Exercise in the fasted state facilitates fibre type-specific intramyocellular lipid breakdown and stimulates glycogen resynthesis in humans

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The effects were compared of exercise in the fasted state and exercise with a high rate of carbohydrate intake on intramyocellular triglyceride (IMTG) and glycogen content of human muscle. Using a randomized crossover study design, nine young healthy volunteers participated in two experimental sessions with an interval of 3 weeks. In each session subjects performed 2 h of constant-load bicycle exercise (\sim 75% $\dot{V}_{O_2,max}$), followed by 4 h of controlled recovery. On one occasion they exercised after an overnight fast (F), and on the other (CHO) they received carbohydrates before (~150 g) and during $(1 g (kg bw)^{-1} h^{-1})$ exercise. In both conditions, subjects ingested 5 g carbohydrates per kg body weight during recovery. Fibre type-specific relative IMTG content was determined by Oil red O staining in needle biopsies from m. vastus lateralis before, immediately after and 4 h after exercise. During F but not during CHO, the exercise bout decreased IMTG content in type I fibres from $18 \pm 2\%$ to $6 \pm 2\%$ (P = 0.007) area lipid staining. Conversely, during recovery, IMTG in type I fibres decreased from $15 \pm 2\%$ to $10 \pm 2\%$ in CHO, but did not change in F. Neither exercise nor recovery changed IMTG in type IIa fibres in any experimental condition. Exercise-induced net glycogen breakdown was similar in F and CHO. However, compared with CHO (11.0 \pm 7.8 mmol kg⁻¹ h⁻¹), mean rate of postexercise muscle glycogen resynthesis was 3-fold greater in F ($32.9 \pm 2.7 \text{ mmol kg}^{-1} \text{ h}^{-1}$, P = 0.01). Furthermore, oral glucose loading during recovery increased plasma insulin markedly more in F (+46.80 μ U ml⁻¹) than in CHO (+14.63 μ U ml⁻¹, P = 0.02). We conclude that IMTG breakdown during prolonged submaximal exercise in the fasted state takes place predominantly in type I fibres and that this breakdown is prevented in the CHO-fed state. Furthermore, facilitated glucose-induced insulin secretion may contribute to enhanced muscle glycogen resynthesis following exercise in the fasted state.

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Fatty acids are important energy substrates in contracting muscles during prolonged exercise. The relative contribution of fatty acids to aerobic energy production is increased as exercise intensity decreases and duration increases. Different triacylglycerol sources contribute to the delivery of fatty acids as fuel to muscles during exercise. The most prominent sources come from subcutaneous and visceral adipose tissue, complemented with triacylglycerols either bound to circulating lipoproteins or stored locally in muscle cells (van der Vusse & Reneman, 1996; Achten & Jeukendrup, 2004). Over the last decade the role of intramyocellular triglycerides (IMTG) in supporting fat oxidation during exercise has been a controversial issue. However, there is accumulating evidence demonstrating that IMTG can significantly contribute to energy provision in muscles during prolonged endurance exercise (Watt *et al.* 2002). Furthermore, the extent of the contribution appears to depend on different factors, including muscle fibre type (Bachmann *et al.* 2001; van Loon *et al.* 2003), training status (Hurley *et al.* 1986; Phillips *et al.* 1996), sex (Roepstorff *et al.* 2002; Steffensen *et al.* 2002) and probably diet (Helge, 2002; Watt *et al.* 2004b). It is well established that high exogenous carbohydrate availability markedly suppresses the rate of fat oxidation during exercise (Coyle et al. 1997). In this respect, recent findings show that glucose ingestion during exercise blunts muscle hormone-sensitive lipase activity, the rate-limiting enzyme of IMTG lipolysis (Watt et al. 2004b). Because carbohydrate intake before and during prolonged endurance exercise is a standard 'ergogenic' procedure, it is interesting to note that virtually all studies investigating exercise-induced IMTG breakdown during prolonged exercise (1-4 h) have been performed in the absence of exogenous carbohydrate supply. In addition, exercise as a rule was performed after an overnight fast (for relevant references see Watt et al. 2002), another factor which predisposes to enhanced fatty acid oxidation (Dohm et al. 1986; Nieman et al. 1987; Knapik et al. 1988). Accordingly, the primary hypothesis of our study was that exercise in the fasted state causes a substantially greater IMTG degradation than exercise performed postprandially in conjunction with carbohydrate intake.

Studies in healthy subjects have shown that increasing IMTG by acute elevation of plasma FFA rapidly impairs insulin-stimulated muscle glucose uptake (Boden *et al.* 2001; Bachmann *et al.* 2001; Itani *et al.* 2002) as well as glycogen synthesis (Chalkley *et al.* 1998). It is well established that postexercise muscle glycogen resynthesis is driven by enhanced insulin-mediated glucose uptake and glycogen synthase activity (Richter *et al.* 1989) which might thus depend on IMTG content. Therefore the second hypothesis of our study was that exercise performed in the fasted state, by virtue of exaggerated IMTG breakdown, could cause a faster rate of postexercise muscle glycogen resynthesis than postprandial exercise.

It is currently believed that the cumulative effects of transient increases in metabolic gene expression during and after repeated bouts of exercise represent the underlying basis for the biochemical adaptations induced by endurance training (Williams & Neufer, 1996; Pilegaard et al. 2000). It has recently been postulated that uncoupling protein 3 (UCP3) serves to protect mitochondria from lipid-induced damage in conditions where fatty acid delivery to the mitochondrial matrix exceeds its capacity for oxidative catabolism (Schrauwen & Hesselink, 2004). Support for this hypothesis comes from the observation that acute or short-term elevation of plasma FFA level either by fasting or administration of a high fat diet (Millet et al. 1997; Samec et al. 1998; Hildebrandt & Neufer, 2000; Hesselink et al. 2003; Pilegaard et al. 2003) increases UCP3 expression at rest. By analogy, recent evidence indicates that the transcriptional activation of the UCP3 gene by acute exercise (Pilegaard et al. 2000; Hildebrandt et al. 2003) can be suppressed by carbohydrate intake (Schrauwen et al. 2002b). Conversely, exercise in rats has been found to attenuate the fasting-induced transcriptional activation of UCP3. Clearly, the interaction of exercise and diet with regard to regulation of UCP3 gene expression in skeletal muscle is poorly understood.

The present study aimed at investigating whether an endurance exercise bout performed in the fasted state, compared with identical exercise performed in combination with an optimal rate of carbohydrate intake to support performance, (1) facilitates degradation of IMTGs and (2) enhances postexercise muscle glycogen resynthesis. Furthermore, the effect of carbohydrate intake on exercise-induced UCP3 gene expression in muscle was investigated.

Methods

Subjects

Nine healthy, physically active men (age: 22.8 ± 0.4 years; body weight (bw): 74.0 ± 2.3 kg) volunteered to participate in the study, which was approved by the local Ethics Committee (K.U.Leuven) and conformed to the Declaration of Helsinki. Subjects gave their written, consent after they were informed in detail of all experimental procedures and risks possibly associated with the experiments. The subjects were habituated to the experimental conditions, which included a 2-h maximal constant-load exercise bout in the fasted state, followed by 4 h of oral carbohydrate loading. Three weeks before the start of the study they participated in two familiarization sessions with a 3-day interval. Upon arrival in the laboratory and after an overnight fast, they performed 2 h of exercise in the fasted state. The subjects cycled on their own race bicycle, which was mounted on a calibrated ergometer (Avantronic Cyclus II, Leipzig, Germany) as previously described (Vandebuerie et al. 1998). During the first familiarization session subjects were allowed to continuously adjust the workload in order to find the maximal workload (in watts) they could maintain for 2 h. This workload was further adjusted during the second familiarization trial and was eventually used during the experimental sessions $(178 \pm 8 \text{ W})$. Other studies on similar subjects in our laboratories have shown that this performance on average corresponds with \sim 75% of $\dot{V}_{O_2,max}$ (range: 67–82% $\dot{V}_{O_2, max}$) (K. De Bock, E. A. Richter, A. P. Russell, B. O. Eijnde, W. Derave, M. Ramaekers, E. Koninckx, B. L'eger, J. Verhaeghe and P. Hespel, unpublished observations). On each occasion, during the initial 4 h recovery period the subjects received the carbohydrate supplements as prescribed by the study protocol (see below). The subjects tolerated the supplements well and none complained of gastrointestinal problems.

Study protocol

According to a balanced and randomized cross-over study design, all subjects participated in two experimental

sessions (F: exercise in the fasted state; CHO: exercise after carbohydrate feeding), with a 3-week period in between. Because muscle triglyceride content can rapidly fluctuate depending on dietary fat content (Bachmann *et al.* 2001), the subjects received a standardized diet containing 3000 kcal day⁻¹ (12552 kJ day⁻¹; 61% carbohydrates, 23% fat, and 15% protein) during 3 days before the start of each session. Subjects were also instructed to abstain from any strenuous physical exercise during this period.

On the morning of the experiments the subjects arrived at the laboratory between 06.00 h and 09.00 h after an 11 h overnight fast. They were assigned to either of two experimental conditions. During CHO half of the subjects received a standardized carbohydrate-rich breakfast (722 kcal (3020.8 kJ): 85% carbohydrates, 4% fat, 11% protein) whereas the others remained fasted. After a 2 h rest period a percutaneous needle biopsy sample was taken from the right vastus lateralis muscle under local anaesthetic (2-3 ml lidocaine) through a 5 mm incision in the skin. Furthermore, 25 ml blood was sampled from an antecubital vein. Thereafter subjects cycled for 2 h at the workload which was determined during the familiarization sessions $(178 \pm 8 \text{ W})$. Before, halfway through and at the end of the exercise bout, oxygen uptake (V_{O_2}) and carbon dioxide output (V_{CO_2}) were measured over a 5-min interval using a breath-by-breath ergospirometry system (Jaeger, Oxycon Alpha). Capillary blood was sampled from a hyperaemic earlobe before and after the first and second hour of exercise. In the CHO condition, subjects received 1 g maltodextrine per kg bw per hour in a flavoured 15% solution during the exercise bout. Conversely, during F they received an equal amount of water which was sweetened (2 ml l⁻¹ cyclamate) and flavoured to be similar in taste and appearance with the maltodextrine solution. At the end of the exercise bout another muscle biopsy and venous blood sample were taken. The muscle biopsy was taken through the same incision as the pre-exercise biopsy but with the needle pointing in another direction. During the following 4 h recovery period, aimed to replete muscle glycogen stores, the subjects rested in the laboratory. Independent of the experimental condition, they received 1.5 g maltodextrin per kg bw (15% flavoured solution) during the initial and last hour of recovery. Between two and three hours of recovery they consumed a pasta meal containing 1.5 g carbohydrates per kg body weight. Furthermore, the subjects were allowed to drink additional water ad libitum. However, the volume consumed during the first trial was recorded and reproduced during the second trial. At the end of the recovery period another muscle and blood sample were taken. The muscle biopsy was taken through a new incision spaced approximately 5 cm from the first.

Analysis of muscle samples

After being freed from any visible non-muscle material, part of the muscle sample was immediately frozen in liquid nitrogen, and the remaining part was mounted in embedding medium (Tissue-Tek, Sakura FineTek, Zoeterwoude, the Netherlands) cooled in isopentane. All samples were stored at -80° C for later analysis. Muscle glycogen content was measured as glucose residues after acid hydrolysis, in freeze-dried muscle tissue using a standard enzymatic flourometric assay (Lowry & Passoneau, 1972).

Fibre typing and Oil red O staining. Serial sections $(4 \,\mu m)$ from biopsy samples collected before, immediately after and 4 h after exercise were laid together on uncoated glass slides. The method to quantify fibre type-dependent intramyocellular lipids stained by Oil red O (ORO), was adapted from Koopman et al. (2001). Briefly, cryosections were fixed for 10 min in 0.1% Triton X-100 added to 4% paraformaldehyde in phosphate-buffered saline (PBS). Slides were rinsed for 2×5 min with wash buffer (0.5% BSA in PBS), treated with 10 mM NH₄Cl and washed again $(2 \times 5 \text{ min})$. Slides were prehybridized in 1% BSA in PBS for 30 min. Thereafter, sections were incubated overnight at 4°C with two primary monoclonal antibodies against human myosin heavy chain I (A4.840 supernatant, Developmental Studies Hybridoma Bank at the University of Iowa, IA, USA) and IIa (N2.261 supernatant, Developmental Studies Hybridoma Bank) to determine muscle fibre type (I and IIa, respectively). Incubation was followed by three 5 min washes with wash buffer, after which the appropriate conjugated antibodies (type I: Alexa Fluor350 antimouse IgG1, Molecular Probes, Leiden, the Netherlands; type IIa: FITC antimouse IgM, Southern Biotechnology Associates, Birmingham, AL, USA) were added. After being washed again $(3 \times 5 \text{ min in wash})$ buffer), Oil red O staining (ORO) was performed. Muscle sections were incubated in formalin for 10 min, washed with deionized water $(3 \times 30 \text{ s})$ before staining for 15 min with the ORO working solution. ORO stock solution was prepared by adding 0.5 g Oil red O (Sigma-Aldrich, St Louis, MO, USA) to 100 ml isopropyl alcohol (99%). Prior to staining, a working solution, containing 30 ml of ORO stock solution and 20 ml of deionized water, was prepared and filtered three times (Millipore, Ireland; prefilter and 0.45 μ m filter size) to remove crystallized ORO. After the immersion in ORO, sections were washed again with deionized water $(3 \times 30 \text{ s})$ and then rinsed under tap water for 7 min. Coverslips were mounted with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA, USA).

Slides were examined using a Nikon E1000 fluorescence microscope (Nikon, Boerhavedorp, Germany) equipped

with a digital camera. Epifluorescence signal was recorded using a Texas red excitation filter for ORO, and FITC and DAPI filter for type I and IIa muscle fibres, respectively. Captured images (× 20 magnification) were processed and analysed using Lucia G software (LIM, Prague, Czech Republic). The Oil red O signal was quantified for each muscle fibre resulting in a total of 168 ± 9 muscle fibres analysed for each muscle cross-section (99 \pm 5 type I and 69 ± 5 type IIa muscle fibres). An intensity threshold representing minimal intensity values corresponding to lipid droplets was set manually and uniformly used for all subjects. Fibre type-specific IMTG content was expressed as the percentage of the measured area that was stained with ORO. The intra-assay coefficient of variation was 14% in type I and 21% in type II fibres. Inter-assay variability was 15% in type I and 27% in type II fibres. To further reduce the analytical error, all samples were analysed in triplicate and mean values were used for data analysis. In addition, all samples from one subject were analysed in the same assay.

Muscle lysate production. Muscle specimens was freeze dried and dissected free of visible blood, connective and fat tissues. Muscles were then homogenized in ice-cold buffer (20 mM Tris base, 50 mM NaCl, 2 mM DTT, 50 mM NaF, 1% Triton X-100, 250 mM sucrose, 5 mM sodium pyrophosphate, 4 μ g ml⁻¹ leupeptin, 6 mM benzamedine, 500 μ M PMSF, 50 μ g ml⁻¹ soybean trypsin inhibitor, pH 7.4) for 20 s using a homogenizer (PT 3100, Brinkmann, NY, USA). Homogenates were rotated end over end for 1 h at 4°C. Lysates were generated by centrifugation (17 500 g) for 1 h at 4°C. Lysates were quick frozen in liquid nitrogen and stored at -80° C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce Chemical Company, IL, USA).

Western blotting. The degree of phosphorylation of α -AMPK (Thr¹⁷²) and acetyl-CoA carboxylase- β (ACC- β ; Ser²²¹) was evaluated using phosphospecific antibodies from Cell Signalling Technology Inc. (Beverly, MA, USA) and Upstate Biotechnology, Inc. (Lake Placid, NY, USA), respectively, as previously described by Hojlund et al. (2004). The ACC-phosphospecific antibody is raised against a peptide corresponding to the sequence in rat ACC- α containing the Ser⁷⁹ phosphorylation site, but the antibody also recognized the human ACC- β when phosphorylated, most likely at the corresponding Ser²²¹. For the detection of α -AMPK phosphorylation (Thr¹⁷²), muscle lysate protein was subjected to SDS-PAGE (7.5% Criterion gradient gel; Bio-Rad Laboratories, Richmond, CA, USA), followed by semidry transfer to PVDF membranes (Immobilon Transfer Membrane, Millipore, Glostrup, Denmark). Immunoreactive bands were visualized with enhanced chemiluminescence (ECL-plus, Amersham Biosciences, Little Chalfont, UK) and detected and quantified using a charge-coupled device image sensor and 1D software (Kodak Image Station, E440CF, Kodak, Glostrup, Denmark).

Glycogen synthase activity. Glycogen synthase (GS) activity was measured on muscle lysate. GS was determined in the presence of 0.17, and 8 mmol l^{-1} glucose-6-phosphate (G6P) and given as the percentage of fractional velocity (% FV) (100 × activity in the presence of 0.17 mmol l^{-1} G6P divided by the activity at 8 mmol l^{-1} G6P) (Jorgensen *et al.* 2004).

RNA extraction and RT-PCR. RNA was extracted from approximately 15 mg of skeletal muscle using a commercially available preparation, peqGOLD Tri-Fast (Peqlab, Germany). Three micrograms of RNA was reverse transcribed to cDNA using Random Hexomer primers and a Stratascript enzyme (Stratagene, the Netherlands). Real-Time PCR was performed using an MX3000p thermal cycler system and Brilliant SYBER Green QPCR Master Mix (Stratagene, the Netherlands). The PCR conditions for all genes consisted of one denaturing cycle at 90°C for 10 min followed by 40 cycles, consisting of denaturing at 90°C for 30 s, annealing at 60°C for 60 s and elongation at 72°C for 60 s. At the end of the PCR the samples were subjected to a melting curve analysis. To control for any variations due to efficiencies of the reverse transcription and PCR all of the genes measured were normalized against β -actin mRNA (Russell *et al.* 2003). All PCR runs were performed in triplicate. The primer sequences for UCP3 and β -actin have been published previously (Arkinstall et al. 2004).

Analysis of blood samples

Venous blood samples were collected into vacuum tubes containing EDTA, lithium heparin and Silica Clot Activator (BD Vacutainer, NJ, USA). Tubes were centrifuged (1500 r.p.m. for 15 min at 4° C) and the supernatant was stored at -80° C until later analysis. Capillary blood samples were collected $(50 \,\mu l)$ into heparin zed glass capillaries and were immediately analysed for blood glucose and lactate concentration using an Analox GM7 analyser (Analox Instruments Ltd, London, UK). Glycerol and FFA concentrations in plasma were measured spectrophotometrically on a Hitachi autoanalyser (Hitachi, Japan). Plasma insulin (Human Insulin, Biotech-IgG, Germany) and adiponectin (Linco Research, St Charles, MO, USA) were assayed using commercially available radioimmunoassay kits. Plasma leptin was measured using an ELISA kit (Linco Research). Serum cortisol was measured using a heterogeneous competitive magnetic separation assay (Technicon Immuno 1 System, Bayer Corporation, Leverkusen, Germany) by detecting the activity of the alkaline phosphatase-cortisol conjugate.

Plasma catecholamines were determined by means of a high performance liquid chromatography method using electrochemical detection.

Data calculations and statistical analyses

From the recorded \dot{V}_{O_2} and \dot{V}_{O_2} , carbohydrate and fat oxidation rates were calculated using the formulae of Peronnet & Massicotte (1991):

Fat oxidation rate = $1.6946\dot{V}_{O_2} - 1.7012\dot{V}_{CO_2}$ Carbohydrate oxidation rate = $4.5850\dot{V}_{CO_2} - 3.2255\dot{V}_{O_2}$

Treatment effects were evaluated using a repeated-measures analysis of variance (ANOVA). Two-way ANOVA was performed to examine interaction between treatment and time. Where the ANOVA yielded a significant effect, a planned contrast analysis was used for *post hoc* comparisons. The strength of association between parameters was analysed by Pearson product moment correlation analysis. A probability level (*P*) equal to or less than 0.05 was considered statistically significant. All data are expressed as means \pm s.E.M.

Results

Intramyocellular triglyceride content (IMTG)

Pre-exercise IMTG, was about 2-fold higher in type I fibres (~18% area covered by lipid staining) than in type IIa fibres (~10%) in either experimental condition. In type I fibres 2 h of exercise in the fasted state (F), but not in the carbohydrate-supplemented state (CHO), decreased IMTG from $18 \pm 2\%$ to $6 \pm 2\%$ (P = 0.007) area covered by lipid staining (Fig. 1*A*). Conversely, in type IIa fibres neither F nor CHO decreased IMTG (Fig. 1*B*). During the 4 h recovery period, IMTG in type I fibres did not change in F, whereas it decreased from $15 \pm 2\%$ to $10 \pm 2\%$ (P = 0.03) in CHO. In either condition, type IIa IMTG did not change during recovery.

Muscle biochemistry and UCP3 expression

Neither pre-exercise nor postexercise muscle glycogen content was significantly different between F and CHO. However, compared with postexercise, by the end of the 4 h recovery period muscle glycogen content had significantly increased in F (P = 0.0001) but not in CHO (Fig. 2A). Mean rate of net glycogen resynthesis during the recovery period was about 3-fold higher in F than in CHO (P = 0.02; F: 32.9 ± 2.7 , CHO: $11.0 \pm 7.8 \text{ mmol kg}^{-1} \text{ h}^{-1}$). Glycogen synthase fractional velocity (FV) before exercise was reduced in F (Fig. 2B). Exercise increased FV in both groups (P = 0.0001) and this increase persisted throughout the recovery period. AMPK activity, measured as the degree of α -AMPK phosphorylation (Fig. 3), increased during exercise and was similar between experimental conditions. Changes in ACC- β phosphorylation status (data not shown) paralleled changes in AMPK phosphorylation status at any time. Compared to CHO, pre-exercise UCP3 mRNA expression was higher in F (P = 0.01). There was a $\sim 50\%$ exercise-induced up-regulation of UCP3 mRNA expression in F, whereas no change was seen in CHO. At the end of the recovery period values were not different from baseline in either group (Fig. 4).

Blood biochemistry

Blood metabolites (Table 1). From the start to the end of the 2 h exercise bout, blood glucose concentration decreased in both experimental conditions (P = 0.001). However, values at any time were lower in F than in CHO (P = 0.001). At the end of the 4 h recovery period,



carbohydrate-fed state on intramyocellular lipid content Intramyocellular lipid content for type I (*A*) and type IIa fibres (*B*) before, immediately after and 4 h after exercise as determined by fluorescence microscopy on Oil red O stained muscle cross-sections. Data provided are expressed as means \pm s.e.m. (n = 9); §P < 0.05versus carbohydrate fed; *P < 0.05 versus pre-exercise; †P < 0.05versus postexercise.

blood glucose was similar between conditions. Plasma FFA before exercise was higher (P = 0.02), and also increased markedly more during exercise in F than in CHO (P = 0.02). After carbohydrate repletion during recovery, FFA levels had returned to similar low values in both groups. The time course of plasma glycerol changes was parallel to the FFA changes.

Hormones (Table 1). Pre- and postexercise plasma insulin concentrations were substantially higher in CHO than in F (P = 0.02). Plasma insulin dropped during exercise (P = 0.01) and increased during recovery (P = 0.001) in both experimental conditions. However, the increase in plasma insulin associated with oral carbohydrate loading during recovery was markedly greater in F than in CHO (P = 0.02). Thus, unlike pre- and postexercise, at the end of the 4 h recovery period, the plasma



Figure 2. Effect of exercise in the fasted *versus* carbohydrate-fed state on muscle glycogen content and glycogen synthase activity

Muscle glycogen content (*A*) and muscle glycogen synthase fractional velocity (*B*) were measured before, immediately after and 4 h after exercise. Data provided are expressed as means \pm s.e.m. (*n* = 9); **P* < 0.05 *versus* pre-exercise; †*P* < 0.05 *versus* postexercise; §*P* < 0.05 *versus* carbohydrate-fed.



Figure 3. Effect of exercise in the fasted *versus* carbohydratefed state on phosphorylation of muscle α -AMPK

Thr¹⁷² phosphorylation on α -AMPK was measured by Western blotting on muscle samples obtained before, immediately after and 4 h after exercise. Data are given as mean arbitrary scanning units \pm s.E.M. (n = 9); *P < 0.05 versus pre-exercise.

insulin level tended to be higher in F than in CHO (P = 0.07). Starting from a similar baseline level, the exercise increased plasma adrenaline in F (P = 0.02) but not in CHO. Values returned to baseline within the 4 h recovery window. In contrast with adrenaline, plasma noradrenaline concentration increased to the same degree with exercise in both groups. Exercise did not impact on plasma cortisol. However after recovery, compared with pre-exercise values plasma cortisol was decreased in F (P = 0.02) but not CHO. Pre-exercise plasma leptin concentration was similar between experimental conditions. However, during exercise plasma leptin level dropped to lower values in F than in CHO (P = 0.01) and remained low during recovery in F whereas it returned to baseline values in CHO. Adiponectin concentrations



Figure 4. Effect of exercise in the fasted *versus* carbohydratefed state on muscle UCP3 mRNA content

Total UCP3 mRNA content in muscle before, immediately after and 4 h after exercise as measured by RT-PCR. Data provided are normalized for β -actin and expressed as means \pm s.e.m. (n = 9); *P < 0.05 versus pre-exercise; †P < 0.05 versus postexercise; §P < 0.05 versus carbohydrate fed.

| | | Pre-exercise | Post-exercise | 4 h recovery |
|---|-----|------------------------------------|---------------------------------------|-------------------------------------|
| Metabolites | | | | |
| Glucose (mmol I ⁻¹) | СНО | $\textbf{6.0} \pm \textbf{0.3}$ | $\textbf{4.1} \pm \textbf{0.2}^{*}$ | $\textbf{6.3} \pm \textbf{0.7}$ |
| | F | $\textbf{4.8} \pm \textbf{0.1} \S$ | $3.5\pm0.1\S^{*}$ | $\textbf{6.5} \pm \textbf{0.7}^{*}$ |
| FFA (μ mol l $^{-1}$) | СНО | 57 ± 9 | $452 \pm \mathbf{98^*}$ | 34 ± 15 |
| | F | $358\pm113\S$ | $1680\pm165\S^*$ | $44\pm18^{*}$ |
| Glycerol (μ mol l $^{-1}$) | СНО | 58 ± 3 | $144\pm23^*$ | 51 ± 6 |
| | F | $77\pm8\S$ | $522\pm54\S^*$ | $54\pm7^{*}$ |
| Hormones | | | | |
| Insulin (μ U ml $^{-1}$) | СНО | $\textbf{36.7} \pm \textbf{9.3}$ | $\textbf{12.1} \pm \textbf{1.8}^{*}$ | $\textbf{26.8} \pm \textbf{3.0}$ |
| | F | $4.6\pm0.9\S$ | $1.3\pm0.5\%$ | $48.0\pm11.8^*$ |
| Leptin (ng ml ⁻¹) | СНО | $\textbf{2.74} \pm \textbf{0.3}$ | $\textbf{2.23} \pm \textbf{0.4}^{*}$ | $\textbf{3.06} \pm \textbf{0.5}$ |
| | F | $\textbf{2.39}\pm\textbf{0.4}$ | $\textbf{1.20} \pm \textbf{0.2} \S^*$ | $1.16\pm0.3\S^*$ |
| Adrenaline (μ g l ⁻¹) | СНО | $\textbf{0.10} \pm \textbf{0.01}$ | $\textbf{0.13} \pm \textbf{0.03}$ | $\textbf{0.09} \pm \textbf{0.01}$ |
| - | F | $\textbf{0.10} \pm \textbf{0.01}$ | $0.26 \pm 0.05 \S^*$ | $\textbf{0.09} \pm \textbf{0.01}$ |
| Noradrenaline (μ g l ⁻¹) | СНО | $\textbf{0.29} \pm \textbf{0.03}$ | $\textbf{0.63} \pm \textbf{0.05}^{*}$ | $0.39\pm0.04^*$ |
| | F | $\textbf{0.24} \pm \textbf{0.03}$ | $\textbf{0.70} \pm \textbf{0.08}^{*}$ | $0.40\pm0.05^{*}$ |
| Cortisol (μ g dl $^{-1}$) | СНО | 17.1 ± 1.6 | $\textbf{16.1} \pm \textbf{1.0}$ | 15.0 ± 1.6 |
| - | F | $\textbf{18.3} \pm \textbf{1.0}$ | $\textbf{20.2} \pm \textbf{3.0}$ | $11.8\pm1.4^{*}$ |

Table 1. Effect of exercise in the fasted versus carbohydrate-fed state on blood metabolites and hormones

Blood metabolites and hormones as measured before, immediately after and 4 h after exercise. Data provided are means \pm s.E.M. (n = 9); *P < 0.05 versus pre-exercise; §P < 0.05 versus carbohydrate fed. See Methods section for further details.

| Table 2. | Effect of | f exercise | in the | fasted | versus | carbo | hyd | rate- | fed | state o | n gas | exch | nange a | and | oxid | ation | rate | calcu | Ilatio | ns |
|----------|-----------|------------|--------|--------|--------|-------|-----|-------|-----|---------|-------|------|---------|-----|------|-------|------|-------|--------|----|
| during t | he 2 h ex | ercise bo | ut | | | | | | | | | | | | | | | | | |

| | | | Time (min) | P value | | | |
|--|-----|-----------------------------------|-----------------------------------|-----------------------------------|-----------|--------|--|
| | | 0 | 60 | 120 | Treatment | Time | |
| Gas exchange | | | | | | | |
| \dot{V}_{O_2} (I min ⁻¹) | CHO | $\textbf{2.6} \pm \textbf{0.1}$ | $\textbf{2.8} \pm \textbf{0.1}$ | $\textbf{2.8} \pm \textbf{0.1}$ | 0.25 | 0.007 | |
| 2 | F | $\textbf{2.7} \pm \textbf{0.1}$ | $\textbf{2.9} \pm \textbf{0.09}$ | $\textbf{2.9} \pm \textbf{0.1}$ | | | |
| | СНО | $\textbf{2.4} \pm \textbf{0.08}$ | $\textbf{2.5} \pm \textbf{0.09}$ | $\textbf{2.4} \pm \textbf{0.06}$ | 0.36 | 0.23 | |
| 2 | F | $\textbf{2.4} \pm \textbf{0.1}$ | $\textbf{2.4} \pm \textbf{0.09}$ | $\textbf{2.3} \pm \textbf{0.08}$ | | | |
| RER | СНО | $\textbf{0.93} \pm \textbf{0.02}$ | $\textbf{0.89} \pm \textbf{0.01}$ | $\textbf{0.88} \pm \textbf{0.01}$ | 0.001 | 0.0002 | |
| | F | $\textbf{0.88} \pm \textbf{0.02}$ | $\textbf{0.84} \pm \textbf{0.01}$ | $\textbf{0.81} \pm \textbf{0.01}$ | | | |
| Oxidation rates | | | | | | | |
| Fat (g min ⁻¹) | СНО | $\textbf{0.29} \pm \textbf{0.11}$ | $\textbf{0.52} \pm \textbf{0.08}$ | $\textbf{0.62} \pm \textbf{0.10}$ | 0.004 | 0.0001 | |
| - | F | $\textbf{0.56} \pm \textbf{0.08}$ | $\textbf{0.77} \pm \textbf{0.05}$ | $\textbf{0.94} \pm \textbf{0.08}$ | | | |
| Carbohydrates (g min ⁻¹) | СНО | $\textbf{2.73} \pm \textbf{0.17}$ | $\textbf{2.35} \pm \textbf{0.14}$ | $\textbf{2.07} \pm \textbf{0.16}$ | 0.003 | 0.0001 | |
| | F | $\textbf{2.17} \pm \textbf{0.23}$ | $\textbf{1.80} \pm \textbf{0.18}$ | $\textbf{1.36} \pm \textbf{0.15}$ | | | |

Oxygen uptake (\dot{V}_{O_2}), carbon dioxide production (\dot{V}_{CO_2}) and respiratory exchange ratio (RER) as measured over 5 min intervals at different time points during exercise. Workload during 2 h exercise bout was fixed at 178 ± 8 Watt. Fat and carbohydrate oxidation rates were calculated using the nonprotein respiratory quotient (see Methods for further details). Values are expressed as means ± s.E.M. (n = 9). No interaction effects were recorded using ANOVA for repeated measurements. *P*-values for overall treatment and time effects are given.

ranged between 10.1 ± 2.4 and $11.2 \pm 2.3 \,\mu$ mol l⁻¹ and did not differ between experimental conditions or time points (results not shown).

Gas exchange

Gas exchange was measured at the start, halfway through and at the end of the 2 h constant-load exercise bout $(178 \pm 8 \text{ W})$ (Table 2). \dot{V}_{O_2} and \dot{V}_{CO_2} were not significantly different between conditions at any time. However, RER consistently was lower in F than in CHO (P = 0.0001). Accordingly, in F the calculated fat oxidation rate was higher (P = 0.03), whilst carbohydrate oxidation rate was lower (P = 0.003). Furthermore, fat oxidation rate gradually increased, while carbohydrate oxidation decreased as the exercise proceeded.

Discussion

The primary hypothesis driving this study was that IMTG breakdown during exercise in the fasted state would be increased when compared with postprandial exercise. In support of this hypothesis, we found that 2 h of submaximal exercise performed after an overnight fast and in the absence of carbohydrate intake during exercise depletes IMTG in type I fibres on the average by $\sim 60\%$ (Fig. 1A), which confirms recent findings by Van Loon et al. (2003). However, the same exercise bout performed with carbohydrate intake before ($\sim 2.5 \text{ g} (\text{kg bw})^{-1}$) and during $(\sim 1 \text{ g} (\text{kg bw})^{-1} \text{ h}^{-1})$ exercise, did not cause type I fibre IMTG content to decrease. Thus, our findings for the first time demonstrate that the effect of prolonged endurance exercise of markedly stimulating IMTG breakdown in type I fibres in the fasted state can be completely inhibited by a high rate of carbohydrate intake before and during exercise. We also found that IMTGs did not significantly decrease in type IIa fibres (Fig. 1B), and this was independent of the dietary regimen used. Given the mixed composition of m. quadriceps, combined with the fact that during submaximal exercise predominantly motor units composed of type I fibres are being recruited, use of a fibre-specific approach can probably be recommended when studying IMTG metabolism in human muscle during exercise, at least if one wants to obtain a reasonable degree of sensitivity to detect changes.

The cellular mechanisms involved in regulation of IMTG metabolism during exercise are poorly understood. It is clear that IMTG breakdown during muscle contractions reflects the balance between lipolysis and FFA re-esterification (Dyck & Bonen, 1998). However, in exercising humans the rate of re-esterification represents only a minor fraction of total IMTG turnover (Sacchetti et al. 2002). Available evidence indicates that IMTG hydrolysis during muscle contractions is probably driven mainly by the enzyme hormone-sensitive lipase (HSL). HSL activity, for a given exercise intensity, is enhanced by an elevated adrenaline: insulin ratio (Donsmark et al. 2004; Watt & Spriet, 2004). In the present study, this ratio was markedly enhanced by exercise in the fasted state (Table 1). By analogy, it was recently shown that glucose ingestion during exercise, resulting in increased insulin versus decreased circulating adrenaline concentration, blunted HSL activity (Watt et al. 2004b).

There has been some recent controversy with regard to the potential role of AMP-activated protein kinase (AMPK) in regulation of IMTG breakdown during exercise (Watt *et al.* 2004*c*; Roepstorff *et al.* 2004). In adipocytes, pharmacological activation of AMPK by the administration of AICAR inhibits β -adrenergic activation of HSL (Sullivan *et al.* 1994). If such a mechanism also applies to skeletal muscle, one could reasonably assume that AMPK activation during exercise in the fasted state could inhibit IMTG degradation. However, as indicated above, compared with exercise in conjunction with adequate carbohydrate intake, IMTG breakdown was increased in the fasted state. Moreover and as expected (Wojtaszewski et al. 2000; Winder, 2002), exercise as such increased the activity of AMPK through increased phosphorylation, which was accompanied by corresponding increase of the phophorylation status of its downstream target, ACC, and these effects were independent of nutritional status. Thus, our current data demonstrate that AMPK is not important in regulation of net IMTG degradation during exercise. This contention is corroborated by the recent findings that the differential activation of AMPK α 2 during exercise in a carbohydrate-depleted versus carbohydrate-repleted condition does not translate into changes in HSL activity or net IMTG breakdown (Roepstorff et al. 2004).

Watt et al. (2004a) recently showed that a pharmacological reduction of plasma FFA availability by the administration of nicotinic acid enhanced IMTG degradation during a 3-h submaximal (~60% $\dot{V}_{O_2,peak}$) exercise bout in the fasted state. They concluded that in circumstances in which plasma FFA availability is unlimited, net IMTG degradation during prolonged exercise is negligible. Our current findings invalidate this conclusion. The high rate of IMTG degradation in type I fibres during exercise in the fasted state coexisted with plasma FFA concentrations which were elevated far above the levels established by exercise in the carbohydrate repleted state (1.7 versus 0.5 mmol l^{-1}). In fact, in the latter condition IMTG breakdown was substantially inhibited. Thus, our findings indicate that the increase in plasma FFA concentration inherent to endurance exercise in normal 'physiological' conditions, does not play a significant role in regulation of net IMTG breakdown. Indeed, in the aforementioned study by Watt and coworkers, pharmacological blockade of the exercise-induced increase in plasma FFA by the administration of nicotinic acid, causing circulating FFA levels during exercise to be abnormally low ($\sim 0.1 \text{ mmol } l^{-1}$), markedly stimulated net IMTG breakdown during exercise. Still the increased plasma adrenaline level due to nicotinic acid intake may conceivably have confounded the presumed causal relationship between reduced plasma FFA level and enhanced IMTG degradation.

We hypothesized that a greater degree of muscle IMTG depletion caused by exercise in the fasting state could facilitate postexercise glycogen resynthesis. In agreement with our hypothesis, we found mean rate of muscle glycogen resynthesis during the initial 4 h recovery period after the fasting exercise bout to be \sim 3-fold higher than after exercise combined with carbohydrate ingestion (Fig. 2), which is compatible with enhanced insulin action. Whether this response involved increased insulin sensitivity is unclear from the present findings.

However, the same amount of carbohydrate intake during recovery resulted in a 2-fold higher systemic insulin level, despite similar blood glucose concentration (Table 1). It is well established that acute exposure of β -cells to a high extracellular FFA concentration can potentate glucose-induced insulin secretion (McGarry & Dobbins, 1999; Deeney et al. 2000; Yaney et al. 2000). In fact, elevated plasma FFA level has been implicated in sustaining insulin secretion during fasting (Dobbins et al. 1998; McGarry & Dobbins, 1999). Thus, FFA potentiation of glucose-induced insulin secretion probably at least partly explains the facilitated muscle glycogen resynthesis following exercise in the fasted state. Surprisingly, this higher rate of glycogen resynthesis occurred in the absence of elevated postexercise glycogen synthase activity (Fig. 2*B*). However, at the end of the 4 h recovery period, increased insulin action probably was overruled by the higher muscle glycogen concentration (Nielsen *et al.* 2001) which had developed by that time.

It has been demonstrated that the excess oxygen consumption (Bahr et al. 1990; Borsheim & Bahr, 2003) during recovery after prolonged endurance exercise is at least partly accounted for by IMTG oxidation (Kiens & Richter, 1998; Bruce et al. 2004). Our current data extend this finding by showing that the degree of postexercise IMTG degradation markedly differs between fibre types and depends on the dietary regimen before and during exercise. During the 4 h recovery period following exercise in conjunction with carbohydrate intake, IMTG content decreased by \sim 30% in type I fibres whereas no significant decrease (-17%) occurred in type IIa fibres. Interestingly, no further IMTG degradation was present after exercise in the fasted state. In resting muscle, low plasma FFA concentration (Chalkley et al. 1998; Boden et al. 2001; Schrauwen-Hinderling et al. 2003), in face of low plasma insulin (Dyck et al. 2001; Watt et al. 2004b), versus high leptin levels (Muoio et al. 1997; Muoio et al. 1999) and high catecholamines (Langfort et al. 1999) stimulate net IMTG oxidation. Such a condition was pertinent during recovery after exercise in the carbohydrate fed state (Table 1). Conversely, following the exercise bout in the fasted state, higher plasma FFA and insulin levels coexisted with low leptin levels and could have inhibited IMTG oxidation. It has been shown that the action of leptin to stimulate net fat oxidation, and thus prevent re-esterification in resting skeletal muscle, is effected by activation of AMPK and downstream inhibition of ACC (Minokoshi et al. 2002). In the current experimental conditions, however, the marked difference in postexercise plasma leptin concentration was not accompanied by different AMPK phosphorylation status between experimental conditions (Fig. 3). This indicates either that leptin is not involved in the regulation of postexercise IMTG-FFA cycling, or that leptin impacts on IMTG-FFA cycling by a signalling pathway which is independent of AMPK.

Data in the literature suggest that FFA import into muscle cells in excess of the potential for mitochondria to oxidize FFA is an important stimulus to increase UCP3 gene transcription. Such a hypothesis is compatible with the close relationship between plasma FFA concentration and UCP3 mRNA content in muscles at rest after fasting, found in the present study (r = 0.73; P = 0.02) and in previous studies (Schrauwen et al. 2002a; Sbraccia et al. 2002). Schrauwen and coworkers also concluded that the increase in UCP3 gene transcription induced by exercise is modulated by the associated elevation of plasma FFA concentration, rather than by contractions per se. This conclusion was based on the observation that suppression of the exercise-induced increase of plasma FFA level by glucose ingestion abolished the exercise-induced rise in UCP3 mRNA content. Accordingly, we found exercise in the fasted state to markedly increase muscle UCP3 mRNA content, whereas an increase was absent during exercise with carbohydrate intake (Fig. 4). However, no correlation was found between plasma FFA levels and UCP3 mRNA content during exercise (r = 0.07) in any condition, indicating a mismatch between plasma FFA level and UCP3 gene transcription during exercise. Presumably contractile activity obliterated the apparent relationship between plasma FFA levels and UCP3 mRNA content found in resting muscle.

In conclusion, the current study shows that IMTG breakdown during prolonged submaximal exercise in the fasted state takes place predominantly in type I fibres. Furthermore, we show for the first time that IMTG breakdown during exercise is completely prevented in the CHO-fed state. In addition, exercise in the fasted state enhances the postexercise insulin response to glucose ingestion, which in turn is likely to contribute to stimulation of postexercise muscle glycogen resynthesis. Finally, carbohydrate ingestion, either before, during or after exercise, is a potent inhibitor of UCP3 gene expression in skeletal muscle.

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Acknowledgements

The authors thank Jørgen F. Wojtaszewski for careful reading and discussion of this manuscript. Betina Bolmgreen is thanked for skilful technical assistance. This study was supported by grants from the Onderzoeksraad K.U. Leuven (grant no. OT04/45), the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (grant no. G.0233.05), the Copenhagen Muscle Research Centre, the Media and Grants Secretariat of the Danish Ministry of Culture, the Danish Diabetes Association, the Novo Nordisk Foundation, the Danish Medical Research Council, and the European

Commission (Integrated Project LSHM-CT-2004-005272). B.O.E. obtained a postdoctoral research fellowship from the Onderzoeksraad K.U. Leuven and W.D. is a recipient of a postdoctoral fellowship from the Flemish Fonds voor Wetenschappelijk Onderzoek Vlaanderen. The monoclonal MHC antibodies, developed by Helen M. Blau, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA 52242, USA).