decrease in carbohydrate oxidation and a reciprocal increase in fat oxidation (Fig. 1). After 1 h of exercise, contributions from muscle glycogen began to decline (Fig. 1), whereas those from blood glucose uptake remained constant. As exercise progressed, more energy was derived from circulating FFA than IMTG. During the first 2 h of recovery, leg $\dot{V}O_2$ returned to resting values, whereas pulmonary $\dot{V}O_2$ remained elevated ($P > 0.05$) by ~50 ml O_2/min compared with rest, which suggests that extramus-

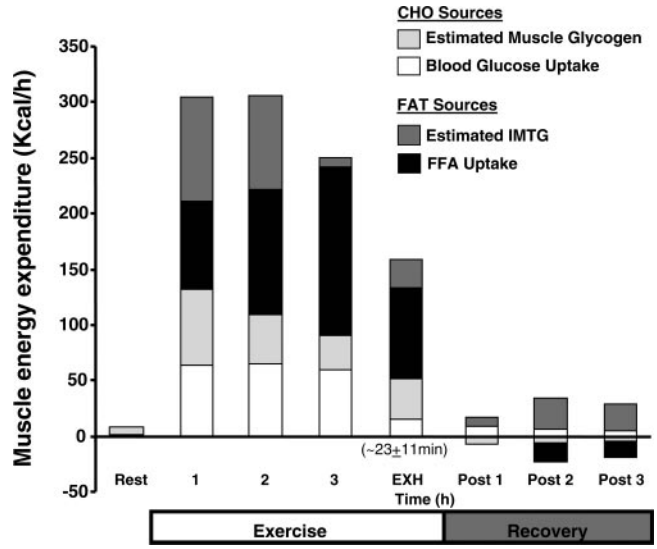

Fig. 1. Muscle energy expenditure during prolonged exercise. Energy derived from blood glucose uptake, estimated muscle glycogen, free fatty acid (FFA) uptake, estimated intramuscular triacylglycerol (IMTG), extramuscular carbohydrate, and extramuscular fat is depicted. Data are presented as mean values (kcal), and muscle energy expenditure is representative of both legs. Exhaustion (EXH) is calculated using the average time to completion of exercise (~3 h and 23 min). CHO, carbohydrate.

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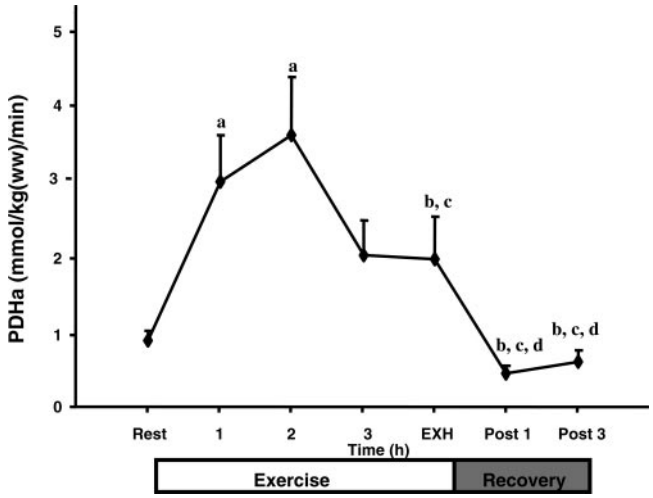

Fig. 2. Pyruvate dehydrogenase activity (PDHa) during prolonged exercise and recovery. Data are means ± SE. Significant difference ($P < 0.05$): [a]from rest; [b]from 1 h of exercise; [c]from 2 h of exercise; [d]from 3 h of exercise.

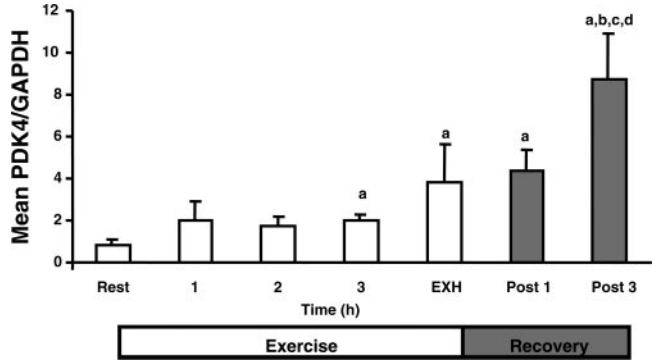

Fig. 3. Ratio changes in PDH kinase 4 (PDK4) gene expression is shown during and after prolonged exercise. Data are means ± SE. GADPH, glyceraldehyde-3-phosphate dehydrogenase. Statistical significance is defined at $P < 0.05$: [a]difference from rest levels; [b]difference from 1 h of exercise; [c]difference from 2 h of exercise; [d]difference from 3 h of exercise.

cular processes may be important in reestablishing energy homeostasis.

PDHa, PDK4 mRNA, pyruvate, and acetyl-CoA. PDHa was elevated ~3.7-fold at 1 h of exercise compared with rest ($P < 0.05$) and reached peak values at 2 h of exercise. Thereafter, PDHa exhibited ~30% decrease at 3 h ($P = 0.16$) and exhaustion ($P < 0.05$) compared with 1 h, and further declined during recovery ($P < 0.05$; Fig. 2). Despite that muscle pyruvate and acetyl-CoA concentrations were unaltered during exercise and recovery (Table 2), net pyruvate production dropped by ~30% (from ~27 to 19 mmol·kg dry $wt^{-1}·h^{-1}$) from 1 to 3 h of exercise, suggesting that pyruvate availability, not PDH activity, was associated with reduced carbohydrate oxidation. PDK4 mRNA was significantly elevated at 3 h of exercise and for the remainder of the protocol, but PDK4 mRNA exhibited a pronounced increase (~7- to 9-fold) in recovery compared with rest (Fig. 3). This increase in PDK4 mRNA suggests specific regulation of the PDK4 gene with prolonged exercise.

Glucose, FFA, and lactate data. Leg glucose uptake was significantly elevated throughout exercise compared with rest, despite the fact that arterial glucose decreased significantly at exhaustion (Fig. 4). Concomitantly, FFA uptake was elevated by 1 h of exercise ($P < 0.05$; Fig. 4), and arterial FFA increased after 2 h of exercise ($P < 0.05$; Fig. 4). During recovery, both leg FFA and glucose uptake returned to resting levels, whereas arterial FFA remained elevated and arterial glucose concentrations returned to resting values. Lactate efflux was modestly elevated ($P > 0.05$) during exercise (Table 3).

Glucagon, insulin, and catecholamine data. Glucagon exhibited an approximately threefold increase at exhaustion ($P < 0.05$; Table 3), implying a notable reliance on liver gluconeogenesis to facilitate glucose homeostasis, while a simultaneous decrease in insulin likely enhanced FFA mobilization ($P < 0.05$; Table 3). Both glucagon and insulin returned to resting levels 3 h postexercise. Norepinephrine and epinephrine were elevated throughout exercise ($P < 0.05$; Table 3) and also facilitated the mobilization of fat to increase circulating FFA.

Amino acid and ammonia data. During exercise, alanine and glutamine release rates were both elevated ~8- to 11-fold from rest and returned to resting rates during recovery (Table 4; $P > 0.05$) with only modest alterations in muscle concentrations (Table 5). Net exchange of glutamine and alanine dropped (~67%) during the first hour of exercise from ~14 to 5.8 mmol/kg dry wt ($P > 0.05$) and ~4.3 to 1.5 mmol/kg dry wt ($P = 0.15$), respectively. Arterial alanine and glutamine were elevated (~47 and ~17%, respectively) in the first 2 h of exercise, coinciding with the net muscle release, and subsequently declined by 3 h ($P < 0.05$; Table 4), indicating that they were cleared by extramuscular tissues.

Muscle glutamate, which is central to forming glutamine and alanine, dropped ~31% at 1 h of exercise compared with rest (12.50 ± 0.89 to 8.72 ± 1.64 mmol/kg dry wt; $P > 0.05$) and maintained these levels for the remainder of exercise and recovery. Glutamate uptake increased approximately fourfold from rest to 1 h of exercise and was accompanied by an ~40% (9 μM) decrease in arterial glutamate in the first hour of exercise ($P < 0.05$). Arterial glutamate remained at this concentration and did not return to rest levels during recovery (Table 4). Total glutamate uptake of ~0.3 mmol/kg dry wt in the first hour of exercise was insufficient to restore the initial ~4 mmol/kg dry wt drop in muscle glutamate (net glutamate consumption = 4.3 mmol·kg dry $wt^{-1}·h^{-1}$; $P < 0.05$). Net consumption was subsequently reduced to ~1.2 mmol·kg dry $wt^{-1}·h^{-1}$ at 2 h of exercise. Although glutamate delivery to the leg and the circulation-to-muscle gradient were constant, the

Table 2. *Muscle pyruvate and acetyl-CoA during prolonged exercise and recovery*

	Rest	1 h	2 h	EXH	Post 1 h	Post 3 h
Pyruvate, mmol/kg dry wt	0.16±0.03	0.21±0.06	0.18±0.03	0.16±0.02	0.16±0.02	0.12±0.02
Acetyl-CoA, mmol/kg dry wt	6.93±1.03	8.75±2.70	6.85±1.69	6.29±2.11	5.97±2.35	7.21±1.60

Values are means ± SE.

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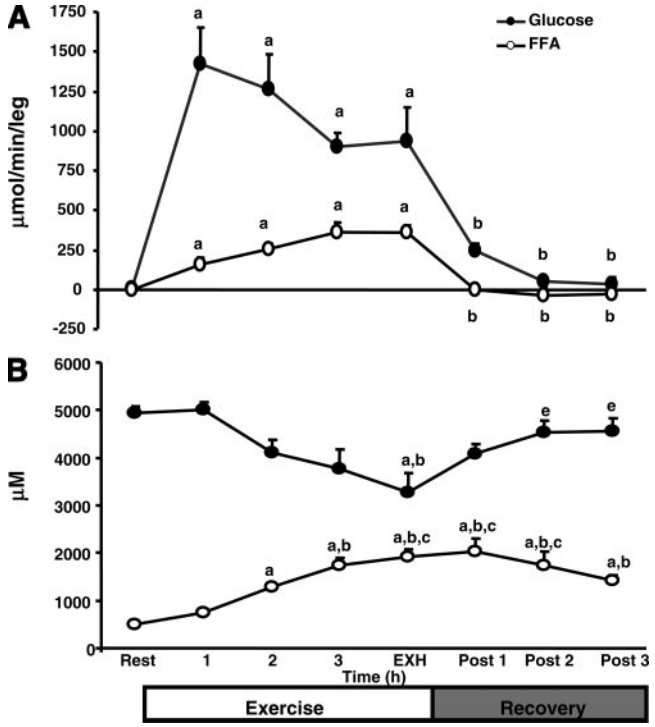

Fig. 4. Leg glucose uptake and FFA uptake (*A*) and arterial glucose and FFA (*B*). Data are means ± SE, where negative numbers reflect release. ●, Glucose data; ○, FFA data. Significant difference ($P < 0.05$): [a]from rest; [b]from 1 h of exercise; [c]from 2 h of exercise; [d]from 3 h of exercise; [e]from EXH.

reduced glutamate net exchange at 2 h suggested that glutamate transport was regulated during exercise.

Despite that alanine, glutamine, glutamate, and BCAA represent more than 50% of the free amino acid pool in muscle, their intramuscular concentrations were minimally altered even with continual exchange between muscle and circulation (Table 5). In early exercise, the majority of arterial amino acid concentrations were modestly elevated from rest concentrations, but most of these amino acids returned to resting levels by 3 h of exercise, and arterial TAA were further reduced during recovery (Table 5; $P < 0.05$). Arterial tyrosine and phenylalanine were elevated at exhaustion, suggesting potential net protein degradation. Net nitrogen balance was also influenced by constant ammonia release during exercise, which was reversed to muscle uptake during recovery (Table 3). Collectively, glutamine, alanine, BCAA, and ammonia demonstrated a relatively large nitrogen release from the muscle (~36 mmol/leg), whereas little nitrogen (primarily glutamate) was taken up (~1.6 mmol/leg) during exercise (Fig. 5). During recovery, glutamine, alanine, and BCAA continued to be released (~6.5 mmol/leg), whereas minimal nitrogen was recovered by glutamate and ammonia uptake (~1.1 mmol/leg).

DISCUSSION

The aim of this study was to investigate the effects of low-intensity, prolonged exercise on pyruvate supply in relation to PDH activity and carbohydrate oxidation. This is the first study, to our knowledge, to examine the time course of interactions of pyruvate production and PDH activity with fat, carbohydrate, and amino acid metabolism during prolonged exercise and recovery. To better understand the changes in substrate utilization during exercise, a moderately low-intensity exercise provided the tool to particularly capture a portrait of the various factors associated with changes in substrate metabolism, from carbohydrate to fat utilization, over the span of a prolonged exercise period. Peak carbohydrate oxidation was observed at 30 min of exercise and declined thereafter, whereas PDH activity reached peak levels at 2 h of exercise and subsequently decreased. This suggests that PDH activity was not integral for decreased carbohydrate oxidation; rather, reduced pyruvate availability may have primarily contributed to the decline in carbohydrate oxidation and ultimately PDH activity. Since none of the known modulators of PDH activity were altered, the regulation of glycogenolysis and glycolysis were likely important in decreasing pyruvate production rates. Despite the attenuated supply of pyruvate, energy was maintained with muscle FFA uptake, which may have also initiated an acute adaptive response to exercise by increasing PDK4 transcription during the exercise bout that led to a more pronounced increase in recovery. The close link between glutamine, alanine, glutamate, and pyruvate metabolism was also evidenced when the peak net exchange of these amino acids corresponded with the greatest net pyruvate production in the first hour of exercise. During the latter part of exercise, the release of alanine and glutamine from muscle, as well as decreased insulin and elevated glucagon, were all integrated in activating gluconeogenesis in extramuscular tissues. These amino acids were also central to nitrogen balance where glutamine and alanine together represented 68% of TAA released during exercise, and glutamate was the predominant amino acid taken up by muscle during exercise and recovery.

Integration of carbohydrate, fat, and amino acid metabolism in substrate oxidation. Carbohydrate oxidation peaked at 30 min of exercise and subsequently declined for the remainder of exercise, whereas PDH activity peaked at 2 h of exercise. This uncoupling between carbohydrate oxidation and PDH activity

Table 3. *Hormone concentrations, lactate flux, and ammonia analysis*

	Rest	1 h	2 h	3 h	EXH	Post 1 h	Post 2 h	Post 3 h
Epinephrine, nM	0.5±0.2	1.2±0.3	5.3±2.1	10.6±2.4[a,b]	17.5±3.7[a–c]	1.7±0.3[d,e]	1.3±0.2[d,e]	1.1±0.3[d,e]
Norepinephrine, nM	1.1±0.2	8.5±1.6[a]	8.9±1.1[a]	10.7±1.4[a]	11.4±1.7[a]	1.7±0.3[b–e]	1.3±0.2[b–e]	1.2±0.2[b–e]
Insulin, pM	31.4±4.8	16.6±6.5	9.1±4.4[a]	9.7±4.0	6.4±3.1[a]	10.1±3.8	18.4±5.2	24.3±6.1
Glucagon, ng/l	94±16	87±11	156±51	153±24	304±39[a–d]	216±57[a,b]	135±29[e]	116±20[e]
Lactate flux, μmol·min^{-1}·leg^{-1}	−16±6	−37±64	−66±74		−30±92	−92±18	−89±20	−74±20
Arterial ammonia, μM	24.4±4.3	61.5±4.3[a]	64.2±8.8[a]	63.6±22.1[a]	61.3±12.7[a]	15.4±4.1[b–e]	10.9±4.6[b–e]	13.9±4.4[b–e]
Venous ammonia, μM	18.3±5.0	72.4±13.3[a]	74.9±12.5[a]	74.1±31.3[a]	76.0±16.0[a]	7.9±3.9[b–e]	8.0±3.9[b–e]	10.4±4.2[b–e]
Ammonia flux, μmol·min^{-1}·leg^{-1}	1.4±1.0	−33.1±16.3	−29.1±9.2	−30.6±2.0	−40.0±5.3[a]	3.2±1.0[e]	1.4±1.0[e]	1.8±1.2[e]

Values means ± SE. Significant difference ($P < 0.05$): [a]from rest; [b]from 1 h of exercise; [c]from 2 h of exercise; [d]from 3 h of exercise; [e]from EXH.

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Table 4. *Arterial concentrations and flux of amino acids during prolonged exercise and recovery*

		Rest	1 h	2 h	3 h	EXH	Post 1 h	Post 2 h	Post 3 h	Statistical Difference, P value
Glu	Art	23.2±1.4	14.3±1.1[a]	12.6±1.1[a]	12.0±1.5[a]	12.5±1.4[a]	11.0±1.1[a,b]	10.3±1.6[a,a]	10.9±1.5[a]	<0.05
	Flux	2.7±0.7	9.9±3.1[a]	5.5±1.4[b]	5.0±2.9	8.3±3.2	2.9±0.6[b]	4.3±0.8	4.6±0.9	<0.05
Ala	Art	89±11	140±13[a]	120±10[a]	118±17	108±12[b]	65±7[b–e]	72±9[b–c]	74±9[b–e]	<0.05
	Flux	−5±1	−30±10	−33±9	−34±8	−43±5	−12±3	−10±4	−10±3	ns
Gln	Art	277±15	324±15[a]	302±9[a]	278±22[b]	252±14[b,c]	182±16[a–e]	181±15[a–e]	197±18[a–e]	<0.05
	Flux	−8±4	−40±25	−56±24	−58±24	−68±21	−16±4	−17±5	−15±5	ns
BCAA	Art	174±9	165±4	155±6	159±10	156±7	185±16	19±17[c,e]	196±14[c,e]	<0.05
	Flux	−2±4	−50±28	−60±31	−79±48	−17±29	−5±6	−9±5	−13±5	<0.05
Thr	Art	38.8±3.1	38.7±2.1	35.3±3.1	31.2±4.2[a,b]	25.5±2.7[a–c]	19.1±2.1[a–e]	18.5±1.5[a–e]	17.4±1.7[a–e]	<0.05
	Flux	−0.4±0.2	−5.8±7.3	−2.9±3.9	−3.8±1.7	1.0±5.7	−0.7±1.3	−2.2±0.6	−2.3±0.5	ns
Tyr	Art	19.0±1.1	23.4±1.1	24.9±0.8[a]	26.6±0.9[a]	25.9±1.2[a]	18.8±1.1[c–e]	18.5±1.0[b–e]	17.5±0.7[b–e]	<0.05
	Flux	−0.7±0.3	−4.6±2.5	−4.7±2.8	−6.3±2.2	−3.5±2.4	−1.1±0.3	−1.1±0.5	−1.2±0.6	0.16
Met	Art	6.4±0.3	8.3±0.6	8.2±0.4	8.0±0.9	7.1±0.6	3.9±0.5[b–e]	4.8±1.0[b–d]	3.8±0.4[a–e]	<0.05
	Flux	−0.7±0.3	−8.8±3.5	−7.7±3.6	−9.4±9.4	−4.1±4.8	−0.5±0.4	−0.2±0.3	−0.6±0.2	<0.05
Phe	Art	16.1±1.2	19.6±1.5[a]	20.5±1.7[a]	20.4±2.1[a]	20.0±1.1[a]	16.7±1.1[b–e]	17.1±1.1[c–e]	18.8±1.4[a,d]	<0.05
	Flux	−0.5±0.1	−2.4±1.7	−2.6±2.4	0.3±5.7	1.0±1.7	−0.5±0.4	−0.2±0.3	−0.7±0.2	ns
Lys	Art	66.9±4.6	75.4±5.4	71.0±3.7	61.8±3.6	58.2±4.4[b]	48.0±4.7[a–c]	49.1±7.9[a–c]	52.6±5.7[b,c]	<0.05
	Flux	0.3±0.7	−6.7±6.2	−4.4±8.4	1.8±6.5	−5.0±2.3	−1.5±0.7	−2.0±1.3	−2.1±1.2	ns
EAA	Art	302±10	307±3	290±4	280±8	266±6	272±17	284±22	288±15	0.06
	Flux	−3.8±3.5	−57.8±46.2	−69.6±40.2	90.4±49.2	−14.3±36.0	−7.9±6.9	−12.2±6.9	−18.3±5.5	0.16
TAA	Art	1,046±28	1,180±36[a]	1,086±35	1,031±74[b]	938±40[a–c]	760±36[a–c]	783±37[a–c]	816±45[a–e]	<0.05
	Flux	−16±6	−143±91	−220±95	−269±100	−118±84	−52±16	−54±16	−58±15	<0.05

Values are means ± SE. Glu, glucose; Ala, alanine; Gln, glutamine; BCAA, branched-chain amino acids; Thr, threonine; Tyr, tyrosine; Met, methionine; Phe, phenylalanine; Lys, lysine; EAA, essential amino acids; TAA, total amino acids; Art, arterial concentrations (μM); Flux, flux of amino acids ($\mu mol \cdot min^{-1} \cdot leg^{-1}$); ns, not significant. Significant difference ($P < 0.05$): [a]from rest; [b]from 1 h; [c]from 2 h; [d]from 3 h; [e]from EXH.

suggests that PDH activity was not regulating the changes in carbohydrate oxidation; rather, reduced carbohydrate oxidation and subsequently PDH activity were likely attributed to decreased pyruvate production, which occurred after 1 h of exercise, independent of changes in muscle pyruvate concentrations. Watt et al. (37, 38) previously suggested that a decrease in RER at the end of a prolonged exercise bout was related to lower PDH activity as a result of elevated PDK activity. However, examining PDH activity and its association with RER at the end of exercise is inappropriate, since RER is known to decline in early exercise. The present study was specifically designed to examine the time course of relevant factors involved in reduced carbohydrate oxidation during prolonged exercise to provide a strong basis for future investigations. With additional time points and the inclusion of flux measurements across the exercising leg, we calculated pyruvate production rate and coupled these changes with carbohydrate oxidation and PDH activity. Although the attenuated rate of pyruvate production was likely to be the key contributing factor to lower carbohydrate oxidation, and subsequently reduced PDH activity, future work is needed to directly investigate the effects of pyruvate availability on carbohydrate oxidation.

The lack of change in the known modulators of PDH activity indicates that they were unlikely to have a major role in PDH regulation. Although PDH phosphatase may be important in the activation of PDH activity at the onset of exercise (17), PDK activity is essential in its downregulation during prolonged exercise (38). However, PDK activity is modulated by changes in pyruvate concentrations and/or cellular energy status, whereby not only was intramuscular pyruvate concentration unaltered in the present study but our laboratory has also shown that adenine nucleotide content as well as ATP-to-ADP ratios were unchanged during prolonged exercise (39). The net production of pyruvate declined from ~27 mmol/kg dry wt in *hour 1* of exercise to ~19 mmol/kg dry wt in *hour 3* and would

Table 5. *Muscle amino acids during prolonged exercise and recovery*

Amino Acids, mmol/kg dry wt	Rest	1 h	2 h	3 h	EXH	Post 1 h	Post 3 h
Glu	12.5±0.9	8.7±1.6	7.9±1.7	10.8±2.8	9.9±1.9	10.6±2.0	8.8±1.7
Ala	11.0±2.7	11.8±2.4	12.1±2.1	16.1±1.9	12.9±2.9	10.7±1.8	11.1±2.6
Gln	60.7±5.1	56.6±2.5	56.9±5.6	56.0±2.0	60.4±6.2	60.6±5.4	45.6±8.2
BCAA	4.5±1.4	3.7±1.2	4.1±1.7	4.3±1.7	4.5±1.7	6.6±1.6	4.5±1.4
Thr	1.2±0.5	1.2±0.5	1.3±0.6	1.6±0.6	1.8±0.7	1.7±0.4	1.3±0.4
Tyr	0.8±0.3	0.7±0.2	0.8±0.3	0.9±0.3	0.9±0.3	0.9±0.3	0.8±0.2
Met	0.4±0.1	0.3±0.1	0.4±0.2	0.5±0.2	0.5±0.2	0.5±0.2	0.5±0.1
Phe	0.8±0.3	0.6±0.2	0.6±0.3	0.7±0.3	0.8±0.3	0.8±0.4	0.8±0.2
Lys	3.0±0.5	2.9±0.5	3.0±0.7	2.8±0.5	3.2±0.6	3.2±0.8	2.7±0.5
EAA	8.7±2.2	7.7±2.0	8.2±2.8	8.3±2.6	9.1±2.8	11.0±2.5	8.5±2.1
TAA	173.7±17.8	168.9±14.5	162.0±28.0	168.0±17.0	184.6±29.5	190.0±15.6	150.1±25.7

Values are means ± SE.

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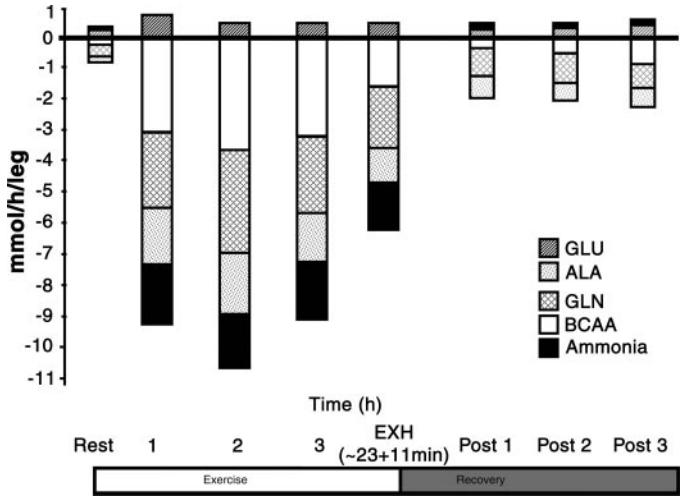

Fig. 5. Summary of net nitrogen balance is presented, whereby mean flux data were used to determine the net changes in key nitrogen carriers, including glutamate (GLU), alanine (ALA), glutamine (GLN), branched-chain amino acids (BCAA), and ammonia, for each hour of exercise and recovery.

have resulted from decreased muscle glycogenolysis, glycolysis, and/or glucose uptake. Muscle glucose uptake was maintained during exercise despite the decrease in arterial glucose, whereas decreased reliance on glycogenolysis (Fig. 1) as well as regulatory factors of glycolytic rate may have been coupled with changes in pyruvate production rate. Although future work is warranted, reduced net pyruvate production was likely modulated by regulatory factors of glycogenolysis and glycolysis, which contributed to decreasing carbohydrate oxidation and subsequently reduced PDH activity.

Despite the decrease in pyruvate supply, energy provision to the working muscles was maintained since TCA cycle flux (i.e., leg $\dot{V}O_2$) was constant. Continual energy provision was exhibited with the significant rise in FFA uptake by muscle, which may have also been associated with decreased PDH activity. In addition, enhanced FFA supply may have instigated a rise in PDK4 mRNA by 3 h of exercise, since it has been suggested in previous work that elevated FFA supply is associated with increased PDK4 gene expression in skeletal muscle (16, 23, 24, 31). Because these transcriptional increases were more pronounced in recovery, which is consistent with previous work (25, 26), they may be indicative of specific adaptive responses associated with prolonged exercise. With a growing reliance on energy derived from FFA uptake, the contracting muscle relied less on IMTG and muscle glycogen as exercise progressed. Enhanced plasma FFA availability was derived from activated lipolysis in adipose tissue as a result of reduced insulin and elevated catecholamine levels and was associated with elevated fat oxidation. Glucose uptake was elevated during exercise, suggesting that a continual reliance on blood-borne glucose for energy was evident. Increased glucagon levels helped to maintain arterial glucose concentrations by activating gluconeogenesis in extramuscular tissues. Thus various tissues integrated diverse signals to facilitate energy provision from carbohydrates and fats for the exercising muscles to accommodate the decrease in net muscle pyruvate production.

Although decreased pyruvate supply resulted in reduced PDH activity, lactate dehydrogenase and alanine aminotransferase, which are near-equilibrium enzymes that also utilize pyruvate as a substrate, were affected differently. Changes in lactate efflux were modest and may have been more important in early exercise (<60 min) when pyruvate production was greatest. In contrast, alanine release from muscle was elevated 8- to 11-fold compared with rest despite that skeletal muscle concentrations were maintained at constant levels. Peak pyruvate production during early exercise coincided with alanine release, and, subsequently, as pyruvate production decreased, alanine release remained constant. It is possible that, since alanine is a gluconeogenic precursor, its constant release may have helped to maintain arterial glucose and to support constant glucose uptake in muscle during the latter phase of exercise. Furthermore, arterial alanine was significantly elevated in early exercise and was reduced to resting levels by 3 h of exercise despite elevated release from the muscle, implying that alanine was cleared by other tissues, presumably the liver. Thus the continuous supply of pyruvate for alanine production suggests an important role for alanine, likely gluconeogenesis, during the latter part of prolonged exercise.

Alanine is intimately coupled with glutamine and glutamate metabolism through the TCA cycle, indicating that other amino acids are associated with pyruvate metabolism. The largest production of glutamine and alanine (~14 and 4.3 mmol/kg dry wt, respectively) and glutamate utilization (~4.1 mmol/kg dry wt) occurred in the first hour of exercise when pyruvate production was greatest (~27 mmol/kg dry wt). Glutamate, alanine, and glutamine net exchanges were subsequently reduced (85, 56, and 54%, respectively) from 1 to 2 h of exercise, suggesting that these amino acids were most dynamic in the first hour of exercise. The potential importance of alanine in early exercise is supported by observations from Gibala et al. (10, 11), where muscle pyruvate and alanine concentrations as well as alanine flux were elevated as early as 5 min of exercise. Thus these data indicate that glutamate, glutamine, and alanine are distinctly linked to pyruvate metabolism and may be important in early exercise regulation.

In addition to pyruvate metabolism, glutamate, glutamine, and alanine had large implications on nitrogen balance during prolonged exercise. Previous work has shown that glutamine, alanine, and BCAA are the predominant amino acids released from muscle during 60–90 min of exercise (5, 10, 21, 32). In the present study, ~36 mmol/leg were released collectively in the form of glutamine, alanine, BCAA, and ammonia during low-intensity exercise, where glutamine and alanine alone represented ~68% of TAA released. With only 1.6 mmol/leg of glutamate taken up during exercise (Fig. 5), the interactions between glutamate, glutamine, alanine, as well as BCAA, reflect the importance of these amino acids in nitrogen and energy balance of exercising muscles. The net release of threonine, tyrosine, methionine, and lysine were elevated throughout exercise, possibly representing continuous muscle protein degradation. Previous studies of shorter exercise duration have reported enhanced release of various amino acids by muscle and corresponding decreases in plasma concentrations (15, 32, 35). As such, the interactions between glutamine, alanine, and glutamate revealed the importance of the these amino acids in both pyruvate metabolism and nitrogen balance in muscle.

Reestablishing homeostasis in the recovery period. During recovery, protein degradation continued with the net release of

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threonine, tyrosine, methionine, and lysine from muscle, which were likely important in reestablishing homeostasis in extramuscular tissues. Greater efflux was evidenced with BCAA, glutamine, and alanine (~6.5 mmol/leg combined), which comprised ~67% of TAA released over the 3-h recovery period. Moreover, nitrogen uptake was only 1.1 mmol/leg (glutamate and ammonia) during the 3-h recovery period. These particular amino acids may have been important to various recovery processes by enhancing protein production and gluconeogenesis in extramuscular tissues. The importance of gluconeogenic amino acids was evident since arterial glucose returned to resting levels by the end of the recovery period. In agreement with previous work (2), the trend in elevated postexercise pulmonary $\dot{V}O_2$ (~50 ml O_2/min), which was 18% greater than preexercise values despite that leg $\dot{V}O_2$ had returned to resting rates, was potentially important in reestablishing homeostasis during recovery. Changes in fat metabolism also suggested a potential role in recovering energy homeostasis because estimated use of IMTG was modestly elevated during recovery as was PDK4 mRNA; these metabolic events may be linked to the further decline in PDH activity during recovery. Kimber et al. (19) demonstrated that, following glycogen-depleting exercise, the circulating fatty acids and triacylglycerol were the primary lipid sources since IMTG levels remained unaltered following 18 h of recovery. However, IMTG use may be somewhat lower than in the present study since subjects in the work of Kimber et al. were fed a high-carbohydrate/low-fat meal following exercise, which may have led to decreased IMTG reliance. Overall, these relationships likely facilitated the attenuation of carbohydrate utilization to enable glycogen resynthesis, potentially demonstrating an acute adaptive response to prolonged exercise. Thus these data illustrate the dynamic relationship between amino acid, carbohydrate, and fat metabolism within muscle and across extramuscular tissues during recovery.

Methodological considerations. Although the techniques and protocol used in this study provided a comprehensive approach to further understanding the regulation of substrate metabolism during prolonged exercise, there are certain factors that should be considered. One must consider that the information attained is descriptive in nature and that future mechanistic studies are needed to further understand substrate regulation in prolonged exercise. Due to the invasive nature of the study, the sample size that was studied warrants careful interpretation of the results presented. Subjects were also studied following an overnight fast, which may have implications on protein degradation and carbohydrate metabolism. However, the introduction of food would confound the results of this study, since many of the parameters measured are highly sensitive to different types of foods (i.e., high- vs. low-carbohydrate meal). For the purpose of this study, nutritional intake before and after exercise was restricted since we were interested in demonstrating regulatory changes in substrate utilization from carbohydrate to fat metabolism during prolonged exercise. Feeding subjects before exercise would exaggerate interindividual variability since the amount of time required to cannulate the femoral artery and vein for each subject before commencing the exercise protocol varies; this could not have been controlled adequately, which would have greater implications on our results.

In summary, decreased carbohydrate oxidation during prolonged exercise was associated with a reduced PDH activity and was attributed to a reduced rate of pyruvate production. None of the known modulators of PDH activity were altered, implying that glycogenolytic and glycolytic regulatory factors may have induced a decrease in carbohydrate oxidation. Increased FFA supply not only provided energy to the exercising muscle but may also have been related to the increased PDK4 mRNA, particularly during recovery, indicating an adaptive response to prolonged exercise. Despite that reduced pyruvate production resulted in lower PDH activity, production of gluconeogenic precursors (alanine and glutamine) was maintained. Pyruvate metabolism was also integrated with other amino acids, such as glutamate and glutamine, in early exercise, which was particularly interesting because these amino acids (alanine, glutamate, and glutamine) demonstrated the most pronounced effects on nitrogen balance.

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