Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise

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Submitted 16 August 2004; accepted in final form 17 September 2004

Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. Am J Physiol Endocrinol Metab 288:E133–E142, 2005. First published September 21, 2004; doi:10.1152/ajpendo.00379.2004.—Intracellular mechanisms regulating fat oxidation were investigated in human skeletal muscle during exercise. Eight young, healthy, moderately trained men performed bicycle exercise (60 min, 65% peak O2 consumption) on two occasions, where they ingested either a high-carbohydrate diet (H-CHO) or a low-carbohydrate diet (L-CHO) before exercise. The data suggest that the cytosolic concentration of citrate and the other involving mitochondrial regulation of CPT-1 have been proposed; one involves malonyl-CoA, and the other involves the cytosolic concentration of carnitine.

Malonyl-CoA is an intermediate in the de novo synthesis of fatty acids (FA) and an allosteric inhibitor of CPT-1 (14). Studies in rat muscle suggest that changes in the glucose supply and energy expenditure of the muscle cell regulate the concentration of malonyl-CoA, in keeping with its need to generate ATP from fat oxidation (28, 32, 33). Two types of regulation of malonyl-CoA have been described: one involving the cytosolic concentration of citrate and the other involving 5'-AMP-activated protein kinase (AMPK) (28, 32). Cytosolic citrate is both an allosteric activator of acetyl-CoA carboxylase (ACC), the key enzyme governing malonyl-CoA synthesis, and a substrate for the malonyl-CoA precursor, cytosolic acetyl-CoA. In rodents and humans, high glucose availability at rest has been shown to elevate the cytosolic citrate concentration and consequently the muscle malonyl-CoA concentration (3, 27, 32, 33). This is likely the mechanism whereby fat oxidation is inhibited by high glucose availability in resting humans (3, 27). AMPK in turn regulates the concentration of malonyl-CoA by phosphorylating and inhibiting ACC (28) and possibly by activating malonyl-CoA decarboxylase (MCD), the major enzyme regulating malonyl-CoA turnover in muscle (34). During muscle contractions in rodents, AMPK is activated and ACC is phosphorylated and consequently inhibited, leading to a decrease in muscle malonyl-CoA concentration, which may induce the increase in fat oxidation at the onset of contraction (28). In contrast, several human studies have failed to show a reduction in the muscle malonyl-CoA concentration during moderate-intensity exercise with normal muscle glycogen stores when fat oxidation is increased from resting levels (7, 17, 18). Still, with low muscle glycogen content, there is a higher AMPK activation during exercise (44), which may decrease the malonyl-CoA level and thereby cause the higher fat oxidation seen during exercise with low vs. high muscle glycogen (44). So far, the muscle malonyl-CoA concentration has never been measured in humans during submaximal exercise with low muscle glycogen content.

FAT AND CARBOHYDRATE are the main sources of fuel for ATP synthesis in human skeletal muscle. The ratio between fat and carbohydrate oxidation during exercise depends on preexercise substrate levels and exercise duration and intensity (12, 40, 42, 44). However, the intracellular determinants of the use of these fuels are unclear.

It has been suggested that the fat oxidation rate in human skeletal muscle during exercise is regulated intracellularly at the entry of long-chain fatty acyl (LCFA)-CoA into the mitochondria (31, 36, 37). The transport of LCFA-CoA across the inner mitochondrial membrane is preceded by the transfer of the acyl moiety to carnitine, a process catalyzed by carnitine palmitoyltransferase 1 (CPT-1), an enzyme located in the outer mitochondrial membrane (23). Two mechanisms for the intracellular regulation of CPT-1 have been proposed; one involves malonyl-CoA, and the other involves the cytosolic concentration of carnitine.

Malonyl-CoA is an intermediate in the de novo synthesis of fatty acids (FA) and an allosteric inhibitor of CPT-1 (14). Studies in rat muscle suggest that changes in the glucose supply and energy expenditure of the muscle cell regulate the concentration of malonyl-CoA, in keeping with its need to generate ATP from fat oxidation (28, 32, 33). Two types of regulation of malonyl-CoA have been described: one involving the cytosolic concentration of citrate and the other involving 5'-AMP-activated protein kinase (AMPK) (28, 32). Cytosolic citrate is both an allosteric activator of acetyl-CoA carboxylase (ACC), the key enzyme governing malonyl-CoA synthesis, and a substrate for the malonyl-CoA precursor, cytosolic acetyl-CoA. In rodents and humans, high glucose availability at rest has been shown to elevate the cytosolic citrate concentration and consequently the muscle malonyl-CoA concentration (3, 27, 32, 33). This is likely the mechanism whereby fat oxidation is inhibited by high glucose availability in resting humans (3, 27). AMPK in turn regulates the concentration of malonyl-CoA by phosphorylating and inhibiting ACC (28) and possibly by activating malonyl-CoA decarboxylase (MCD), the major enzyme regulating malonyl-CoA turnover in muscle (34). During muscle contractions in rodents, AMPK is activated and ACC is phosphorylated and consequently inhibited, leading to a decrease in muscle malonyl-CoA concentration, which may induce the increase in fat oxidation at the onset of contraction (28). In contrast, several human studies have failed to show a reduction in the muscle malonyl-CoA concentration during moderate-intensity exercise with normal muscle glycogen stores when fat oxidation is increased from resting levels (7, 17, 18). Still, with low muscle glycogen content, there is a higher AMPK activation during exercise (44), which may decrease the malonyl-CoA level and thereby cause the higher fat oxidation seen during exercise with low vs. high muscle glycogen (44). So far, the muscle malonyl-CoA concentration has never been measured in humans during submaximal exercise with low muscle glycogen content.
Carnitine is a substrate for CPT-1. Consequently, the availability of cytosolic carnitine may limit fat oxidation. Another function of carnitine is to buffer accumulated acetyl-CoA in a reaction catalyzed by carnitine acetyltransferase (CAT) (26). It has been proposed that, under exercise conditions with high glycolytic flux and therefore excess formation of acetyl-CoA compared with its utilization by the tricarboxylic acid cycle, carnitine serves to buffer the excess acetyl-CoA, which leaves less carnitine available to CPT-1 (40, 43). By this mechanism, carnitine may play a role in adjusting the rate of fat oxidation inversely to the rate of carbohydrate oxidation. Accordingly, in humans it was shown that fat oxidation and muscle carnitine concentration were tightly coupled during incremental exercise: with increasing exercise intensity, they both decreased, while, on the other hand, carbohydrate oxidation increased (40).

The present study investigated intracellular mechanisms to regulate fat oxidation in response to altered carbohydrate availability in human skeletal muscle during submaximal exercise. Healthy male subjects performed 60 min of submaximal bicycle exercise with either high (H-CHO) or low (L-CHO) preexercise muscle glycogen. During the two exercise protocols, with expected similar energy output but marked differences in the relative fat and carbohydrate oxidation, we measured in the same setup the malonyl-CoA, carnitine, acetylcarnitine, and acetyl-CoA concentrations, the pyruvate dehydrogenase (PDH) and AMPK activity, and the ACCβ Ser221 phosphorylation in skeletal muscle to examine the intramuscular mechanisms that regulate the ratio between fat and carbohydrate oxidation during exercise. To rule out marked differences between H-CHO and L-CHO conditions in blood-borne substrate and hormone levels and their possible confounding effects on the interpretations of the results, we allocated a light meal 4.5 h before exercise and infused glucose intravenously during exercise to keep the arterial blood glucose concentrations nearly constant and similar between the two conditions. By this approach, the levels of several other circulating metabolites and hormones also were kept nearly similar between H-CHO and L-CHO.

**Materials and Methods**

**Subjects.** Eight young, healthy, moderately trained men [age, 26 ± 1 yr; height, 1.80 ± 0.02 m; body mass, 76 ± 3 kg; body mass index, 23.5 ± 0.7 kg/m²; body fat, 14.6 ± 1.9%; peak O2 consumption (VO2 peak), 4.1 ± 0.2 l/min; VO2 peak/body mass, 53.7 ± 1.6 ml·kg⁻¹·min⁻¹] were recruited to participate in the study. All subjects were nonsmokers and were engaged in 4–6 h/wk of regular exercise training such as running, bicycling, and resistance training. Before inclusion into the study, subjects were fully informed about the nature and possible risks of the study and gave their written consent. The study was approved by the Copenhagen Ethics Committee (KF-01-078/01) and carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki II). The results presented in this study are part of a larger study on the regulation of lipid metabolism during exercise in human skeletal muscle that has, in minor part, been published previously (30, 31).

**Preexperimental testing.** All subjects initially performed an incremental exercise test on a bicycle ergometer (Monark 839 Electronic Ergometer; Monark Exercise Sweden) to determine peak O2 uptake (VO2 peak). Respiratory measurements were carried out with the Douglas bag technique. Percent body fat was measured by dual-energy X-ray absorptiometry (DEXA; Lunar, Madison, WI; DPX-IQ v. 4.6.6).

**Experimental design.** Subjects underwent two experimental protocols separated by 2–3 wk. In both protocols, subjects completed a bout of glycogen-depleting bicycling followed by a controlled diet for the rest of the day (Fig. 1, day 1). In one protocol the controlled diet consisted primarily of fat (L-CHO), and in the other protocol primarily of carbohydrate (H-CHO) (see Glycogen depletion bout). The next day, subjects performed a bicycle exercise test for 60 min at ~65% VO2 peak (Fig. 1, day 2). The order of the L-CHO and H-CHO protocols was randomized and stratified.

**Dietary control.** On the 3 days preceding the first protocol, subjects determined their habitual energy and nutrient intake by a self-reported dietary record. All food and beverage intakes were weighed to the accuracy of 1 g and recorded. Subsequently, the energy intake and composition of the habitual diet were calculated by means of a computer database (Dankost 2000; Danish Catering Center, Copenhagen, Denmark). On the 3 days preceding the second protocol, subjects followed a diet identical to the one ingested before the first protocol.

**Glycogen depletion bout.** On the day of the glycogen depletion bout, subjects arrived at the laboratory at 7:30 AM. All subjects

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**Fig. 1.** Schematic diagram showing the experimental protocol. Each protocol consisted of 2 experimental days. Day 1: glycogen depletion and dietary manipulation. Day 2: exercise experiment. All subjects completed 2 protocols [high (H-CHO) and low (L-CHO) preexercise muscle glycogen].
abstained from any heavy physical activity on the day before the glycogen depletion bout. A breakfast meal was served that contained 25% of each subject's daily energy intake and consisted of 30% energy (30 E%) as fat, 55 E% as carbohydrate, and 15 E% as protein. At 10 AM, subjects initiated the glycogen depletion bicycle bout, which consisted of alternating periods of continuous and intermittent exercise with a few short bouts of arm cranking in between, as previously described (31). The glycogen depletion bout lasted for 3–4.5 h and was well tolerated by all subjects. To determine the muscle glycogen concentration after the glycogen depletion protocol, a muscle biopsy was obtained under local anesthesia from the vastus lateralis muscle by the needle biopsy technique. For the following 6 h, subjects were allowed to move freely around at the laboratory. Meals were served immediately after the biopsy procedure and 1, 2, 3.5, and 5 h after completion of the glycogen depletion bout. Then subjects were given a light snack to ingest at 11 PM and left the laboratory. The controlled diet during the recovery period consisted of 85 E% fat, 2 E% carbohydrate, and 13 E% protein in L-CHO and 8 E% fat, 80 E% carbohydrate, and 12 E% protein in H-CHO. The total energy intake during this period was calculated as the individual daily energy intake (DEI), as reported from the dietary record, minus the breakfast (25% DEI) plus the estimated extra energy consumption during the bicycle exercise to secure that subjects were in energy balance. During the glycogen depletion bout and for the rest of the day, subjects were allowed to drink water ad libitum.

**Exercise experimental protocol.** Subjects arrived at the laboratory at 7:30 AM having abstained from any physical activity during the morning. A light breakfast was served that contained 10% of the individual DEI and consisted of 4 E% fat, 78 E% carbohydrate, and 18 E% protein. After 30 min of rest in the supine position, Teflon catheters were inserted under local anesthesia into the femoral artery and vein, and femoral venous blood was drawn from the femoral artery and vein, and femoral venous blood was obtained under local anesthesia from the vastus lateralis muscle by the needle biopsy technique. For the following 6 h, subjects were served immediately after the biopsy procedure and 1, 2, 3.5, and 6 h after completion of the glycogen depletion bout. Then subjects were offered water ad libitum and stored at −80°C for subsequent biochemical analysis. Seventy-five to eighty milligrams wet weight were freeze-dried and dissected free of all visible adipose tissue, connective tissue, and blood under a microscope. The dissected muscle fibers were pooled and then divided into subpools for the respective analyses. Malonyl-CoA content and PDH activity in its active form (PDHα) were measured on 25 and 10 mg of wet muscle tissue, respectively, which were not freeze dried.

**Muscle glycogen.** The glycogen concentration was determined on 2 mg dry wt by a fluorometric method (13).

**Muscle lysates.** Muscle lysates were prepared from 6 mg dry wt of freeze-dried and dissected muscle tissue as described previously (10). Due to the limited amount of tissue, muscle lysates were only prepared from biopsies obtained at rest and 60 min of exercise.

**Western blotting.** Phosphorylation of α-AMPK Thr172 and ACCβ Ser21 was determined by Western blotting on muscle lysates. The lysates were boiled in Laemml buffer before being subjected to SDS-PAGE and immunoblotting. Primary phosphospecific antibodies were rabbit anti-α-AMPK Thr172- and ACCβ Ser21-antibodies as described previously (45). Secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark). Antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL+; Amersham Biosciences) and quantified by a Kodak Image Station E440CF (Kodak, Glostrup, Denmark). The anti-α-AMPK Thr172- and ACCβ Ser21-antibodies detected a single band at ~63 kDa as expected. With the phosphospecific ACCα Ser79 antibody, which probably recognizes the equivalent Ser79 in human ACCβ in the phosphorylated state, a single band was detected at ~260 kDa as expected.

**AMPK activity.** α-Isomorph-specific AMPK activity was determined in immunoprecipitates from muscle lysates as described previously (44). Briefly, immunoprecipitates were prepared from 200 μg of muscle lysate protein using anti-α- or anti-β-AMPK antibodies as described previously (46) (kindly donated by Dr. D. G. Hardie, University of Dundee, Dundee, Scotland). AMPK activity was measured in the immunoprecipitates using SAMS-peptide (HMRASGSLHLVKKR, 200 μM) as previously described (45).

**Malonyl-CoA.** The muscle malonyl-CoA concentration was measured as described previously (15), modified to human muscle tissue (7). In short, 25 μg of muscle tissue (wt wt) were homogenized in 250 μl of perchloric acid (10%) and neutralized with NaOH. Malonyl-CoA was determined in the neutralized muscle homogenates by...
measuring malonyl-CoA-dependent incorporation of [3H]acetyl-CoA into palmitate catalyzed by FA synthase (kindly donated by Dr. J. Knudsen, University of Southern Denmark). Each sample was determined in duplicate. The coefficient of variation (CV) between three single determinations on the same muscle extract was 18%, and the CV between two different muscle extracts, determined in duplicate, from the same biopsy was 11%.

PDHαs. Due to limited amount of muscle tissue, PDHα activity was only determined on the biopsies obtained at rest and at 60 min of exercise. The frozen muscle tissue (10 mg) was homogenized in 300 μl of buffer (pH 7.8) containing 200 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 50 mM Tris·HCl, 50 mM NaF, 5 mM dichloroacetate, and 0.1% Triton X-100. Subsequently, PDHα activity was determined by a radioisotopic assay first described by Constantinidis et al. (6) and modified by Putman et al. (25). The CV between five single determinations on the same muscle extract was 17%, and the CV between four different muscle extracts from the same biopsy determined in duplicate was 17%.

Acetyl-CoA, acetylcarnitine, and free carnitine. Six milligrams dry weight of freeze-dried and dissected muscle tissue were extracted with 0.6 M perchloric acid and neutralized with 2 M KHCO₃. Acetyl-CoA, acetylcarnitine, and free carnitine concentrations were determined by radioisotopic assays (4). The CV between five single determinations on the same muscle extract was 3.9, 3.1, and 5.8% for acetyl-CoA, acetylcarnitine, and free carnitine, respectively. The CV between four different muscle extracts, determined in duplicate, from the same biopsy was 11.9, 8.1, and 6.2% for acetyl-CoA, acetylcarnitine, and free carnitine, respectively. Recovery of acetyl-CoA, acetylcarnitine, and carnitine added to muscle extracts was 90, 96, and 99%, respectively.

Statistics. Data are presented as means ± SE. For variables independent of time, a paired t-test was performed to test for differences between L-CHO and H-CHO. For variables measured before and after exercise as well as variables measured before and during exercise, a two-way analysis of variance (ANOVA), with repeated measures for the effect of time was found, significant pairwise differences were performed using Tukey’s post hoc test. Unless otherwise stated, a probability of 0.05 was used as the level of significance.

RESULTS

Workload. During the 60-min bicycle exercise test, subjects exercised at 180 ± 7 W in both conditions. In L-CHO the systemic VO₂ averaged 2.8 ± 0.1 l/min during exercise, whereas in H-CHO the systemic VO₂ averaged 2.5 ± 0.1 l/min during exercise (P < 0.001). The %VO₂ peak during the bicycle exercise test averaged 68 ± 1 and 62 ± 1% in L-CHO and H-CHO, respectively (P < 0.001).

Intravenous glucose infusion. The glucose infusion rate during exercise averaged 39 ± 5 and 15 ± 6 μmol·kg body mass⁻¹·min⁻¹ in L-CHO and H-CHO, respectively (P < 0.01).

Cardiovascular parameters. Heart rate was 62 ± 2 and 56 ± 2 beats/min (bpm) at rest in L-CHO and H-CHO, respectively (P < 0.05), and increased (P < 0.001) at initiation of exercise to a plateau level averaging 163 ± 5 and 152 ± 6 bpm during exercise in L-CHO and H-CHO (P < 0.001). At rest, femoral venous blood flow was 0.51 ± 0.09 and 0.41 ± 0.02 l/min in L-CHO and H-CHO, respectively (not significant [NS]). At onset of exercise, femoral venous blood flow increased (P < 0.001) to 6.5 ± 0.4 and 6.1 ± 0.4 l/min in L-CHO and H-CHO, respectively (NS), and remained at this level throughout exercise.

Fat and carbohydrate oxidation. RER and leg RQ did not differ significantly from each other in either condition. At rest, RER was 0.76 ± 0.02 and 0.83 ± 0.03 and leg RQ was 0.75 ± 0.01 and 0.85 ± 0.03 in L-CHO and H-CHO, respectively. Within the first 10 min of exercise, RER increased (P < 0.01) to 0.82 ± 0.02 and 0.91 ± 0.01 in L-CHO and H-CHO, respectively, and leg RQ increased (P < 0.01) to 0.85 ± 0.02 and 0.93 ± 0.02 in L-CHO and H-CHO, respectively. No further significant changes were seen in RER or leg RQ during exercise. There was a main effect of condition on RER and on leg RQ, both being lower in H-CHO than in L-CHO (P < 0.001).

The leg fat oxidation rate across the leg did not differ significantly between L-CHO and H-CHO at rest (Fig. 2). In H-CHO the leg fat oxidation rate increased (P < 0.05) from rest to exercise, and an even larger increase (P < 0.001) was seen in L-CHO. The leg fat oxidation rate differed significantly between L-CHO and H-CHO at all exercise time points (P < 0.001). The leg carbohydrate oxidation rate increased (P < 0.001) from rest to exercise in both conditions. A main effect of condition was observed in the leg carbohydrate oxidation rate (P < 0.01).

Blood metabolite concentrations. At rest, the arterial concentration of plasma FA was 674 ± 110 and 596 ± 112 μM in L-CHO and H-CHO, respectively. It decreased (P < 0.05) at initiation of exercise to 468 ± 61 and 334 ± 34 μM at 10 min in L-CHO and H-CHO, respectively, after which it increased (P < 0.05) continuously during exercise to 703 ± 58 μM in L-CHO and 618 ± 58 μM in H-CHO at 90 min. A main effect of condition was observed on arterial plasma FA concentration, it being slightly higher in L-CHO than in H-CHO (P < 0.01).

The arterial blood glucose concentration at rest was 4.9 ± 0.2 and 5.1 ± 0.1 mM in L-CHO and H-CHO, respectively (NS). During exercise, it was kept nearly constant because of the intravenous glucose infusion.

Circulating hormones. At rest, the arterial plasma insulin concentration was 6.6 ± 1.1 and 6.5 ± 0.6 μU/ml in L-CHO and H-CHO, respectively (NS). In both conditions, the arterial plasma insulin concentration decreased (P < 0.001) continuously during exercise to 3.4 ± 0.2 and 4.2 ± 0.3 μU/ml at 60 min of exercise in L-CHO and H-CHO, respectively. The
arterial plasma insulin concentration was lower in L-CHO than in H-CHO at 20, 30, 50, and 60 min of exercise ($P < 0.05$).

At rest, the arterial plasma epinephrine concentration was 0.60 ± 0.10 and 0.54 ± 0.08 nM in L-CHO and H-CHO, respectively (NS). At initiation of exercise, it increased ($P < 0.001$) to 1.79 ± 0.19 and 1.54 ± 0.19 nM at 10 min in L-CHO and H-CHO, respectively, whereby a further continuous increase ($P < 0.001$) occurred to 2.92 ± 0.49 and 2.05 ± 0.31 nM at 60 min of exercise in L-CHO and H-CHO, respectively. During exercise, the arterial plasma epinephrine concentration did not differ significantly between L-CHO and H-CHO.

The arterial plasma norepinephrine concentration at rest was 1.22 ± 0.29 and 1.49 ± 0.29 nM in L-CHO and H-CHO, respectively (NS). At onset of exercise, it increased ($P < 0.001$) and averaged 14.88 ± 1.39 and 11.04 ± 0.87 nM in L-CHO and H-CHO, respectively, during exercise. The arterial plasma norepinephrine concentration was higher in L-CHO than in H-CHO at 10 min ($P < 0.05$) and at 20, 40, 50, and 60 min ($P < 0.01$).

**Muscle glycogen.** The glycogen concentration in the vastus lateralis muscle immediately after the glycogen depletion bout was 35 ± 10 and 84 ± 42 mmol/kg dry wt in L-CHO and H-CHO, respectively (NS) (Fig. 3). After the following dietary manipulation and immediately before the 60-min bicycle exercise test, the glycogen concentration had increased ($P < 0.001$) to 197 ± 21 and 504 ± 25 mmol/kg dry wt in L-CHO and H-CHO, respectively. During the first 30 min of exercise, a significant decrease ($P < 0.01$) was observed in the muscle glycogen concentration in both conditions, but the rate of glycogen breakdown was 46% higher in H-CHO than in L-CHO ($P < 0.05$). From 30 to 60 min of exercise, the glycogen concentration decreased further ($P < 0.001$) in H-CHO but not in L-CHO (NS). At rest and at 30 and 60 min of exercise, the glycogen concentration in the vastus lateralis muscle was lower in L-CHO than in H-CHO ($P < 0.001$).

**AMPK and ACC.** $\alpha$-AMPK Thr$^{172}$ phosphorylation in the vastus lateralis muscle at rest did not differ significantly between H-CHO and L-CHO (Table 1). From rest to 60 min of exercise, a 306% increase ($P < 0.01$) occurred in $\alpha$-AMPK Thr$^{172}$ phosphorylation in L-CHO, whereas no significant change was observed in H-CHO. At 60 min of exercise, $\alpha$-AMPK Thr$^{172}$ phosphorylation was 126% higher ($P < 0.01$) in L-CHO than in H-CHO.

<table>
<thead>
<tr>
<th>Table 1. $\alpha$-AMPK Thr$^{172}$ phosphorylation and activity</th>
<th>Rest</th>
<th>60 min of Exercise</th>
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<tbody>
<tr>
<td><strong>L-CHO</strong></td>
<td><strong>H-CHO</strong></td>
<td><strong>L-CHO</strong></td>
</tr>
<tr>
<td>$\alpha$-AMPK Thr$^{172}$ phosphorylation, AU</td>
<td>8 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>$\alpha_1$-AMPK activity, pmol-mg prot$^{-1} \cdot$ min$^{-1} \cdot$ 16</td>
<td>2.7 ± 0.4</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>$\alpha_2$-AMPK activity, pmol-mg prot$^{-1} \cdot$ min$^{-1} \cdot$ 16</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
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Values are means ± SE. $\alpha$-AMPK Thr$^{172}$ phosphorylation and activity in the vastus lateralis muscle before and at 60 min of bicycle exercise at 65% peak $O_2$ consumption with low (L-CHO) or high (H-CHO) preexercise muscle glycogen. AMPK, AMP-activated protein kinase; CHO, carbohydrate; AU, arbitrary units; prot, protein. *Different from H-CHO, $P < 0.01$. †Different from rest, $P < 0.01$. ††Borderline-significant main effect of exercise, $P = 0.06$. §Borderline significantly different from rest, $P = 0.06$.

$\alpha_1$-AMPK activity in the vastus lateralis muscle did not differ significantly between conditions at rest or at 60 min of exercise (Table 1). A borderline-significant main effect of exercise was observed in $\alpha_1$-AMPK activity, which increased from rest to 60 min of exercise ($P = 0.06$).

$\alpha_2$-AMPK activity was similar between L-CHO and H-CHO at rest (NS) (Table 1). In L-CHO a 238% increase ($P < 0.01$) occurred from rest to 60 min of exercise, whereas the exercise-induced 119% increase in $\alpha_2$-AMPK activity in H-CHO only reached borderline statistical significance ($P = 0.06$). At 60 min of exercise, $\alpha_2$-AMPK activity was 62% higher in L-CHO than in H-CHO ($P < 0.01$).

ACCo Ser$^{221}$ phosphorylation did not differ significantly between L-CHO and H-CHO at rest or at 60 min of exercise, but it increased ($P < 0.001$) approximately sixfold from rest to exercise irrespective of condition (Fig. 4A).

**Malonyl-CoA.** At rest the malonyl-CoA concentration in the vastus lateralis muscle did not differ significantly between L-CHO (0.32 ± 0.03 µmol/kg wet wt) and H-CHO (0.30 ± 0.03 µmol/kg wet wt) (Fig. 4B). From rest to 30 min of exercise, there was a 13% decrease ($P < 0.05$) in the muscle malonyl-CoA concentration irrespective of condition. The lower malonyl-CoA concentration persisted throughout exercise (0 vs. 60 min, $P < 0.05$). During exercise there was no significant effect of condition on the muscle malonyl-CoA concentration.

**PDH.** At rest PDHa activity was lower in L-CHO (0.17 ± 0.06 mmol·min$^{-1}$·kg wet wt$^{-1}$) than in H-CHO (0.52 ± 0.10 mmol·min$^{-1}$·kg wet wt$^{-1}$) ($P < 0.01$) (Fig. 5). In both conditions, PDHa activity was higher after 60 min of exercise than at rest ($P < 0.001$). After 60 min of exercise, PDHa activity was still lower in L-CHO (0.86 ± 0.12 mmol·min$^{-1}$·kg wet wt$^{-1}$) than in H-CHO (1.48 ± 0.21 mmol·min$^{-1}$·kg wet wt$^{-1}$) ($P < 0.01$).

**Acetyl-CoA.** The resting acetyl-CoA concentration in the vastus lateralis muscle did not differ significantly between L-CHO (18.8 ± 2.6 µmol/kg dry wt) and H-CHO (13.5 ± 3.0 µmol/kg dry wt) (Fig. 6A). In L-CHO it was ~35% lower ($P < 0.05$) during exercise than at rest, whereas in H-CHO it was ~70% higher at 30 min ($P < 0.001$) and at 60 min ($P < 0.01$) of exercise than at rest. The muscle acetyl-CoA concentration was lower in L-CHO than in H-CHO at 30 min (49%, $P < 0.001$) and 60 min (45%, $P < 0.01$) of exercise.
Carnitine and acetylcarnitine. At rest, the acetylcarnitine concentration in the vastus lateralis muscle was higher in L-CHO (8.1 ± 0.9 mmol/kg dry wt) than in H-CHO (4.4 ± 1.0 mmol/kg dry wt) (P < 0.01) (Fig. 6B). In L-CHO it did not change significantly from rest to exercise, whereas it increased (P < 0.001) ~138% in H-CHO. At 30 and 60 min of exercise, the muscle acetylcarnitine concentration was ~37% lower in L-CHO than in H-CHO (P < 0.01).

The free carnitine concentration in the vastus lateralis muscle at rest was lower in L-CHO (16.1 ± 1.4 mmol/kg dry wt) than in H-CHO (20.2 ± 1.3 mmol/kg dry wt) (P < 0.01) (Fig. 6C). In L-CHO it did not change significantly from rest to exercise, whereas it decreased (P < 0.001) ~46% in H-CHO. The muscle free carnitine concentration was higher in L-CHO than in H-CHO at 30 min (55%, P < 0.001) and 60 min (43%, P < 0.01) of exercise.

DISCUSSION

The intracellular mechanisms regulating the ratio between fat and carbohydrate oxidation in human skeletal muscle during exercise have not yet been fully clarified. A likely site of regulation of fat oxidation is CPT-1 at the entry of LCFA-CoA into mitochondria. The present study is the first to simultaneously measure several factors related to the possible role of malonyl-CoA and carnitine in regulation of fat oxidation at CPT-1 in humans during exercise. Exercise was performed under two conditions at comparable work loads but with marked differences in the relative fat and carbohydrate oxidation rates. The latter was accomplished by manipulating the muscle glycogen content to high (H-CHO) or low (L-CHO) levels before a 60-min bicycle exercise bout at 65% VO2peak.

It was previously suggested that low preexercise muscle glycogen content might be associated with high AMPK activity in rodents during muscle contraction in vitro (8) and in humans during exercise (44). However, in the latter study, circulating epinephrine was also dependent on muscle glycogen content (44), wherefore the possible effect of muscle glycogen on α2-AMPK activity may have been confounded by adrenergic stimulation of α2-AMPK. Adrenergic stimulation of AMPK has been demonstrated in 3T3-L1 adipocytes (47) and has also been reported for rat skeletal muscle (19). In the present study, circulating epinephrine did not differ significantly between conditions, and, still, α2-AMPK activity during exercise was markedly higher in L-CHO than in H-CHO (Table 1). This suggests that altered muscle glycogen alone can influence α2-AMPK activity in human skeletal muscle during exercise independently of adrenergic stimulation, in agreement with studies in rodent muscle (8). On this basis, AMPK could be a likely candidate mediating the effect of muscle glycogen on fat oxidation through regulation of ACC activity and malonyl-CoA formation, as suggested earlier (34, 41). However, the similar ACCβ Ser221 phosphorylation and malonyl-CoA concentration between L-CHO and H-CHO in the present study (Fig. 4) suggest that the effect of muscle glycogen on fat oxidation occurs primarily by other mechanisms in human skeletal muscle during exercise.

AMPK Thr172 phosphorylation closely paralleled α2-AMPK activity in the present study (Table 1), indicating that the markedly higher α2-AMPK activity during exercise in L-CHO than in H-CHO was due to a higher stimulatory effect on AMPK by an upstream AMPK kinase that phosphorylated α2-AMPK on Thr172. However, altered muscle glycogen content did not influence the large increase in ACCβ Ser221 phosphorylation seen with exercise (Fig. 4A), suggesting that allosteric regulation of AMPK, which is not detected in the AMPK activity assay, may have overridden the covalent regulation of AMPK by an upstream AMPK kinase. This is in contrast to a previous study in which α2-AMPK activity and ACCβ Ser221 phosphorylation were closely associated during exercise with low vs. high muscle glycogen (44). The major difference between that study (44) and the present study was that, in the present study, subjects ingested a preexercise meal and had

![Fig. 4. Acetyl-CoA carboxylase (ACC) phosphorylation and malonyl-CoA concentration in the vastus lateralis muscle at rest and during 60 min of bicycle exercise at 65% VO2peak with L-CHO or H-CHO. A: ACCβ Ser221 phosphorylation. Arb units, arbitrary units. #Main effect of exercise, P < 0.001. B: malonyl-CoA concentration. w.w., Wet weight. †Different from rest, P < 0.05.](http://ajpendo.physiology.org/)

![Fig. 5. Pyruvate dehydrogenase activity in the active form (PDHa) in the vastus lateralis muscle at rest and at 60 min of bicycle exercise at 65% VO2peak with L-CHO or H-CHO. *Main effect of condition (L-CHO vs. H-CHO), P < 0.01. #Main effect of exercise, P < 0.001.](http://ajpendo.physiology.org/)
Ser221 phosphorylation. If so, the in vivo dissociation between measured glucose and epinephrine levels, where differences in α2-AMPK activity did not translate into differences in ACC activity (8).

Despite markedly different α2-AMPK activity during exercise between H-CHO and L-CHO (Table 1), ACCβ Ser211 phosphorylation and malonyl-CoA concentration did not differ significantly between L-CHO and H-CHO (Fig. 4). Still, the fat oxidation rate was higher during exercise with low muscle glycogen even though 160% more glucose was infused intravenously under this condition. This suggests that the AMPK-ACC-malonyl-CoA pathway is not likely to mediate the regulation of fat oxidation by muscle glycogen availability in human skeletal muscle during prolonged exercise. This conclusion is also supported by other human exercise models where no association between malonyl-CoA content and fat oxidation was obtained in skeletal muscle during prolonged moderate-intensity exercise (17) or during graded-intensity exercise (7, 18). However, compartmentalization of malonyl-CoA may occur in skeletal muscle, i.e., concentrations of malonyl-CoA in the vicinity of CPT-1 may vary without any detectable changes in the measurement of total muscle malonyl-CoA concentration. In resting human skeletal muscle, changes in malonyl-CoA concentration have occurred with opposite changes in fat oxidation. Thus a significant negative correlation was obtained between muscle malonyl-CoA content and fat oxidation rate in healthy middle-aged men during a two-step euglycemic-hyperinsulinemic clamp (3). Furthermore, in healthy, young individuals, the inhibition of fat oxidation induced by hyperglycemia with hyperinsulinemia occurred together with a threefold increase in muscle malonyl-CoA concentration (27). These results suggest that, in resting human skeletal muscle, the downregulation of fat oxidation by insulin may be mediated by malonyl-CoA.

The absolute increase in fat oxidation rate that occurred from rest to exercise in both conditions in the present study (Fig. 2) was associated with increased ACCβ Ser211 phosphorylation and decreased malonyl-CoA concentration (Fig. 4). This suggests that inactivation of ACCβ by AMPK and the consequent decrease in malonyl-CoA concentration from rest to exercise may have relieved malonyl-CoA inhibition of fat oxidation in both conditions (Fig. 7). There was a tendency (P = 0.10) for malonyl-CoA to decrease more from rest to exercise in L-CHO than in H-CHO. This could have been due to lower availability of cytosolic acetyl-CoA, the precursor of malonyl-CoA, in L-CHO than in H-CHO. Alternatively, the activity of MCD, the enzyme catalyzing the turnover of malonyl-CoA, may have differed between the two conditions. It has been suggested that AMPK phosphorylates and activates MCD during muscle contraction (19, 34). Consequently, the higher α2-AMPK activity in L-CHO than in H-CHO might have induced higher malonyl-CoA turnover in L-CHO via an effect on MCD. The tendency toward a more pronounced decrease in muscle malonyl-CoA

![Fig. 6. Acetyl-CoA, acetylcarnitine, and carnitine concentrations in the vastus lateralis muscle at rest and at 30 and 60 min of bicycle exercise at 65% \( V_{\text{O2 peak}} \) with L-CHO or H-CHO. A: acetyl-CoA, B: acetylcarnitine. C: carnitine. Different from L-CHO, *P < 0.01 and **P < 0.001. Different from rest, #P < 0.05, 1P < 0.01, and 2P < 0.001.](http://ajpendo.physiology.org/)

...as spatial dissociation of AMPK and ACC or phosphatase activity acting on ACCβ Ser211. Those factors may in some cases render ACCβ Ser211 phosphorylation a not-so-optimal predictor of in vivo α2-AMPK activity. Altogether, the present study suggests that during exercise, covalent regulation of α2-AMPK on Thr172 is glycogen dependent, but that ACCβ Ser211 phosphorylation does not parallel α2-AMPK activity when circulating glucose and epinephrine levels are standardized. This is supported by findings in isolated contracting rodent skeletal muscle with high and low muscle glycogen levels, where differences in α2-AMPK activity did not translate into differences in ACC activity (8).
by exercise in L-CHO than in H-CHO may have contributed to the difference between conditions in fat oxidation rate during exercise (Fig. 7). However, the increase in absolute fat oxidation rate by exercise was much more marked in L-CHO than in H-CHO. Therefore, factors other than malonyl-CoA seem to be more important in “fine tuning” the fat oxidation rate during prolonged exercise with different muscle glycogen stores. Availability of carnitine, a necessary substrate for CPT-1, could be one such factor. The importance of carnitine in fat oxidation is evidenced by the fact that, in the pathology of lipid storage myopathy, a marked reduction in skeletal muscle carnitine content by 85% was associated with a 75% reduction in mitochondrial generation of acetyl-CoA and, secondarily, acetyl carnitine. Whole tissue malonyl-CoA levels are reduced by exercise due to increased phosphorylation (inactivation) of ACC by AMP-activated protein kinase (AMPK), which is activated moderately by exercise. The combination of reduced free carnitine content and reduced malonyl-CoA content leads to a moderate increase in fat oxidation rate from rest to exercise. Whole tissue malonyl-CoA levels are reduced by exercise due to increased phosphorylation (inactivation) of ACC by AMPK, which is activated markedly by exercise. The reduction in malonyl-CoA may be slightly more pronounced compared with high-glycogen individuals because of the lower concentration of acetyl-CoA, the source from which malonyl-CoA is formed by ACC. The combination of unchanged free carnitine content and reduced malonyl-CoA content leads to a marked increase in fat oxidation rate from rest to exercise. Muscle concentration of pyruvate was not measured in the present study but was previously shown to increase more from rest to exercise with high than with low muscle glycogen (25). The muscle concentration of citrate, the source of the acetyl-CoA from which malonyl-CoA is generated (35), was not measured in the present study but was previously shown not to differ during exercise with high and low muscle glycogen, although an increase with exercise occurred (11). AcCoA, acetyl-CoA; MaCoA, malonyl-CoA; FACoA, fatty acyl-CoA; CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase-1/2 system; AMPK, 5′-AMP-activated protein kinase; CL, citrate lyase; +, activating effect; −, inhibiting effect.

Fig. 7. Metabolic events that may determine the fat oxidation rate in response to muscle glycogen availability in human skeletal muscle during exercise. A: individuals with high muscle glycogen. Exercise activates glycogenolysis, glycolysis, and PDH, leading to markedly increased mitochondrial generation of acetyl-CoA and, secondarily, acetyl carnitine. The latter results in a decrease in mitochondrial malonyl-CoA and, consequently, cytosolic carnitine. Whole tissue malonyl-CoA levels are reduced by exercise due to increased phosphorylation (inactivation) of ACC by AMPK, which is activated moderately by exercise. The combination of reduced free carnitine content and reduced malonyl-CoA content leads to a moderate increase in fat oxidation rate from rest to exercise. B: individuals with low muscle glycogen. The increases in glycogenolytic and glycolytic rates and PDH activity by exercise are less than in individuals with high glycogen. Consequently, mitochondrial acetyl-CoA and acetyl carnitine generation appears to be lower, and no decrease in whole tissue free carnitine content occurs from rest to exercise. Whole tissue malonyl-CoA levels are reduced by exercise due to increased phosphorylation (inactivation) of ACC by AMPK, which is activated markedly by exercise. The reduction in malonyl-CoA may be slightly more pronounced compared with high-glycogen individuals because of the lower concentration of acetyl-CoA, the source from which malonyl-CoA is formed by ACC. The combination of unchanged free carnitine content and reduced malonyl-CoA content leads to a marked increase in fat oxidation rate from rest to exercise. Muscle concentration of pyruvate was not measured in the present study but was previously shown to increase more from rest to exercise with high than with low muscle glycogen (25). The muscle concentration of citrate, the source of the acetyl-CoA from which malonyl-CoA is generated (35), was not measured in the present study but was previously shown not to differ during exercise with high and low muscle glycogen, although an increase with exercise occurred (11). AcCoA, acetyl-CoA; MaCoA, malonyl-CoA; FACoA, fatty acyl-CoA; CAT, carnitine acetyltransferase; CPT, carnitine palmitoyltransferase-1/2 system; AMPK, 5′-AMP-activated protein kinase; CL, citrate lyase; +, activating effect; −, inhibiting effect.
muscle carnitine content and fat oxidation rate were markedly higher with low muscle glycogen (25). Taken together, the present and previous studies suggest a relation between muscle free carnitine availability and fat oxidation rate in human skeletal muscle during exercise.

Protein oxidation was not measured in the present study, and, consequently, absolute rates of fat and carbohydrate oxidation across the leg were calculated using the nonprotein respiratory quotient (20). Protein oxidation may have differed between L-CHO and H-CHO and, in that case, would have slightly confounded the fat and carbohydrate oxidation rates reported in Fig. 2. However, during moderate-intensity exercise, protein usually covers <5% of oxidative metabolism (22). Therefore, the fact that protein oxidation was ignored in the present study has probably not influenced the results and interpretations to any major extent.

Several potentially confounding blood metabolites and hormones were kept almost similar between L-CHO and H-CHO in the present study due to a light carbohydrate-rich preexercise meal and a variable intravenous glucose infusion during exercise. Thus the higher preexercise muscle glycogen content seems to have been the primary cause of the higher PDH activity seen in H-CHO vs. L-CHO (Fig. 5). In accordance, it has previously been suggested that PDH activity is sensitive to the muscle glycogen level (21, 25). PDH activity seems to be important in limiting glycolysis during exercise (24, 38, 42).

In the present study, the high PDH activity during exercise in H-CHO may have, consequently, enhanced glycolytic flux and production of acetyl-CoA from carbohydrate compared with L-CHO. The accumulation of acetyl-CoA in H-CHO compared with L-CHO most likely decreased the muscle free carnitine content via the carnitine acyltransferase equilibrium (26). If muscle free carnitine is an important regulator of the fat oxidation rate during exercise, then PDH, acetyl-CoA, and acetyl-carnitine could have linked the regulation of fat oxidation to carbohydrate availability and glycolytic flux (Fig. 7). The present findings therefore support the hypothesis that regulation of fat oxidation in response to carbohydrate availability in human skeletal muscle during exercise is at least partly mediated via the availability of free carnitine to CPT-1.

In conclusion, the present study is the first to show a decline in muscle malonyl-CoA concentrations from rest to moderate-intensity exercise in humans, which may contribute to the increase in absolute fat oxidation at the onset of exercise. However, malonyl-CoA does not seem to be a major factor in fine tuning fat oxidation in human skeletal muscle during prolonged exercise with different preexercise muscle glycogen content, since muscle malonyl-CoA concentrations did not depend on muscle glycogen levels despite marked differences in fat oxidation rates between low and high muscle glycogen conditions. On the other hand, the present findings support the assertion that the availability of free carnitine to CPT-1 may participate in regulation of fat oxidation in human skeletal muscle during prolonged moderate-intensity exercise, since muscle carnitine and fat oxidation rate were both lower during exercise with high compared with low glycogen.

ACKNOWLEDGMENTS

We thank Dr. Jens Knudsen, University of Southern Denmark, for kindly donating the purified fatty acid synthase and Dr. D. Grahame Hardie, University of Dundee, Scotland, for kindly donating the α-isotform-specific AMPK antibodies. We are grateful to Dr. Henriette Pilegaard, University of Copenhagen, Denmark, for help on the PDHa assay. We acknowledge the skilled technical assistance of Irene Bech Nielsen, Betina Bolmgren, and Winne Taagerup.

GRANTS

This study was supported by The Danish National Research Foundation (grant no. 504-14), The Copenhagen Muscle Research Centre, The Novo Nordisk Research Foundation, The Danish Diabetes Association, a Research and Technological Development Project (QLG1-CT-2001-01488) funded by the European Commission, The Danish Sports Research Council, The Danish Medical Research Council, and a Hallas Møller Fellowship from The Novo Nordisk Foundation (J. F. P. Wojtaszewski).

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