Substrate kinetics in patients with disorders of skeletal muscle metabolism

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THE 8 ORIGINALS PAPAERS ARE:

I. Ørngreen MC, Olsen DB, Vissing J. Exercise tolerance in carnitine palmitoyltransferase II deficiency with IV and oral glucose. Neurology 2002;59: 1046-1051.

II. Ørngreen MC, Ejstrup R, Vissing J. Effect of diet on exercise tolerance in carnitine palmitoyltransferase II deficiency. Neurology 2003;61(4):559-61.

III. Ørngreen MC, Zacho M, Hebert A, Laub M, Vissing J. Patients with severe muscle wasting are prone to develop hypoglycemia during fasting. Neurology 2003;61(7):997-1000.

IV. Ørngreen MC, Nørgaard MG, Sacchetti M, van Engelen BGM, Vissing J. Fuel utilization in patients with very long-chain acyl-CoA dehydrogenase deficiency. Ann Neurol 2004;56(2):279-283.

V. Ørngreen MC, Dunø M, Ejstrup R, Christensen E, Schwartz M, Sacchetti M, Vissing J. Fuel utilization in subjects with carnitine palmitoyltransferase 2 gene mutations. Ann Neurol 2005;57(1):60-66.

VI. Ørngreen MC, Nørgaard MG, van Engelen BG, Vistisen B, Vissing J. Effects of IV glucose and oral medium-chain triglyceride in patients with VLCAD deficiency. Neurology, 2007;69(3):313-5.

VII. Ørngreen MC, Schelhaas HJ, Jeppesen TD, Akman HO, Wevers RA, Andersen ST, Ter Laak HJ, van Diggelen OP, DiMauro S, Vissing J. Is muscle glycogenolysis impaired in X-linked phosphorylase b kinase deficiency? Neurology, 2008;70(20):1876-82.

VIII. Ørngreen MC, Jeppesen TD, Andersen ST, Taivassalo T, Hauerslev S, Preisler N, Haller RG, van Hall, Vissing J. Fat metabolism during exercise in patients with McArdle disease. Neurology 2009;72(8):718-724.

In the text, these publications are referred to by their Roman numerals.

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INTRODUCTION

The main purpose of the following studies was to investigate pathophysiological mechanisms in fat and carbohydrate metabolism by stabile isotope technique in patients with metabolic myopathies and in patients with severe muscle wasting. Furthermore, the intention was to treat the same defects with potential diets and supplementations.

Metabolic myopathies refer to a group of hereditary muscle disorders caused by specific enzymatic defects due to defective genes. The disorders can be subdivided into two major groups consisting of disorders of glycogen and glucose metabolism and disorders of lipid metabolism (figures 1 and 2).1 Both groups present with the same two main clinical presentations: 1) Acute, recurrent episodes with exercise intolerance, muscle cramps and pain that in severe occasions results in skeletal muscle fibre breakdown (rhabdomyolysis) and myoglobinuria. The metabolic myopathy patients studied were all in this group with dynamic exercise-related symptoms. 2) Static, often progressive muscle weakness, sometimes simulating dystrophic, inflammatory, and neurogenic processes. The above-mentioned clinical presentations are most often seen in adults and older children. Newborns and infants exhibit severe multisystem disorders characterized by episodes of hypoglycaemia, encephalopathy, and sudden death.1

To differentiate between defects in fat and glucose metabolism with dynamic symptoms, it should be determined whether the exercise-related symptoms occur within the first 10 minutes of exercise or after longer duration of exercise. In the beginning of exercise energy production depends on muscle glycogenolysis and glycolysis, and symptoms in that period point towards glycogenoses. In longer duration exercise at low-intensity, energy production depends on fatty acids oxidation and symptoms developing at this state point towards a disorder of lipid metabolism. Symptoms in patients with metabolic myopathies occur primarily during exercise and are caused by a mismatch between energy demands and the energy supply from muscle metabolic pathways that generate ATP. 2 At rest and during dynamic exercise, such as walking, running or cycling, energy primarily comes from ATP synthesized from muscle glycogen, blood glucose and free fatty acids (FFA). 3,4 The types of substrates oxidized depend on intensity and the duration of exercise. 1) At low intensity (<65% of maximal oxygen uptake (VO_2max)), energy primarily comes from fatty acid oxidation.3,4 2) At moderate intensity, ATP is regenerated by high-energy phosphates, followed by muscle glycogen breakdown during the first 5 to 10 minutes of exercise. As an indication of glycogen breakdown, lactate rises sharply in the first 10 minutes of exercise. Lactate levels then drop as muscle triglycerides and blood-borne fuels are used for energy production.5-8 3) During high intensity exercise (> 75% of VO_2max) muscle carbohydrate oxidation is the major fuel source.4 However, carbohydrates from muscle and hepatic glycogenolysis and blood glucose are far more limited than fat and can support high-intensity exercise less than one hour. 3,4 4) During anaerobic exercise, ATP production primarily relies on the ADP phosphorylation by the creatine kinase reaction and anaerobic glycogenolysis. 3 Anaerobic glycolysis plays a smaller role in the total energy production, and is usually only activated during anaerobic conditions such as sustained isometric contraction or high-intensity exercise >90% of VO_2max when blood flow and oxygen delivery to exercising muscle are reduced.1 During fasting periods, energy production relies on gluconeogenic products. Hepatic glycogen stores are depleted after 24 hours of fasting, at which time glucose production is maintained almost exclusively by gluconeogenesis in the liver.6 During the first days of fasting, glucose production is kept relatively high to supply enough glucose to the brain. This phase of fasting has been phrased “the gluconeogenic phase”.3 In this phase, the fraction of gluconeogenesis derived from amino acids is unknown, but the contribution has been estimated to account for about half of the gluconeogenesis in the first 24 hours of fasting.6 The most important amino acid for gluconeogenesis is alanine released by skeletal muscle.6,8 As fasting continues, the brain adapts to combustion of ketone bodies and the relative contribution of muscle protein to gluconeogenesis becomes less than that of glycerol derived from fat combustion. This phase, that starts after four to seven days of fasting, has been phrased “the protein conservation phase”.3

We used nutritional interventions and metabolic studies with stable isotope technique and indirect calorimetry, both at rest and during exercise, in patients with metabolic myopathies to get information of the metabolism of the investigated diseases, and to gain knowledge of the biochemical pathways of intermediary metabolism in human skeletal muscle.9

The following studies emphasize the importance of skeletal muscle in production of energy, both when skeletal muscle lack important metabolic enzymes (metabolic myopathies), and when skeletal muscle is dystrophic and muscle mass is low.

MATERIAL AND METHODS

Patients

In the following, we have studied substrate kinetics in patients with metabolic myopathies, more specifically the glycogenoses; McArdle disease and Phosphorylase b Kinase deficiency and the FAO disorders; Carnitine Palmitoyltransferase (CPT) II deficiency and Very Long-Chain Acyl-CoA Dehydrogenase (VLCAD) deficiency. Furthermore, glucose homeostasis was investigated in patients with low muscle mass caused by Duchenne Muscular Dystrophy (DMD), Spinal Muscular Atrophy (SMA) and Congenital Myopathy (CM).

Disorders of fatty acid oxidation (Figure 1)

Carnitine palmitoyltransferase II deficiency

The mitochondrial carnitine palmitoyl transfereases are required to transport long-chain fatty acids from the cytoplasm into the mitochondria for β-oxidation.10,11 CPT I resides in the outer mitochondrial membrane, and CPT II in the inner mitochondrial membrane. CPT II deficiency is one of the most common inborn errors of fatty acid oxidation (FAO),1 impairing the transport of long-chain fatty acids into the mitochondrial matrix.12 The most common mutation is the (p.Ser113Leu).13 The condition was recognized in 1973 as the first disorder of FAO.14 Mutations in the *CPT2 gene* can result in two phenotypes; a rare lethal neonatal form with seizures, hypoketotic hypoglycemia and hepatomegaly, and a more prevalent adult myopathic form that is the most common cause of rhabdomyolysis and myoglobinuria.1,14-16 The muscular form of the disease is always associated with some residual functional CPT II activity, whereas mutations, which abolish enzyme activity, are associated with the lethal early-onset form. Symptoms are provoked by fasting, prolonged exercise and febrile episodes.7 Disease severity generally correlates with residual CPT II enzyme activity.17,18 In the myopathic type, residual CPT II activity is usually 10-25% of normal.15

Very long-chain acyl-CoA dehydrogenase deficiency

One of the more recently described inborn errors of FAO is very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. VLCAD was identified in 1992 as a key enzyme of mitochondrial long-chain fatty acid β-oxidation.19 Mutations in the *VLCAD gene* causes three phenotypes: (1) a fatal infantile form presenting with hypertrophic cardiomyopathy, hepatocellular disease, and hypoketotic hypoglycemia,20 (2) a childhood onset and milder form with Reye-like disease and hypoketotic hypoglycemia,20 and (3) a juvenile- or adult onset myopathic form characterized by muscle pain, rhabdomyolysis, and myoglobinuria often associated with prolonged exercise or fasting.21-23 As in CPT II deficiency genotype correlate with the clinical phenotype.24

The first myopathic cases of VLCAD deficiency were reported in 1993. 21,25 Less than 200 patients have been described so far.

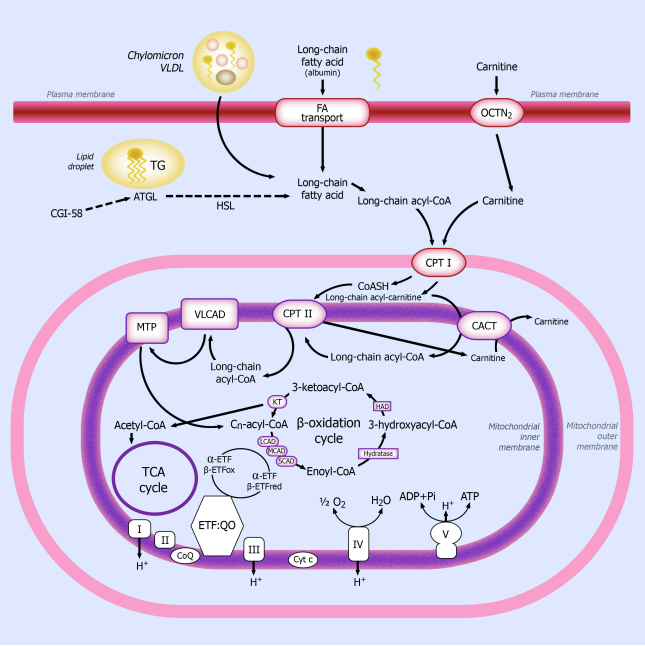

Figure 1. Fatty acid oxidation pathway. ATGL, adipose triglyceride lipase; ATP, adenosine-5'-triphosphate; CACT, carnitine/acylcarnitine translocase; CGI-58, comparative gene identification-58; CoA, coenzyme A; CoASH, coenzyme A; CoQ, coenzyme Q; CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; Cyt c, cytochrome c oxidase; ETF, electron-transfer flavoprotein; ETF-QO, ETF coenzyme Q oxidoreductase; HAD, L-3-hydroxyacyl-CoA dehydrogenase; HSL, hormone-sensitive lipase; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, mitochondrial trifunctional protein; OCTN2, sodium-dependent carnitine transporter; SCAD, short-chain acyl-CoA dehydrogenase; TCA, tricarboxylic acid; VLCAD, very long-chain acyl-CoA dehydrogenase; VLDL, very-low-density lipoprotein; I, respiratory chain complex I; II, respiratory chain complex II; III, respiratory complex III; IV, respiratory complex IV; respiratory chain complex V.

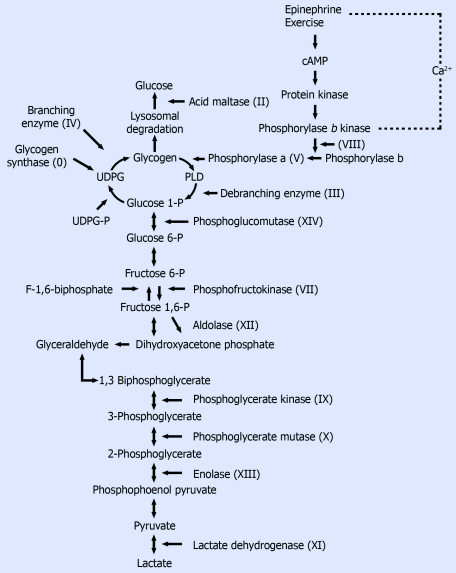

Figure 2. Glycogenolytic pathway. Roman numerals refer to the muscle glycogenoses caused by the enzymes denoted: Acidmaltase (II), debrancher (III), brancher (IV), phosphorylase (V), phosphofructokinase (VII), phosphorylase b kinase (VIII), phosphoglycerate kinase (IX), phosphoglycerate mutase (X), lactate dehydrogenase (XI), aldolase (XII), enolase (XIII), phosphoglucomutase (XIV).

Glycogenoses (Figure 2)

McArdle disease

Patients with myophosphorylase deficiency (McArdle disease) have a block of muscle glycogen breakdown due to mutations in the PYGM gene on chromosome 11 that encodes muscle glycogen phosphorylase.26 The disease is autosomal recessively inherited, and nearly all genotypes cause complete enzyme deficiency. This severely limits anaerobic and aerobic exercise capacity,27 and the maximal O_2 uptake is between 35 and 50% of normal in patients with McArdle disease.28 The patients experience exercise intolerance, muscle fatigue and exercise-induced muscle contractures, muscle pain, rhabdomyolysis and myoglobinuria, which can lead to acute renal failure.27 Patients with McArdle disease develop a pathonomonic phenomenon after the first 5-8 minutes of exercise called the second wind. In the first minutes of exercise McArdle patients have an energy crisis due to the blocked muscle glycogenolysis and low availability of extra-muscular fuels.29

Interestingly, a small amount of residual myophosphorylase activity almost normalizes the oxidative capacity and virtually eliminates the spontaneous second wind.30

Phosphorylase b kinase deficiency

Phosphorylase b kinase (PHK) deficiency is a rare glycogen storage disease (GSD type VIII), which impairs glycogen breakdown by decreasing the phosphorylation of glycogen phosphorylase from the inactive (b) to the active (a) form. The functional PHK molecule is a polymer with four subunits: α, β, γ and δ. Subunits α and β regulate phosphorylation, γ is the catalytic subunit, and δ is identical to calmodulin and confers Ca2+ sensitivity to the enzyme.27,31 For the α and γ subunits, tissue-specific isoforms are known for muscle (αM, γM), liver (αL) and testis (γT).32,33 The inheritance is X-linked for the α subunit,33, 34 and autosomal for β, γ and δ subunits.34

Beside the one patient included in this thesis, only six patients with mutations in the gene encoding the X-linked muscle-specific a subunit (PHKA1) have been reported.35-40 Since the biochemical defect of PHK deficiency affects the function of myophosphorylase, patients with PHK deficiency are expected to clinically mimic patients with McArdle disease. In agreement with this, the symptoms of exercise intolerance, myoglobinuria and exercise-induced muscle contractures and pain reported in patients with PHK deficiency resemble those observed in McArdle disease, although they seem to be less severe.

Patients with low muscle mass

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked recessively inherited disease caused by mutations of the dystrophin gene leading to loss of dystrophin.41 DMD affects approximately 1:3,500 boys, and each year around 20,000 children are born with

DMD worldwide. DMD was first described in the 19th century, and debuts with a progressive proximal muscular dystrophy with characteristic pseudohypertrophy of the calves before the age of 3.41 In the beginning of their teens, patients become wheel chair bound. Cardiac and smooth muscle is affected as well, and results in cardiomyopathy and respiratory involvement.41

Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessively disease characterized by degeneration of the anterior horn cells of the spinal cord, leading to symmetric muscle weakness and atrophy.42

SMA is the second most common lethal, autosomal disease in Caucasians after cystic fibrosis.42 SMA type II is an intermediary form of SMA with onset within the first years of living and survival until adolescent or later.42

Congenital myopathy

The patients in our study were originally diagnosed with central core disease, which is a subtype of congenital myopathy (CM). Further genetic analysis, however, disclosed that one of the patients was affected by Selenoprotein deficiency caused by mutation in SEPN1, formerly known as Rigid spine syndrome.43 Rigid spine syndrom is characterized by contractures in spinal extensors causing limitations in movement of the spine and thoracic cage and patients often develop musculoskeletal deformities such as scoliosis resulting in respiratory difficulties and need for respiratory support.43

Design

Study I, II and V.

We investigated glucose and fat metabolism in four patients homozygous and three subjects heterozygous for mutations in the *CPT2 gene*, and compared results with those found in five healthy controls. Furthermore, fuel supplementation studies were performed in five patients with CPT II deficiency.

Study III.

The purpose of the study was to investigate the effect of prolonged fasting in patients with low muscle mass. We investigated the effect of 23 hours of fasting on glucose-turnover by stabile isotope technique and protein breakdown as indicated by plasma alanine and neurohormonal changes in 13 patients with low muscle mass: 2 CM, 7 DMD and 4 SMA II patients. The muscle wasting was so severe that patients were wheelchair-bound and required respiratory support.

Study IV and VI.

In study IV, FAO was investigated by indirect calorimetry and use of stable isotope technique in two Dutch patients with genetically and biochemical well-defined VLCAD deficiency and five healthy subjects. Furthermore, we investigated whether these patients could benefit from different fuel supplementation.

Study VII.

We investigated muscle metabolism in a PHK-deficient patient and compared the results with findings in healthy controls and patients with McArdle disease. We used a forearm ischemic handgrip test to study lactate and ammonia production, and cycle ergometry to determine maximal oxidative capacity, fat metabolism and a potential presence of a second wind phenomenon. Furthermore, we investigated the effect of IV glucose on exercise tolerance.

Study VIII.

We investigated substrate kinetics of glucose and palmitate by indirect calorimetry and use of stable isotope technique in eleven patients with myophosphorylase deficiency.

Exercise physiologic testing

The exercise physiologic tests were carried out to 1) test the maximal oxygen uptake and 2) investigate systemic metabolism.

Pre-experimental preparations

In all studies (I-VIII, except II) subjects were instructed to follow a standardized carbohydrate-rich diet three days prior to the experiment containing 65% carbohydrate, 15% protein and 20% fat.44 To determine the workload for the experiments (study I, II, IV-VIII), all subjects completed a maximal incremental exercise test. In the maximal incremental test, workload was increased every other minute until exhaustion. To ensure the correct workload for the test day the patients completed a 20 minutes constant workload test at the desired intensity after one hour of rest from the maximal test.

Experimental settings for cycle ergometry studies to investigate systemic responses

Experiments started around 9.00 a.m. Subjects had a meal two hours before exercise testing (IV, V, VII, VIII) or fasted from midnight (I, II, III and VI). Two catheters were inserted in arm veins, one in the cubital vein for infusion and one in the distal cephalic vein for blood sampling. A heating pad wrapped around the hand was used to arterialize the venous blood samples.45

In all studies, except study III, subjects were studied on a cycle ergometer (MedGraphics CPE2000, St. Paul, MN), a Tunturi cycle ergometer or a Sahva recumbent bike. Heart rate and the level of perceived exertion (Borg Scale)46 were monitored every other minute during exercise. Gas exchanges were measured by Med Graphics CPX/D cardiopulmonary exercise test system or a gas and airflow analyzer (Quark b2, Cosmed, Italy).

Stable isotopes

Stable isotopes were used as tracers to investigate carbohydrate and fat metabolism. A tracer is a compound that is chemically and functionally identical to the naturally occurring compound (the tracee), but is distinct in the number of neutrons, which enables detection. By following the fate of a tracer in the body, information can be obtained regarding the metabolism of the tracee.47 For example, carbon-12, carbon-13 and carbon-14 are three isotopes of the carbon atom with mass numbers 12, 13 and 14, respectively. The 12, 13 and 14 refer to carbon atoms of atomic masses. The atomic number of carbon is 6, which means that every carbon atom has 6 protons, so that the neutron numbers of these isotopes are 6, 7 and 8, respectively. Stable isotopes can be used as tracers. The most commonly used tracers are the 13C, 2H, 15N and 18O.47 A stable isotope is non-radioactive,

totally harmless and can be infused without any health risks in humans. 12C is the most abundant isotope and represents 98.89% of the total carbon, whereas 13C only represents 1.11% and therefore is useful as a naturally occurring stable isotope. Corn has a different number for carbon isotopes ratios than most plants, with a higher share of 13C. Therefore, it is important to instruct subjects not to ingest any sort of corn products the days before experiments using 13C tracer dilution technique.

A primed, constant rate infusion of [U-13C]-palmitate (0.0026 mg kg-1 min-1) and [6,6-2H2]-glucose (0.73 µmol min-1 kg-1), primed by a 0.085 mg kg-1 NaH13CO3 and 17 µmol/kg [6,6-2H2]-glucose) was delivered by a Gemini PC2 pump (IMED, San Diego, CA). Two hours after the start of the isotope infusion, subjects cycled for 40 min. at a workload of 50-60% of VO_2max as determined beforehand. In study VIII, control subjects exercised at the same absolute workload performed by the patients with whom they were matched. In study I, II, IV and V, control subjects exercised at the same relative workload (50-60% of VO_2max). Blood and expired gases for isotope measurements were collected at 10 min intervals at rest and during exercise. Expired air was collected in a 15 L Douglas bag, and 10 ml air samples from the bags were injected into vacutainer tubes for analyses of $13CO_2$ enrichment.

When measuring palmitate oxidation by quantifying the rate of excretion of 13C-labeled CO_2 and concentration of 13C-labeled plasma palmitate, as we have done in these studies, a proportion of the labeled CO_2 that is produced via oxidation, will not be excreted in blood or breath, but will be trapped in the tissues. Furthermore, some of the labeled palmitate will be fixed in other metabolites via isotopic exchange reactions, mainly in the TCA cycle.48 Therefore, there is a need to correct oxidation rates for the "loss" of tracer. This correction factor can be measured via an acetate correction trial.48 On a separate day, two of the McArdle patients and two of the healthy subjects completed such an acetate correction trial. The study protocol was identical to the one described above, except the infused tracer was [1,2-C13]-acetate. The purpose of this study was to measure an acetate correction factor specific for our McArdle patients and healthy controls. The measured values for the acetate correction factor were similar both between the two patients with McArdle disease and between groups. At rest and during exercise, the acetate correction factor was only slightly higher in the patients with McArdle disease compared with the healthy controls (rest: 30% vs. 20%; exercise: 80% vs. 75%). Thus, acetate correction factors were collected from the literature in all the other studies of fat metabolism.49

Preparation of tracers

The glucose, acetate and bicarbonate tracers (99% enriched; Cambridge Isotope Laboratories, MA, USA) were dissolved in 0.9% saline. The palmitate tracer (98% enriched; Cambridge Isotope Laboratories, MA, USA) was dissolved in heated, sterile water and passed through a 0.2 µm filter and mixed with 5% human serum albumin.

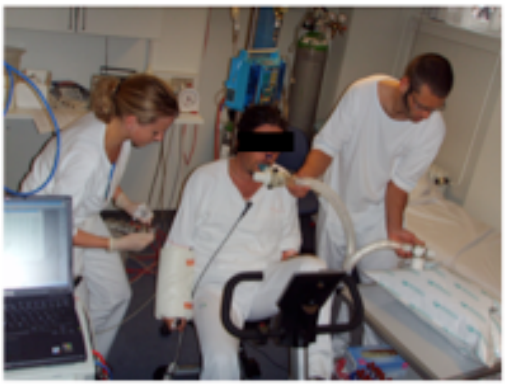

Figure 3. Illustration of a stable isotope experiment.

Calculations

Whole body glucose and palmitate rates of appearance (Ra) (µmol min^{-1}) and rates of disappearance (Rd) (µmol min^{-1}) were calculated using the non-steady-state equations of Steele adapted for stable isotopes.17,18

$$R_a = \frac{F - V[(C_2 + C_1)/2][(E_2 - E_1)/(t_2 - t_1)]}{(E_2 + E_1)/2}$$

$$R_d = R_a - pV[(C_2 - C_1)/(t_2 - t_1)]$$

where F is the infusion rate (µmol kg^{-1} min^{-1}), V= 0.04L/kg for palmitate and V=0.145 L/kg for glucose is the distribution volume for the tracers, C_1 and C_2 are the tracee concentrations (mmol l^{-1}) at times 1(t_1) and 2(t_2). E_1 and E_2 are the plasma enrichments at t_1 and t_2.

Breath enrichments are expressed as the tracer/tracee ratio (TTR):

$$TTR_{breath} = (^{13}C/^{12}C)_{sa} - (^{13}C/^{12}C)_{bk}$$

where sa = sample and bk= background value.

Whole body plasma palmitate oxidation was calculated by considering pulmonary $13CO_2$ production and applying an acetate correction factor (rest=20, exercise=75) determined under similar exercise intensities.19,20

Palmitate oxidation (µmol min^{-1}) was calculated as:

$$Rox = \frac{(VCO_2 \times TTR_{breath}/16)}{(TTR_{palmitate} \times \text{acetate correction factor}/100)}$$

Whole body calculations:
Indirect calorimetry:

Whole body carbohydrate oxidation
=
$4.585 \times VCO_2 - 3.226 \times VO_2 \times 17.5$

Whole body fatty acid oxidation
=
$1.695 \times VO_2 - 1.701 \times VCO_2 \times 39$

Where, VO_2 = oxygen uptake (µmol min^{-1}), VCO_2= expired carbon dioxide (µmol min^{-1})

Respiratory exchange rate (RER)
=
VCO_2/VO_2

RER increases progressively from ~0.7 at rest to 1.0 with maximal exercise, indicating a shift in oxidative substrate from primarily lipid to virtually total carbohydrate.50

Handgrip

A catheter in the cubital vein provided blood for the analyses of lactate and ammonia concentrations. The subjects squeezed the handgrip dynamometer at intended maximal voluntary contraction force during each contraction, alternating between contractions for 1 second and resting for 1 second. The exercise lasted 1 minute and was performed while the blood circulation was blocked by inflating a blood pressure cuff on the upper arm to 250 mmHg. The cuff was released immediately after exercise. Blood samples were drawn at rest, after 1, 2 and 5 min of handgrip-exercise.

Statistics

Values are mean ± SE. A p value < 0.05 (two-tailed testing) was considered significant. Differences between patients and healthy controls were assessed by paired and unpaired Student's t-test. Due to the small number of carriers of a single *CPT2 gene* mutation and the patient with Phosphorylase b kinase deficiency, statistical testing was not performed. Changes and differences are only referred to as such when a consistent change was present in all three subjects with single *CPT2 gene* mutations or if the PHK patient was outside reference interval from the healthy controls.

RESULTS

Disorders of lipid metabolism in skeletal msucle (I, II, IV, V, and VI)

Substrate kinetics (IV, V). The rather low residual enzyme activity in patients with CPT II deficiency and VLCAD deficiency ensures a normal FAO at rest, but FAO is severely impaired during prolonged, low-intensity exercise at 50% of VO_{2max}, figure 4. Subjects carrying a single mutation in the *CPT2 gene* had an intermediate increase in palmitate oxidation during exercise, and two of the single *CPT2 gene* mutation carriers, who displayed symptoms of CPT II deficiency, had a FAO comparable to the patients, figure 4.

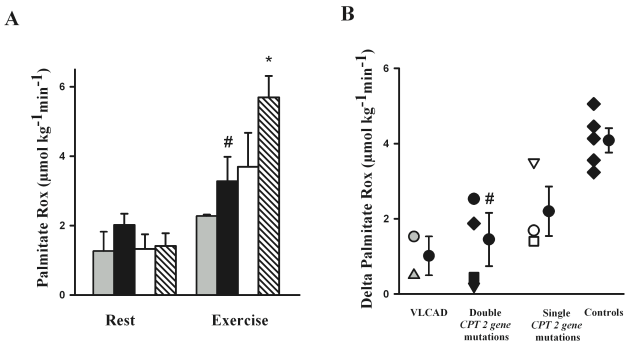

Figure 4. Palmitate oxidation at rest and during exercise at 50% of VO_{2max} (A), and delta palmitate oxidation (exercise–rest) (B) in two patients with VLCAD deficiency (grey), four patients with double *CPT2 gene* mutations (black), three subjects with a single *CPT2 gene* mutation (white), and five healthy controls (shaded). Filled circle with error bars indicate the mean value±SE for each group.
*Different from rest, P<0.05.
#Different from healthy, P<0.05.

Glucose disposal did not change significantly during exercise in healthy controls or patients with CPT II or VLCAD deficiencies, table 1. However, total carbohydrate oxidation (CHO) increased in all groups, indicating exercise-induced muscle glycogenolysis, table 1.

	VLCAD (rest)	VLCAD (exercise)	Double *CPT 2 gene* mutations (rest)	Double *CPT 2 gene* mutations (exercise)	Single *CPT 2 gene* mutations (rest)	Single *CPT 2 gene* mutations (exercise)	Control (rest)	Control (exercise)
Total CHO (mmol min^{-1})	0.2±0.1	8.7±0.4	1.2±0.2	8.6±0.9*	0.8±0.1	6.3±0.6§	1.2±0.3	7.9±1.6*
Total FAO (µmol kg^{-1} min^{-1})	6.7±3.2	6.0±0.9	6.0±1.3	8.0±1.9	3.8±1.0	15.5±1.7	5.7±0.9	20.1±2.0
$Rd_{Glucose}$ (µmol kg^{-1} min^{-1})	11±3	15±4	26±6	26±4	24±5	28±3	23±2	30±4
$Ra_{Palmitate}$ (µmol kg^{-1} min^{-1})	3.7±0.6	3.7±0.4	1.9±0.2	4.0±0.4*	2.0±0.8	4.0±0.7§	2.6±0.3	5.7±0.9*
Plasma palmitate (µmol L^{-1})	224±64	331±88	102±5	177±6*	108±73	253±58	162±37	278±38*
Plasma FFA (µmol L^{-1})	591	777±15	371±58	542±70*	302±95	624±85§	487±63	786±91*
Plasma glycerol (µmol L^{-1})	89±7	140±17	70±7	166±27*	83±28	194±23§	84±18	257±46*
Plasma alanine (µmol L^{-1})	369±17	442±29	308±32	366±84¶	336±26	390±33§	391±31	398±20
Plasma glucose (mmol L^{-1})	5.1±0.1	4.7±0.2	5.2±0.3	4.9±0.2	5.1±0.4	5.0±0.3	4.9±0.3	4.9±0.3
Plasma lactate (mmol L^{-1})	0.9±0.1	1.5±0.0	0.8±0.1	2.4±1.0*	1.1±0.1	1.4±0.1§	1.2±0.1	2.1±0.5*
Plasma norepinephrine (nmol L^{-1})	3.0±0.2	3.5±0.5	2.0±0.3	3.1±0.4¶	1.4±0.2	3.7±1.3§	2.7±0.4	7.9±1.5
Plasma insulin (pmol L^{-1})	36±4	21±1	76±18	39±5¶	97±15	26±8§	42±7	20±2*

Table 1. Indirect calorimetry, substrate turnover, plasma metabolites and hormones in two patients with VLCAD deficiency, four patients compound heterozygous for CPT 2 gene mutations, three carriers of single CPT 2 gene mutations and five healthy control subjects at rest and at the end of the exercise period.
Values are mean ± SE. * Different from rest, P<0.05. § Different from rest in all three subjects heterozygous for the CPT 2 gene mutation. ¶ Different from healthy, P<0.05.
CHO=carbohydrate oxidation; FAO=fatty acid oxidation; RdGlucose = rate of disappearance (glucose utilization); RaPalmitate = rate of appearance palmitate (palmitate production); FFA=free fatty acids. Number of observations for RaGlucose was two for the VLCAD deficiency group and three for the other groups.

In line with the impaired FAO in the CPT II and VLCAD patients, RER increased similarly with that of the healthy controls, but did not lower with time as in healthy subjects indicating that the patients depended on carbohydrate oxidation, figure 5.

Hormones and metabolites, table 1. Exercise-induced increases in plasma free fatty acid (FFA) and plasma glycerol, were blunted in VLCAD patients, whereas responses of palmitate, lactate, glucose, and insulin were similar between healthy controls and patients with VLCAD deficiency and CPT II deficiency.

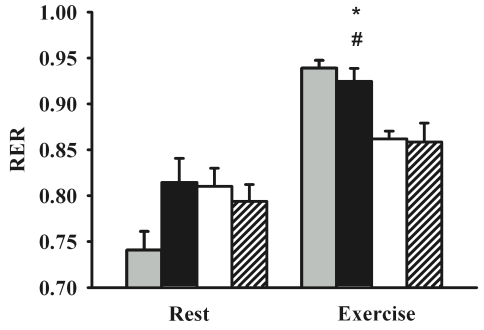

Figure 5. Respiratory exchange rate (RER) at rest and during exercise at 50% of VO_{2max} in two patients with VLCAD deficiency (grey), four patients with double *CPT2 gene* mutations (black), three subjects with a single *CPT2 gene* mutation (white), and five healthy controls (shaded).
*Different from rest, P<0.05.
#Different from healthy, P<0.05.

Fuel supplements (I, II, VI). After a seven-day high carbohydrate diet, five CPT II patients improved exercise tolerance as indicated by lowering of heart rate and Borg score. Furthermore, four of these patients improved exercise tolerance and cycled for a longer time with IV glucose. However, a supplement of oral glucose consisting of 25 grams of glucose ten minutes before exercise had no beneficial effect and worsened exercise tolerance with higher heart rate and shorter exercise duration in the same patients with CPT II deficiency, figure 6.

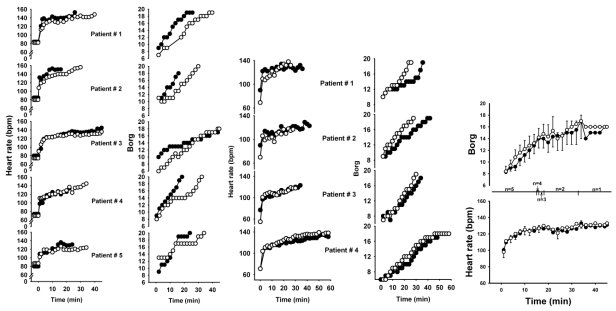

Figure 6. Treatment of patients with double *CPT 2 gene* mutations. A. Heart rate and score of perceived exertion (Borg) with IV glucose (o) vs. IV placebo (•) in five patients with CPT II deficiency during constant workload exercise of 50% of VO_{2max} to exhaustion. B. Heart rate and score of perceived exertion (Borg) after seven days carbohydrate rich diet (•) vs. seven days fat rich diet (o) in four patients with CPT II deficiency during constant workload exercise of 50% of VO_{2max} to exhaustion. C. Heart rate and score of perceived exertion (Borg) with oral glucose (o) vs. oral placebo (•) in five patients with CPT II deficiency during constant workload exercise of 50% of VO_{2max} to exhaustion.

We found no effect on exercise tolerance of oral medium chain triglyceride emulsion supplementation or IV glucose infusion in the patients with VLCAD deficiency, figure 7.

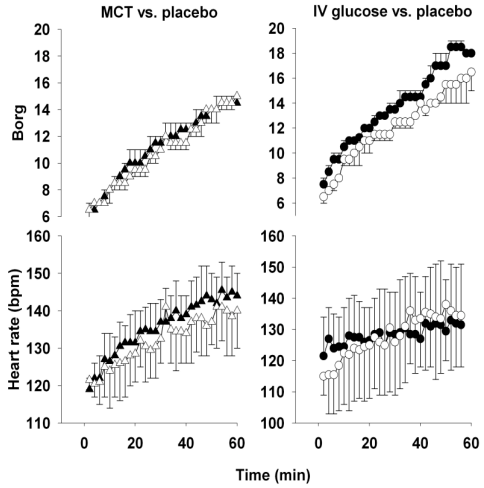

Figure 7. Heart rate and score of perceived exertion (Borg) with oral medium chain triglyceride (△) vs. placebo (▲) and IV glucose (○) vs. placebo (●) in two patients with VLCAD deficiency at rest and during constant workload exercise at 50% of VO_{2max}.

Glycogenoses (VII, VIII)

Heart rate and rate of perceived exertion (Borg score). The second wind phenomenon was found in all patients with McArdle disease and in none of the healthy subjects or the patient with PHK deficiency, figure 8.

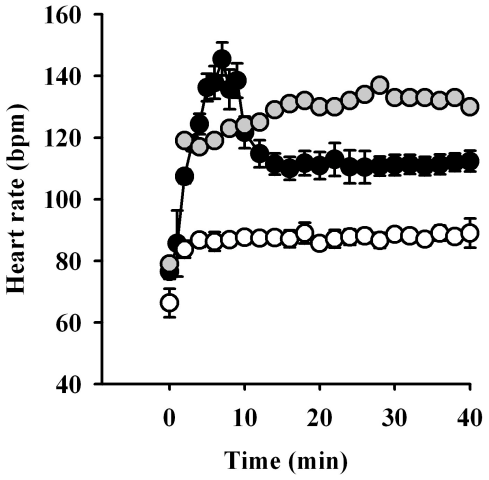

Figure 8. Heart rate at rest and during exercise illustrating the second wind phenomenon in 11 patients with McArdle disease (●) compared to 11 healthy controls (○) and one patient with PHK deficiency (●).

Substrate kinetics (VII, VIII). Systemic substrate turnover showed, that palmitate utilization, oxidation and plasma concentrations increased to a greater extent in patients with McArdle disease vs. healthy subjects, figure 9.

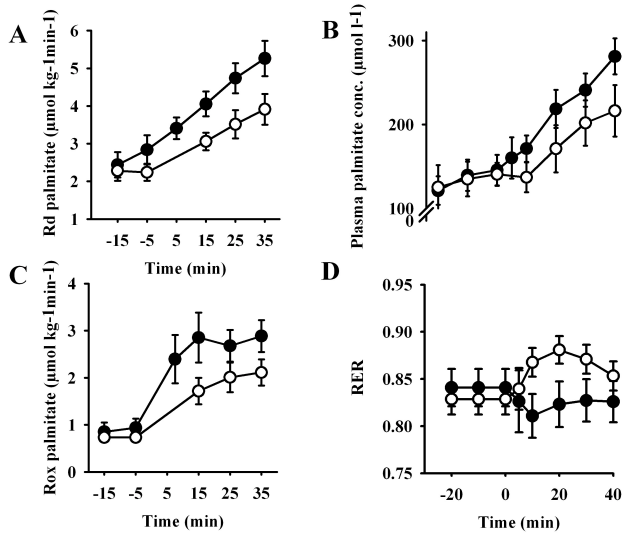

Figure 9. A. Palmitate rate of disappearance (Rd), B. Plasma palmitate concentration, C. Palmitate oxidation (Rox), and D. Respiratory exchange rate (RER) at rest and during exercise at 50-60% of VO_{2max} in 11 patients with McArdle disease (●) and 11 healthy controls (○). Healthy controls were exercising at the same absolute workload as the McArdle patients.

Similarly, substrate turnover studies in the PHK patient showed that palmitate lipolysis, utilization and plasma concentration was higher and total CHO lower in the PHK patient during exercise vs. healthy subjects (figure 10 and table 3).

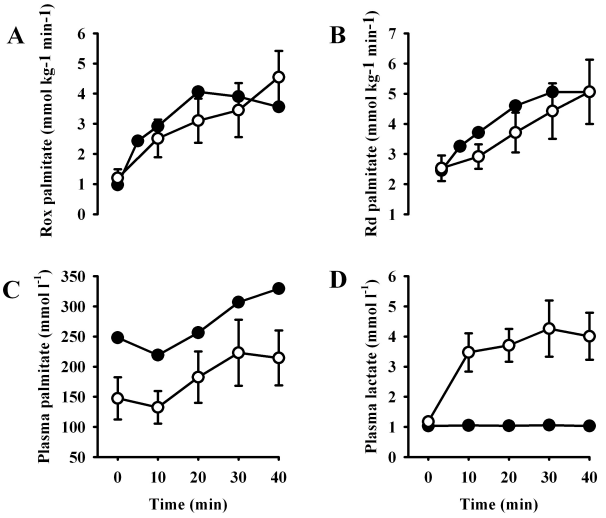

Figure 10. A. Palmitate oxidation (Rox), B. Palmitate rate of disappearance (Rd), C. Plasma palmitate concentration, and D. Plasma lactate at rest and during exercise at 60% of VO_{2max} in one patient with PHK deficiency (●) and 9 healthy subjects (o).

	McArdle (rest)	McArdle (exercise)	Control (rest)	Control (exercise)
VE/VO_2	29.6±1.6*	23.5±1.0*¶	29.2±2.4	24.7±1.4¶
Total CHO (mmol min^{-1})	1.3±0.3	3.2±0.6	1.0±0.2	3.7±0.5
Total FAO (µmol kg^{-1} min^{-1})	4.2±0.7	15.3±2.4	3.7±0.4	9.0±1.0
$Ra_{Palmitate}$ (µmol kg^{-1} min^{-1})	2.9±0.4	4.9±0.4	2.3±0.3	3.8±0.4
Plasma FFA (µmol L^{-1})	454±97	696±51¶	367±59	466±46
Plasma glycerol (µmol L^{-1})	178±30	260±20*	127±14	160±10
Plasma alanine (µmol L^{-1})	419±62*	357±22*	259±30	236±11
Plasma glucose (mmol L^{-1})	5.74±0.09	5.08±12¶	5.47±0.15	5.18±0.08¶
Plasma lactate (mmol L^{-1})	0.94±0.06	0.72±0.03¶	0.87±0.03	0.76±0.03¶
Plasma epinephrine (pmol L^{-1})	34±18	133±31¶	46±22	80±28
Plasma insulin (nmol L^{-1})	84±22	29±5¶	32±8	24±4

Table 2. Mean oxygen consumption, mean plasma metabolites and mean plasma hormones in 11 patients with McArdle disease and 11 healthy subjects at rest and during exercise at 50-60% of VO_{2max}. Healthy controls were exercising at the same absolute workload as the McArdle patients.
Values are mean ± SE. Exercise refers to mean values of the last 20 minutes of exercise, except for the results for plasma epinephrine and plasma insulin, where samples were taken at the end of exercise. N=6 patients with McArdle disease and 7 healthy subjects for plasma glycerol and alanine.
*Different from healthy, P<0.05.
¶Different from rest, P<0.05.
VE=ventilation exchange; VO_2=oxygen consumption; CHO=carbohydrate oxidation; FAO=fatty acid oxidation; RaPalmitate = rate of appearance palmitate (palmitate production); FFA=free fatty acids.

In line with this, respiratory exchange rate did not change with exercise, indicating that energy during exercise at 50-60% of VO_{2max} primarily comes from FAO in patients with McArdle disease. We found augmented fat oxidation with the onset of a second wind, but further increases in FFA availability, as exercise continued did not result in further increases in FAO, figure 11 and table 2.

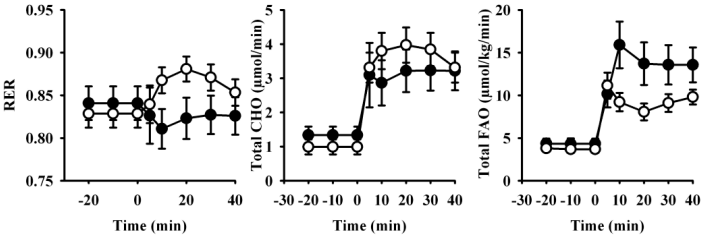

Figure 11. Indirect calorimetry results: Total fatty acid oxidation (FAO), total carbohydrate oxidation (CHO) and respiratory exchange ratio (RER) at rest and during exercise at 50-60% of VO_{2max} in ten patients with McArdle disease (●) and eleven healthy controls (○). Healthy controls were exercising at the same absolute workload as the McArdle patients.

	PHK (rest)	PHK (exercise)	Control (rest)	Control (exercise)
RER	0.68	0.92±0.01	0.84±0.05	0.87±0.01
RF	23	32±3	13±3	28±1
VE/VO_2	29	29±1	29±1	23±0
Total CHO ($mmol\ min^{-1}$)	0.1	8.1	1.4±0.5	10.7±2.1
Total FAO ($\mu mol\ kg^{-1}\ min^{-1}$)	6.5	8.4	2.1±0.7	21.6±8.6
$Ra_{Palmitate}$ ($\mu mol\ kg^{-1}\ min^{-1}$)	2.5	5.0±0.1	2.6±0.4	4.3±1.0
Plasma FFA ($\mu mol\ L^{-1}$)	496	655±69	375±86	482±57
Plasma glycerol ($\mu mol\ L^{-1}$)	610	782±27	113±19	227±25
Plasma alanine ($\mu mol\ L^{-1}$)	340	357±6	384±27	495±16
Plasma glucose ($mmol\ L^{-1}$)	6.3	5.6±0.4	6.5±0.1	5.6±0.1
Plasma epinephrine ($pmol\ L^{-1}$)	78	382	47±3	301±110
Plasma insulin ($nmol\ L^{-1}$)	99	35	33±10	17±7

Table 3. Oxygen consumption, plasma metabolites and plasma hormones in one patient with PHK deficiency and 9 healthy subjects at rest and during exercise at 60% of VO_{2max}.
Values are mean ± SE. Exercise refers to mean values of the last 20 minutes of exercise, except for the results for plasma epinephrine and plasma insulin, where samples were taken at the end of exercise.
RER=respiratory exchange rate; RF=respiratory frequency; VE=ventilation exchange; VO_2=oxygen consumption; CHO=carbohydrate oxidation; FAO=fatty acid oxidation; RaPalmitate = rate of appearance palmitate (palmitate production); FFA=free fatty acids.

Metabolites (VII). During forearm ischemic exercise the patient with PHK deficiency did not develop muscle contractures, and there was a normal increase of plasma lactate and ammonia, figure 12. However, plasma lactate did not change during dynamic, sub-maximal exercise in contrast to the 4-fold increase in healthy subjects, figure 10D.

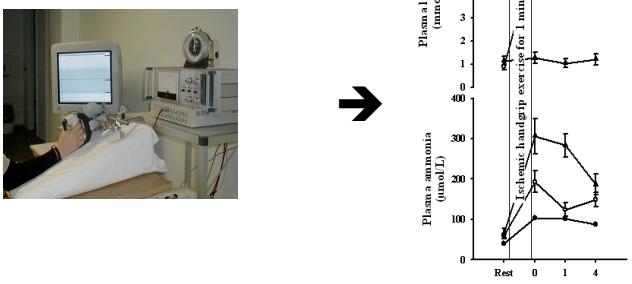

Figure 12. Plasma lactate and ammonia concentrations before and after ischemic handgrip exercise in the patient with PHK deficiency (●), seven patients wtih McArdle disease (○) and nine healthy subjects (▲).

Fuel supplements (VII). IV glucose administration appeared to improve exercise tolerance in the patient with PHK deficiency, but not to the same extent as in McArdle patients, figure 13.

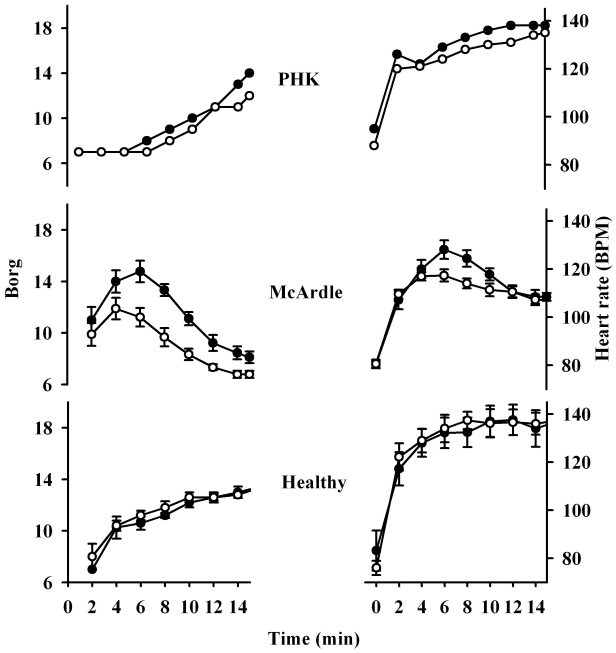

Figure 13. Perceived exertion (Borg scale) and heart rate during exercise at 60% of VO_{2max} with IV glucose (o) or saline (•) in the patient with PHK deficiency, seven patients with McArdle disease, and five healthy subjects.

Genetics and muscle glycogen. Sequencing of PHKA1 showed a novel pathogenic mutation (c.831G>A) in exon 7. In the patient with PHK deficiency, serum creatine kinase levels were mildly elevated on several occasions (400U/l; normally <200U/l). Furthermore, muscle glycogen content was elevated (231µg/mg protein; normal range 30-180).

Patients with low muscle mass

Muscle mass. Muscle mass as percentage of body weight was 4±1% (DMD), 6±1% (spinal muscular atrophy (SMAII)), 9±1% (congenital myopathy (CM)) and 37±3% (healthy).

Metabolites and substrate kinetics.

All SMA and CM patients, and one DMD patient, but no healthy subjects developed hypoglycaemia during fasting.

The gluconeogenic amino acid, alanine, and total glucose production was consistently lowest in patients who developed hypoglycaemia, and during fasting the glucose production fell below the estimated utilization of glucose by the brain in these patients, figure 14.

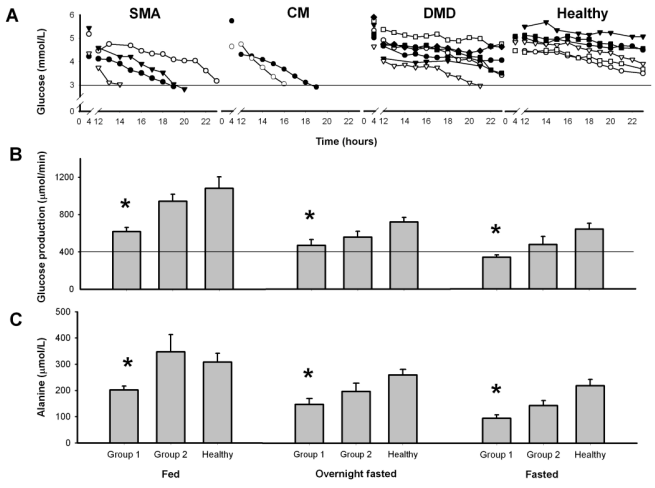

Figure 14. Part A shows individual plasma glucose concentrations during 23 hours of fasting in thirteen patients with muscle wasting due to neuromuscular diseases and in six healthy subjects. Symbols of the subjects correspond to those provided in table 1. Part B shows total hepatic glucose production and part C shows plasma alanine concentration in neuromuscular patients with muscular atrophy who developed hypoglycemia during fasting (Group 1, n = 7 persons), and those who did not (Group 2, n = 6) and in six healthy subjects (Healthy). Values in the fed state were evaluated 4 hours after the last meal, values at overnight fasted were evaluated 12 hours after the last meal and values for the fasted state were obtained at the end of the study. The horizontal line in figure B depicts estimated total glucose utilization of the brain. Results are means ± SE. * Group 1 different from healthy (P<0.05). SMA = spinal muscular atrophy; CM = congenital myopathy and DMD = Duchenne muscular dystrophy.

DISCUSSION AND CONCLUSION

Skeletal muscle has an important role in metabolism. The presented series of scientific experiments have attempted to clarify some of the pathophysiological mechanisms underlying disorders of skeletal muscle metabolism and attempted to develop treatment for the related symptoms in the patients. Furthermore, our results have contributed to the understanding of skeletal muscle metabolism in vivo. Glucose and fat homeostasis will be discussed in separate sections, and results from the above-mentioned studies under these headings.

Glucose homeostasis.

Lipid metabolism disorders.

The blunted increase of fat oxidation and the high RER during exercise in the CPT II and VLCAD patients together with the increased glucose disposal and total CHO, indicates that their energy deficit was covered by carbohydrate metabolism. This is further indicated by study I where we found that blood-born glucose from an IV glucose infusion is an important fuel for patients with CPT II deficiency. Similar findings were demonstrated in a patient with neutral lipid storage disease,51 and the findings are supported by fasting studies in a patient with CPT II deficiency and carnitine deficiency where fasting worsened exercise performance.52 However, patients cannot acutely carbohydrate load themselves by oral glucose ingestion because of lower glucose availability and higher insulin levels, that inhibit muscle glycogenolysis. On the other hand, a seven-day carbohydrate-rich diet improved exercise tolerance compared with a fat-rich diet in patients with CPT II deficiency. Oppositely, IV glucose did not improve exercise tolerance in patients with VLCAD deficiency. This might be explained by the short exercise period together with the mild affection of the two patients with VLCAD deficiency. However, it cannot be excluded that patients with more severe defects of VLCAD may benefit from the studied fuel supplements.

Glycogenoses.

It has been known since the 1960s that IV glucose improves exercise tolerance in patients with McArdle disease. Interestingly, glucose infusion also improved slightly the exercise tolerance of our patient with PHK deficiency, as indicated by the glucose-induced increase in VO_{2max} and maximal workload, and by the lowering of heart rate and perceived exertion. This beneficial response, albeit much smaller than that observed in McArdle patients, indicates an energy gap that probably explains the symptoms of exercise intolerance in the PHK patient. Besides patients with McArdle disease and PHK deficiency, glucose-induced improvements of exercise tolerance have also been seen in another form of glycogenoses; in a patient with phosphoglucomutase deficiency.53 However, the second wind and the glucose-induced second second-wind phenomenon is so far pathonomonic for patients with McArdle disease,54 and has not yet been found in other glycogenoses.1 carbohydrate-rich diet has also recently been shown to improve exercise tolerance in patients with McArdle disease.55 In McArdle disease, this is supported by the finding of up-regulation of the glucose transporter protein, GLUT4, and the rate-limiting enzyme of glycolysis, phosphofructokinase.56 In contrast, patients with Phosphofructokinase deficiency do not benefit from IV glucose infusion, since their defect result in a complete defect of muscle glycolysis, and therefore lipids represent the primary fuel available for muscular work.57 Oppositely, patients with Phosphofructokinase deficiency improved exercise tolerance after an overnight fast.

Patients with mitochondrial myopathy have a comparable low oxidative capacity as compared to patients with McArdle disease. Nevertheless, substrate kinetic studies in patients with mitochondrial myopathy indicate that these patients have a normal balanced fat and carbohydrate oxidation during moderate-intensity exercise. Hence, manipulating dietary fat and carbohydrate content is not a feasible therapeutic treatment for these disorders.58.

Since the discovery of PHK deficiency, it has been debated whether this enzyme deficiency is merely a neutral metabolic variant or patients are truly symptomatic.32 Reasons for this uncertainty include: 1) lack of molecular characterization in most patients; 2) non-specific symptoms, such as exercise intolerance and myalgia; and 3) normal lactate production during ischemic exercise in several patients.

The effect of IV glucose in the PHK patient could not be reproduced in two other patients with PHK deficiency that we studied recently.40 These two patients were milder affected and had a normal glucose production and utilization. Oppositely, the first patient we studied had a lower total CHO oxidation compared with healthy controls. This might result in energy deficit that was alleviated by IV glucose as indicated by the lowered Borg score and heart rate during exercise.

Our findings clearly demonstrate that X-linked PHK deficiency is a mild metabolic myopathy ranging from near normal to mildly affected patients, characterized by impaired lactate production during moderate-intensity dynamic exercise, mild elevations of plasma creatine kinase and muscle glycogen content, and a possible improvement of exercise tolerance with intravenous glucose infusion.40

The relative mild symptoms of X-linked PHK deficiency resemble three other glygocogenoses: 1) the partial glycolytic defect, phosphoglycerate mutase deficiency;33 2) the newly identified glycogenoses type VIX; phosphoglucomutase deficiency59 and 3) the patients with McArdle disease who retain some residual myophosphorylase activity.30 During submaximal exercise, patients with McArdle disease and the PHK patient have similar responses, with an almost absent lactate production. At peak exercise, however, the two conditions have very little in common, because alternative activation pathways, described below, rescue myophosphorylase activity, and thereby maximal oxidative capacity is near normal in PHK deficiency.

In contrast to the severely impaired lactate production during cycle exercise, the PHK-deficient patient produced normal amounts of lactate during an ischemic handgrip exercise. These findings may seem difficult to reconcile, but are probably explained by different activation pathways for myophosphorylase. Phosphorylase b can be activated not only via phosphorylation of specific serine residues by PHK, but also directly by higher Ca^{2+} levels.60 Myophosphorylase can also be activated directly by high AMP levels.60 During isometric, anaerobic exercise, both Ca^{2+} and AMP levels increase markedly in the cytosol of muscle cells, which may explain the normal muscle glycogenolysis in the PHK deficient patient under this condition. This discrepancy in capacity for glycogen breakdown during anaerobic and aerobic exercise in PHK deficiency can also explain why these patients, in contrast to McArdle patients, develop very few symptoms during anaerobic exercise. The results indicate that an ischemic forearm exercise test is not diagnostically helpful in patients with PHK deficiency.

Patients with low muscle mass.

Glucose homeostasis is impaired in patients with low muscle mass, and our findings showed that these patients are prone to develop hypoglycemia during prolonged fasting. The findings indicate that hypoglycaemia developed in these patients because there was no muscle mass (protein pool) to feed gluconeogenesis with gluconeogenic amino acids.

All studied patients had approximately the same absolute muscle mass, but it was preferentially patients with a low body weight who developed hypoglycaemia. This can most likely be explained by the similar brain size in studied subjects. Post-mortem brain analysis and imaging studies have found either normal or just slightly lower brain volumes in DMD and SMA patients compared with healthy subjects.61-64 The brain has a high and constant glucose utilization during the first 24 hrs of fasting.65,61 The switch to a higher ketone combustion occurs gradually after the first days of fasting, so that 50% of the energy metabolism in the brain after 2-3 weeks of fasting comes from ketone oxidation.65,66 Assuming that the liver constituted 1.5% of the body mass, patients with a low body weight had lower hepatic glycogen stores to support glucose metabolism of the brain, and as a result developed hypoglycemia. The patients in this study are probably more comparable to fasted children than to fasted healthy, adult subjects, because children like the neuromuscular patients in this study have a relatively large brain to body ratio. Accordingly, the progression from primary dependence on glucose to dependence on fat is more rapid in fasted children vs. healthy adults.67

This study also indicated that development of hypoglycemia is independent of disease type, and relates to the low muscle and body mass per se, since hypoglycemia developed in patients with so variable neuromuscular diseases as DMD, SMA, and congenital myopathy. This suggests that the findings may be applied to other neuromuscular conditions associated with low muscle and body mass. We therefore believe that hypoglycemia in this broad patient group is clinically significant, and should be a concern for caretakers and doctors who follow these patients. Besides hypoglycemia caused by irregular food intake and fever, another major concern is fasting for surgery in this patient group. Operations for scoliosis, tendotomy and tracheotomy are commonly performed in the patients, and the rate of per- and post-operative complications are high.68 Some complications relate to the use of certain anesthetics and muscle relaxants,69,70 but others include acute hyperkalemia68 that may be associated with low blood glucose. Our findings suggest that plasma glucose should be monitored perioperatively in neuromuscular patients with low muscle mass and prevented by intravenous glucose during the fasting period.

Fat homeostasis.

Disorders of lipid metabolism.

In patients with the FAO disorders CPT II and VLCAD deficiencies, enzyme activity ensures a normal FAO at rest, but residual enzyme activity is not sufficient to increase FAO during exercise. Even though patients are able to compensate partly for their impaired fat oxidation by increasing carbohydrate combustion, particularly muscle glycogenolysis, during exercise, they still have an energy deficit that results in their symptoms of exercise intolerance. Interestingly, FAO was also impaired during exercise in a patient with the muscular form of Neutral Lipid Storage Disease (NLSDM).51 Unlike VLCAD and CPT II deficiencies, the blocked FAO in the NLSDM patient appears to be caused by low concentration of FAs and absence of exercise-induced increase in plasma palmitate as a result of the defect in mobilization of fatty acids from triglycerides. In contrast FA availability is normal in VLCAD and CPT II deficiencies, and in these conditions FAO is impaired because of defective transport of fatty acids into the mitochondria (CPT II deficiency) or defect of fatty acid β-oxidation (VLCAD deficiency). The findings in these studies suggest that the mechanism behind exercise-induced rhabdomyolysis and muscle pain in CPT II and VLCAD deficiencies relates to a more rapid depletion of muscle glycogen stores. For obvious ethical reasons, patients in our studies were not pushed to the limit where muscle symptoms emerged. For the same reason, the exercise session was limited to 60 min.

Another possible mechanism of muscle symptoms in CPT II and VLCAD deficiency, besides energy shortage, could be a progressive accumulation of unmetabolized intermediates of FFA during exercise. This theory is further supported by the absence of rhabdomyolysis episodes triggered by exercise, fasting or fever

in NLSDM, which could be due to the absence of potentially toxic effect of accumulated long chain fatty acids in this disease.

FFA uptake is dependent on extracellular FFA concentrations and transporters that are activated by muscle contractions.71 In VLCAD patients, increasing FFA concentrations and muscle contractions in the absence of increases in total FFA oxidation may well have led to intracellular accumulation of long-chain acyl-CoAs and acylcarnitine esters that in experimental models destabilize the sarcolemma.72 Interestingly, not only exercise-induced FAO, but also fat mobilization (palmitate and glycerol production), was low in patients with CPT II and VLCAD deficiencies. The lower mobilization of FFA in patients apparently was not caused by lower sympathetic activation during exercise as inferred by plasma insulin and heart rate responses. On the other hand, the lower FAO during exercise in CPT II and VLCAD patients was not caused by the lower rate of lipolysis per se, because the availability of FFA, as inferred by plasma FFA levels, was high during exercise. These findings suggest that there may be a regulatory feedback link between the intramuscular fatty acid availability and the systemic mobilization of FFA.

Interestingly, the two subjects with the single E454X and D213G mutations had palmitate oxidation values during exercise that were comparable to findings in patients carrying two *CPT 2 gene* mutations. When interviewed, these two carriers had actually experienced myopathic symptoms in their daily lives. The fact that in vivo oxidation of long-chain fatty acids is severely impaired in symptomatic carriers of *CPT 2 gene* mutations, indicates a dominant negative effect of single *CPT2 gene* mutations. Theoretically, this could be because of loss of function of the protein due to the tetrameric folding of the protein that might slightly change even with one mutation. Furthermore, it seems like it is mutation specific, since the one subject with the S113L mutation had a normal increase of palmitate oxidation during exercise. Furthermore, the family history supports this theory.

Theoretically, MCT could be beneficial in VLCAD deficiency as seen in children with Long-chain 3-hydroxy acyl CoA dehydrogenase deficiency73, because MCT bypasses the metabolic block, and the breakdown products of MCT enter the hepatic portal vein just minutes after ingestion.74 However, our study shows that MCT ingestion does not improve exercise tolerance in patients with VLCAD deficiency. This might relate to the mild metabolic defect of the patients we studied, leaving the patients with a normal maximal oxidative capacity. Furthermore, the fuel supplements produce hyperinsulinemia, which inhibits muscle glycogenolysis, which is crucial for the beta-oxidation defects.

Glycogenoses.

Patients with McArdle disease and PHK deficiency are able to increase FAO during exercise. However, FAO did not increase further to cover the energy deficit occurring during exercise in the patients. This is despite the fact that both patient groups had plenty of plasma palmitate available. The relative impairment of FAO in patients with McArdle disease is most likely found in the intermediary metabolism of the TCA cycle. Furthermore, our studies indicate that even a small glycogenolytic defect may cause a lower flux of substrates to the TCA cycle, and thus impair FAO.37,38,75 The impaired FAO is most likely caused by a combination of a low substrate flux of pyruvate to the TCA cycle, and low concentration of TCA intermediates. A low flux through the TCA cycle is followed by a diminished formation of mitochondrial NADH and $FADH_2$ which limits oxidative phosphorylation and O_2 consumption due to the reduced rate of formation of mitochondrial reducing equivalents.28

The limited oxidative capacity in McArdle patients before the spontaneous second wind depends on and fluctuates with changing availability of extra muscular fuels.54 The second wind phenomenon results in a marked drop in perceived exertion and heart rate. This is attributable to an increase in oxidative capacity due to increased energy availability of extra-muscular fuels in combination with glucose derived from hepatic glycogenolysis.54, 76,77 The steep increase of fat oxidation in the first fifteen minutes of exercise indicates that fat too is an important metabolite in the process of initiating the second wind in the McArdle patients. A similar increase is seen in lactate oxidation, (Ørngreen et al; unpublished data). During exercise patients with McArdle disease are able to partly compensate for the blocked glycogenolysis by mobilizing and burn more hepatic glucose and fat from adipose tissue. Despite the high availability of FFA's in the McArdle patients during exercise, FAO cannot increase further. This indicates that FAO is impaired and that fat supplementation probably doesn't improve exercise tolerance in patients with McArdle disease. These findings are in contrast to the two case studies, reporting a beneficial effect of respectively fat supplementation and overnight fast in patients with McArdle disease.28 However, in line with our results another study on McArdle patients showed that intra-lipid infusion did not improve exercise tolerance.78

Patients with low muscle mass. Hypoglycaemia developed in patients although the hormonal signal for glucose production was higher, i.e. higher catecholamines and lower insulin/glucagon ratio. As a result of this higher sympathetic activation, lipolysis appeared higher in patients based on plasma FFA levels. Interestingly, patients developed hypoglycaemia although the contribution of glycerol to gluconeogenesis seemed to be higher in patients vs. healthy as indicated by the FFA/glycerol ratios, which further emphasizes the role of muscle as a key supplier of gluconeogenic substrates during fasting.

Limitations

A limitation of technically complicated studies in rare patients, like the above-presented studies, is the low number of patients one can enroll. However, the results of the studies are convincing since findings were very uniform in all subjects in each study. The low sample size increases the risk of false positive and false negative results in the treatment studies. Furthermore, these studies are in increased risk of bias, since medical staff was not blinded, and patients were not blinded in the diet study as well.

Clinical implications and perspectives.

We found that patients with CPT II deficiency improved exercise tolerance by IV glucose and by a carbohydrate-rich diet. However, the diet is the only treatment that can be done outside the hospital, and still the diet cannot cover the total energy deficit these patients suffer from. A powerful therapy would theoretical-

ly be if it was possible to increase the residual enzyme activity in patients with FAO disorders. Results pointing in this direction have been published in recent years. In vitro-studies suggests that PPAR receptor agonists (fibrates) can up-regulate residual enzyme activity of carnitine palmitoyltransferase (CPT) II and very long-chain acyl-CoA dehydrogenase (VLCAD) in patients deficient in these enzymes.1 79-81 PPAR is a transcription factor, belonging to the superfamily of steroid-thyroid hormone receptors, which are able to modify CPT II and VLCAD gene expression.82 83 These data provided the first evidence for a possible pharmacological effect of PPAR agonists on FAO defects in humans, and recent studies showed a marked increase in vitro FAO capacities.80 84 The effect was caused by the drug-induced up-regulation of CPT II mRNA/VLCAD mRNA and protein levels.80 85 Furthermore, a 6-month treatment with Bezafibrate increased scores of life quality on the SF-36 survey in six patients with muscle pain and rhabdomyolysis episodes due to CPTII deficiency.79 These results strongly suggested that treatment with the lipid-regulating drug Bezafibrate improves fatty acid oxidation in the muscle of patients with fatty acid oxidation disorders such as CPT II and VLCAD deficiencies, paving the way for a treatment of these metabolic myopathies.

However, we recently conducted another study showing that treatment with Bezafibrate does not improve FAO in vivo in patients with CPT II and VLCAD deficiencies, and thus does not allow to alleviate consistently the symptoms related to these diseases (Ørngreen et al, unpublished data). Despite the promising findings of a Bezafibrate-induced increase in palmitoyl L-carnitine oxidation levels by up to 284% in cultured fibroblasts of patients with CPT II deficiency,79 our findings showed that this in vitro efficacy measure does not translate into a clinically meaningful effect in vivo. We predicted that the patients should at least increase palmitate oxidation 1.7-fold more than normal, based on previous assessments of FAO in healthy subjects and patients homozygous or heterozygous for CPT 2 and VLCAD gene mutations. However, FAO was unaffected by Bezafibrate, which was consistent in all ten patients we studied. The lack of effect could potentially be explained by counteracting mechanisms in vivo, which are not active in vitro. One such potential mechanism is the Bezafibrate-induced reduction in substrate availability for FAO. Thus, Bezafibrate significantly lowered the plasma concentrations of palmitate and other FFAs. Since the level of FAO is known to be directly related to the plasma concentration of FFA,86 Bezafibrate could have a negative influence on FAO rates. On the other hand, lowering of FFA could also limit the potential toxic effect of accumulated long chain fatty acids in patients with VLCAD deficiency, and to some extent CPT II deficiency, thus decreasing the episodes of rhabdomyolysis. However, the only episode of myoglobinuria during this trial occurred in a patient on Bezafibrate treatment.

These results might end up having an important impact on future clinical researches aiming to evaluate the effect of fibrates, not only for these two metabolic myopathies, but also for more frequent diseases such as Huntington disease or mitochondrial disorders, for which recent studies demonstrated pre-clinical, in vitro metabolic improvement with Bezafibrate.87-89

Furthermore, the activation of PPAR receptors has been demonstrated to cause upregulation of OCTN2, leading to an increase of intracellular carnitine concentrations in animal models.90-92 Patients with primary carnitine deficiency have absent or low residual OCTN2 enzyme activity, and Bezafibrate might be a potential candidate for treating these patients as well.90 Recently Resveratrol, a natural polyphenol, has been reported to induce mitochondrial FAO capacities in human fibroblasts.93 The effect on FAO in vivo in patients with FAO disorders would also be interesting to know.

An anaplerotic diet might have a beneficial effect on activities of daily living measured by the SF-36 in patients with FAO disorders.94 These results suggest that the triheptanoin produced TCA intermediate oxaloacetate and the glycolytic product acetyl-CoA improves activities of daily living. Hence, energy metabolism is seriously compromised by the inability to fuel the TCA cycle by beta-oxidation in patients with FAO disorders.

Other patients than patients with FAO disorders might benefit from an anaplerotic diet. An anaplerotic diet might be very useful in patients with glycogenolytic disorders, especially in patients with McArdle disease, where it seems like the TCA cycle flux is impaired due to impaired anaplerosis.

SUMMARY

The main purpose of the following studies was to investigate pathophysiological mechanisms in fat and carbohydrate metabolism and effect of nutritional interventions in patients with metabolic myopathies and in patients with severe muscle wasting.

Yet there is no cure for patients with skeletal muscle disorders. The group of patients is heterozygous and this thesis is focused on patients with metabolic myopathies and low muscle mass due to severe muscle wasting. Disorders of fatty acid oxidation (FAO) are, along with myophosphorylase deficiency (McArdle disease), the most common inborn errors of metabolism leading to recurrent episodes of rhabdomyolysis in adults. Prolonged exercise, fasting, and fever are the main triggering factors for rhabdomyolysis in these conditions, and can be complicated by acute renal failure. Patients with low muscle mass are in risk of loosing their functional skills and depend on a wheel chair and respiratory support.

We used nutritional interventions and metabolic studies with stable isotope technique and indirect calorimetry in patients with metabolic myopathies and patients with low muscle mass to get information of the metabolism of the investigated diseases, and to gain knowledge of the biochemical pathways of intermediary metabolism in human skeletal muscle.

We have shown that patients with fat metabolism disorders in skeletal muscle affecting the transporting enzyme of fat into the mitochondria (carnitine palmitoyl-transferase II deficiency) and affecting the enzyme responsible for breakdown of the long-chain fatty acids (very long chain acyl-CoA dehydrogenase deficiency) have a normal fatty acid oxidation at rest, but enzyme activity is to low to increase fatty acid oxidation during exercise. Furthermore, these patients benefit from a carbohydrate rich diet. Oppositely is exercise capacity worsened by a fat-rich diet in these patients. The patients also benefit from IV glucose, however, when glucose is given orally just before exercise, exercise

capacity is worsened, most likely due to the sympatho-adrenergt response, that increases heart rate and blocks gluconeogenesis. Substrate turnover studies in patients with McArdle disease and Phosphorylase b kinase deficiency showed that palmitate lipolysis, utilization and plasma concentration was higher and total CHO lower in the patients during exercise vs. healthy subjects. In patients with low muscle mass glucose homeostasis is impaired, and our findings showed that these patients are prone to develop hypoglycemia during prolonged fasting.

The following studies emphasize the importance of skeletal muscle in production of energy, both when skeletal muscle lack important metabolic enzymes (metabolic myopathies), and when skeletal muscle mass is low.

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