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Training with low muscle glycogen enhances fat metabolism in well-trained cyclists

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Running title: Training with low muscle glycogen

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Abstract

Purpose: To determine the effects of training with low muscle glycogen on exercise performance, substrate metabolism, and skeletal muscle adaptation. **Methods:** Fourteen well-trained cyclists were pair-matched and randomly assigned to HIGH or LOW-glycogen training groups. Subjects performed 9 aerobic training (AT; 90 min at 70% VO_2max) and 9 high-intensity interval-training sessions (HIT; 8 x 5 min efforts, 1 min recovery) during a 3-wk period. HIGH trained once daily, alternating between AT on day 1 and HIT the following day, whereas LOW trained twice every second day, firstly performing AT and then 1 h later performing HIT. Pre and post-training measures were a resting muscle biopsy, metabolic measures during steady state cycling (SS), and a time trial (TT). **Results**: Power output during HIT was 297 ± 8 W in LOW compared with 323 ± 9 W in HIGH ($P<0.05$), however, TT performance improved by ~10% in both groups ($P<0.05$). Fat oxidation during SS increased after training in LOW (from 26±2 to 34±2 μmol/kg/min, $P<0.01$). Plasma FFA oxidation was similar before and after training in both groups but muscle-derived triacylglycerol oxidation increased after training in LOW (from 16±1 to 23±1 μmol/kg/min, $P<0.05$). Training with low muscle glycogen also increased β-hydroxyacyl-CoA-dehydrogenase protein content ($P<0.01$). **Conclusion**: Training with low muscle glycogen reduced training intensity and, in terms of performance, was no more effective than training with high muscle glycogen. However, fat oxidation was increased after training with low muscle glycogen, which may have been due to enhanced metabolic adaptations in skeletal muscle.

Key words: Training adaptation, skeletal muscle, substrate metabolism, exercise performance

Introduction

Paragraph 1

Carbohydrate (CHO), mainly muscle glycogen, is the predominant substrate utilized during prolonged exercise at intensities above 65% of maximal oxygen uptake (VO_2max) (36, 43, 44), and glycogen depletion is closely linked with the development of fatigue. Consuming a CHO-rich diet can increase muscle glycogen content (3, 14, 23), delay the onset of fatigue (3), and maintain performance during periods of intensive training (1). Therefore, endurance athletes consume large amounts of CHO in the belief that training longer and/or more intensely, as a result of increased glycogen availability, will promote superior training adaptations (15).

Paragraph 2

Regular endurance training induces a number of adaptations that enhance performance, including an increase in VO_2max and a shift in substrate metabolism from CHO to lipid oxidation (17, 27). Such changes are largely the result of increased mitochondrial volume (20), increased capillary density (27), and greater activity of oxidative enzymes such as citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) (11, 27). These adaptations occur as a result of an accumulation of specific proteins and thus altered gene expression is considered to be an important process underlying the adaptive response to endurance exercise (12). Interestingly, it appears that altered substrate availability might play a role in the regulation of exercise-induced gene expression. For example, commencing an acute bout of endurance exercise with low muscle glycogen induces a greater increase in the transcriptional activity of several metabolic genes when compared to exercise performed with normal glycogen stores (10, 24, 33).

Paragraph 3

Furthermore, Hansen et al. (12) reported that untrained subjects who completed 10 wk of aerobic training, 50% of which commenced with low muscle glycogen, had more pronounced increases in resting muscle glycogen content and CS activity when compared with the same volume of training performed with normal glycogen concentrations. Training with low muscle glycogen also resulted in a twofold greater increase in exercise capacity (time to fatigue at 90% peak power output) when compared with the same volume of training undertaken with normal glycogen. These findings demonstrate that under certain circumstances training with low muscle glycogen can be beneficial. However, a number of details make it difficult to extrapolate these findings. First, the subjects were untrained and it is not yet known if training with low muscle glycogen will translate into improved adaptations in already well-trained athletes. Second, the subjects performed a fixed amount of work in each training bout even though higher glycogen stores would allow more exercise and/or higher intensities. Third, the results are from single leg kicking exercise and not a competitive sport. Considering these points, further research is clearly needed before glycogen depleted training can be recommended as a practical strategy to enhance training adaptations and performance in already well-trained athletes.

Paragraph 4

One recent study (49), using a similar training model to that of the Hansen study (i.e. training twice every second day vs. once daily), but in well-trained cyclists, found that self-selected training intensity was reduced when high-intensity interval training (HIT) commenced with low muscle glycogen. However, after completing the training

period, time trial cycling performance was improved to a similar extent in high and low glycogen groups. Another interesting observation was that training with low muscle glycogen resulted in higher rates of whole-body fat oxidation during exercise, whereas training with high muscle glycogen had no effect on substrate metabolism. The aim of the present study was twofold. Firstly, we aimed to confirm the recent findings of Yeo et al. (49). Secondly, using stable isotope tracers, we aimed to examine in greater detail the reported changes in substrate metabolism after training with low muscle glycogen. Specifically, by infusing [U-^{13}C]palmitate and [6,6-^{2}H$_2$]glucose we were able to quantify plasma and muscle-derived CHO and fat oxidation and thus gain a better understanding of adaptations in substrate metabolism after training with low muscle glycogen.

Method

Paragraph 5

Subjects

Fourteen endurance-trained male cyclists who had not undertaken any high-intensity interval training in the 4 wk prior to the investigation were recruited to participate in this study. Subject characteristics can be seen in Table 1. Subjects were informed of the potential risks involved with the experimental procedures before providing their written consent. The study was approved by the Local Research Ethics Committee (Solihull REC, UK).

Insert Table 1 here

Paragraph 6

Preliminary testing

Prior to baseline measurements, subjects visited the laboratory on two separate occasions. During the first visit, subjects performed an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) for determination of VO_2max and maximum power output (Wmax). The test began at 95 W, followed by 35 W increments every 3 min thereafter. Gas exchange measurements were performed during the final min of each stage using traditional Douglas bag techniques, with VO_2 and VCO_2 calculated using conventional equations. Approximately 3-5 days later, subjects returned to the laboratory, where they were infused with [1,2-^{13}C]acetate during 60 min rest and 60 min cycling at 70% VO_2max. Expired breath samples were collected every 15 min for measures of VO_2, VCO_2 and $^{13}CO_2$ enrichment. The acetate infusion was performed in order to determine an individual acetate recovery factor for each subject to correct [U-^{13}C]palmitate oxidation rates for carbon label retention within the

bicarbonate pools and by way of isotopic exchange reactions within the tricarboxylic acid cycle (acetate recovery was on average 89%). After 2 h of infusion, and on completion of the cycle task, the acetate infusion was stopped and subjects began a 60 min time trial. Subjects were later pair-matched for VO_2max, Wmax, TT performance and training history, and randomly assigned to either HIGH or LOW-glycogen training groups.

Paragraph 7

General experimental design

The experiment consisted of three separate phases: *1*) baseline measures, *2*) a 3 wk training intervention, and *3*) post-training measures.

Paragraph 8

Baseline measures

Muscle biopsy

A muscle biopsy (~80 mg) was obtained from the Vastus Lateralis under local anesthetic (2% lidocaine), using the percutaneous needle-biopsy technique (2) modified for use with suction (9). Samples were immediately frozen in liquid nitrogen and stored at -80°C until later analysis. All muscle biopsies were taken in the morning after an overnight fast, and 48 h after the last exercise bout to avoid the transient effect of exercise on transcriptional activity which can last up to 24 h (47).

Paragraph 9

Experimental trial

This experiment was performed at least 48 h after subjects underwent the muscle biopsy procedure to ensure that there would be no difficulties in completing the exercise task. Subjects reported to the laboratory in the morning after an overnight fast. Teflon catheters (Venflon, Becton Dickinson, Plymouth, UK) were inserted into the antecubital veins of each arm to allow stable isotope infusion and repeated blood sampling. After baseline blood and expired breath samples were obtained subjects received a $[6,6\text{-}^2H_2]$glucose (priming dose 26 µmol/kg; continuous rate 0.7 µmol/kg/min) and $[U\text{-}^{13}C]$palmitate infusion (no prime; continuous rate 16.5 nmol/kg/min) by means of calibrated syringe pump (Asena GS Syringe Pump, Alaris Medical Systems, Basingstoke, UK). Subjects then rested in a semi-supine position for 60 min, allowing sufficient time for isotopic equilibrium to be reached, before commencing steady-state cycling for 60 min at 70% VO_2max. Blood and expired breath samples were collected at the end of the resting period and at 15-min intervals during exercise for determination of isotopic enrichments, metabolite concentrations, and measures of VO_2 and VCO_2.

Paragraph 10

On completion of the 60 min steady-state cycle test, the ergometer was adjusted to the cadence dependant (linear) mode and subjects completed a set amount of work (1017 ± 73 kJ) as fast as possible. The total amount of work to be performed was calculated using the formula:

Total work (J) = 0.75 x Wmax x 3600 s.

Paragraph 11

The only information available to the subjects during the time trial was elapsed work and percentage of work performed (i.e. 0% at the start and 100% on completion). No blood or respiratory measures were taken and every effort was made to minimize any possible distractions. These are standard testing procedures used in our laboratory (7).

Paragraph 12

Training intervention

Training consisted of 9 aerobic training (AT) and 9 high-intensity interval training (HIT) sessions spread over a 3 wk period. All training sessions took place in the laboratory under the careful supervision of the experimenters. AT was performed on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) and consisted of 90 min continuous cycling at ~70% VO_2max. HIT was performed on the subjects' own bicycle, attached to a stationary trainer, fitted with power measuring SRM-cranks (Schoberer Rad Messtechnik, Julich, Germany), and consisted of a 20 min warm-up followed by 8 x 5 min "all-out" efforts interspersed with 1 min of recovery. The metabolic demands of this type of training have been reported elsewhere (43). This setup, using a stationary trainer and the subjects' own bicycle, was chosen for the self-paced HIT sessions because it enabled the subject to instantly adjust power output through changing gear and/or pedaling speed. SRM cranks were factory calibrated prior to the start of the study, and the zero offset was checked on a daily basis according to the manufacturer instructions.

Paragraph 13

In order to manipulate pre-exercise muscle glycogen content, each group trained according to different schedules. HIGH trained once daily, alternating between AT on day 1 and HIT the following day, whereas LOW trained twice every second day, firstly performing AT and then 1 h later performing HIT. All training sessions were performed in the morning after an overnight fast, and subjects continued to fast until the entire training session was completed. Water intake was allowed during training sessions. We did not take muscle biopsies to confirm muscle glycogen content, however, it was assumed that the long (20-24 h) recovery period in HIGH would allow adequate time for glycogen resynthesis between each training session (providing sufficient CHO intake), whereas the short (1 h) recovery period in LOW would ensure that HIT began with reduced muscle glycogen. This assumption is confirmed by a recent study using exactly the same training intervention and a similarly trained population of elite male cyclists (48). In that study, subjects training twice every second day began HIT with 50% less muscle glycogen than subjects training just once daily. Self-selected power output during HIT was used as a measure of training intensity.

Paragraph 14

Post-training measures

The nature and timing of post-training measures was identical to that of baseline measures. A muscle biopsy sample was obtained 48 h after the final training session, using the same leg as the baseline sample but separated by 3-5 cm from the first incision. A further 48 h later, subjects performed a 60 min steady-state cycle test at the same absolute workload as baseline measures (i.e. ~70% of pre-training VO_2max). This was immediately followed by a time trial designed to last ~60 min.

Paragraph 15

Diet control

The nutritional status of subjects was controlled for 24 h before the experimental measures (muscle biopsy, 60 min steady-state cycle test and time trial). Subjects were provided with a standard diet consisting of 67.5% CHO (8 g/kg body mass), 13.5% protein and 19% fat. Throughout the 3 wk training intervention subjects were asked to maintain a high CHO diet and were given detailed instructions on how to achieve this.

Paragraph 16

Analyses

Blood samples (10 mL) were collected into EDTA-containing tubes (Becton Dickinson, Plymouth, UK) and centrifuged at 3000 rpm for 10 min at 4ºC. Aliquots of the plasma were immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Plasma glucose (Glucose HK, ABX Diagnostics, UK) and free fatty acid (FFA) (NEFA-C Kit, Alpha Laboratories, UK) concentrations were analyzed using a semi-automatic analyser (Cobas Mira Plus, ABX, UK).

Paragraph 17

Expired breath samples were analyzed for $^{13}C/^{12}C$ ratio by gas chromatography continuous-flow isotope ratio mass spectrometry (GC-IRMS) (Trace GC Ultra; IRMS Delta Plus XP; both Thermo Finnigan, Herts, UK). For determination of plasma palmitate kinetics, FFA were extracted from plasma, isolated by solid phase extraction, and derivatized to their methyl esters. Palmitate concentration was

determined on an analytical GC equipped with flame ionization detection (FID), using heptadecanoic acid as the internal standard, and the tracer-to-tracee (TTR) ratio of [U-^{13}C]palmitate was determined using GC-combustion IRMS. After derivatization with heptafluorobutyric acid, plasma enrichment was determined by electron ionization GC-mass spectrometry (GC, Agilent 6890N; MS, Agilent 5973N; both Agilent Technologies, Stockport, UK).

Paragraph 18

Glycogen content

A section of muscle (~20 mg) was freeze dried and dissected free of connective tissue, blood and other non-muscle tissue. Samples were then homogenized using pestle and mortar, and 500 µL of 1 M hydrochloric acid was added to 2-3 mg portions and heated at 100°C for 3 h. After cooling to room temperature, samples were neutralized with 250 µl of a tris/KOH mixture (1.44 g tris and 12 g KOH in 100 mL of distilled water saturated with KCL), centrifuged at 3000 rpm for 10 min at a temperature of 4ºC and the resulting supernatant analyzed enzymatically for glucose concentration (Glucose HK, ABX Diagnostics, UK).   Muscle glycogen concentrations were expressed as millimoles of glucosyl units per kg dry mass (mmol/kg dm).

Paragraph 19

Protein content

Antibodies

The following primary antibodies were used at a 1:1000 dilution. Rabbit polyclonal fatty acid translocase CD36 (FAT/CD36, AbCam: ab36977), mouse monoclonal cytochrome *c* oxidase subunit 2 (COX2, Molecular Probes: A11142), subunit 5

(COX5, Molecular Probes: A21351), chicken polyclonal antibody recognizing β-hydroxyacyl-CoA-dehydrogenase (β-HAD, AbCam: ab37673), rabbit polyclonal antibody recognizing eukaryotic elongation factor 2 (eEF2: Cell Signalling: #2332) and a mouse monoclonal antibody for GLUT4 (Santa Cruz Biotechnology: sc-53566).

Paragraph 20

Tissue preparation

Muscle samples were powdered on dry-ice, weighed and aliquoted into 1.5 mL eppendorf tubes for subsequent analysis. Approximately 30 mg of powdered tissue was added to 250 μL of lysis buffer (50 mM Tris pH 7.5; 250 mM Sucrose; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 1 mM NaVO4; 50 mM NaF; 0.10% DTT; 0.50% PIC). The samples were mixed at 1200 rpm and 4ºC for 30 min and then homogenized on ice for ~30 sec at slow speed (Polytron), the lysate centrifuged for 5 min at 12,000 rpm and the supernatant removed for protein determination. Protein concentration was determined using the DC protein assay (BioRad).

Paragraph 21

Western Blot

Equal aliquots of muscle protein were solubilised in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; 5% β-mercaptoethanol), and the samples were boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5 or 15% acrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Protran, Whatman, Dassel, Germany) at 100 V for 1 h. The membrane was blocked for 1 h in 3% milk in Tris-buffered saline + 0.1% Tween (TBST). Membranes were

incubated overnight at 4°C with appropriate primary antibody in TBST (1:1000 dilutions). The membrane was then washed 3 times in TBST before incubation for 1 h at room temperature with peroxidase-conjugated IgG secondary antibody in TBST (1:10,000 dilution; Pierce, Rockford, IL). Antibody binding was detected using enhanced chemiluminescence HRP substrate detection kit (Millipore, Billerica, MA) and imaging and band quantification were carried out using a ChemiGenius Bioimaging Gel Doc System (Syngene, Cambridge, UK). All individual pre and post samples were run on the same gel to minimize gel-to-gel variation whilst protein content was normalized to eEF2, which served as an internal control.

Paragraph 22

Calculations

Rates of whole body CHO and fat oxidation were calculated using stoichiometric equations (22), with the assumption that protein oxidation was negligible:

CHO oxidation (g/min) = 4.210 x VCO_2 - 2.962 x VO_2

Fat oxidation (g/min) = 1.695 x VO_2 - 1.701 x VCO_2

Rates of appearance (Ra) and disappearance (Rd) of glucose and palmitate were calculated using the single pool non-steady-state equations of Steele (39), modified for use with stable isotopes (46):

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

$$Rd = Ra - V\left(\frac{C_2 - C_1}{t_2 - t_1}\right)$$

Where F is the infusion rate (μmol/kg/min); V is the volume of distribution for glucose or palmitate (0.16 and 0.04 L/kg, respectively); C_1 and C_2 are the plasma glucose or palmitate concentrations (μmol/L) at time points t_1 and t_2, respectively; and E_1 and E_2 are plasma glucose or palmitate enrichments (TTR) at time points t_1 and t_2, respectively. $^{13}CO_2$ production ($Pr^{13}CO_2$; mol/min) from the infused palmitate was calculated as:

$$Pr^{13}CO_2 = (TTRCO_2 \times VCO_2)/(k \times Ar)$$

Where $TTRCO_2$ is the breath $^{13}C/^{12}C$ ratio at a given time point; VCO_2 is carbon dioxide production (L/min); k is the volume of 1 mol of CO_2 (22.4 L/mol); and Ar is the fractional recovery of ^{13}C label recovery in breath CO_2 after infusion of labeled acetate, calculated as:

$$Ar = [(TTRCO_2 \times VCO_2)/(k \times 2F)]$$

Where F is infusion rate of $[1,2-^{13}C]$acetate (mol/min). Plasma palmitate oxidation (Rox; mol/min) can then be calculated as:

$$\text{Rox palmitate} = \text{Rd palmitate} \times (Pr^{13}CO_2/F \times 16)$$

Where Rd palmitate is the rate of disappearance of palmitate (mol/min); F is the palmitate infusion rate (mol/min); and 16 is the number of carbon atoms in palmitate. Total FFA oxidation was calculated by dividing plasma oxidation by the fractional

contribution of plasma palmitate to total plasma FFA concentration (plasma palmitate concentrations were on average 24% of total plasma FFA). Muscle-derived triacylglycerol (TG) use was estimated by subtracting plasma FFA oxidation from total fat oxidation. However, it should be mentioned that these stable isotope methods do not differentiate between muscle- and lipoprotein-derived TG use. Nonetheless, these methods are routinely used to study exercise metabolism as the contribution of lipoprotein-derived TG oxidation to energy expenditure is assumed to be minimal. Rd glucose was taken to represent plasma glucose oxidation because 100% of Rd glucose is oxidized during exercise (21). Therefore, muscle glycogen oxidation was calculated by subtracting plasma glucose oxidation from total CHO oxidation. Calculations of tracer kinetics and substrate metabolism were performed over the final 30 min of exercise (30 – 60 min period).

Paragraph 23

Statistical analysis

All data were analyzed using a two-factor ANOVA, with one between subject factor (group; HIGH *vs.* LOW) and one within subject factor (time; pre-training *vs.* post-training). The level of significance was set at $P < 0.05$ and significant interactions were followed up with Tukey's honestly significant difference *post hoc* test. All data are presented as means ± standard error of the mean (means ± SE) unless otherwise stated.

Results

Paragraph 24

Performance data

Self-selected training intensity

HIT power output increased throughout the training period (main effect for time, $P <$ 0.001, Fig. 1). Average power output was 323 ± 9 W in HIGH compared with 297 ± 8 W in LOW (main effect for group, $P < 0.05$). This represents 87 ± 1 and 78 ± 2% Wmax for HIGH and LOW groups, respectively.

Insert Fig 1 here

Paragraph 25

Time trial performance before and after training

After training, mean power output during the time trial increased from 271 ± 13 to 298 ± 13 W in HIGH and from 278 ± 11 to 307 ± 10 W in LOW (main effect for time, $P < 0.001$, Fig. 2). Time taken to complete the task decreased from 62.10 ± 1.49 to 56.37 ± 1.17 min in HIGH and from 61.90 ± 1.12 to 56.12 ± 1.22 min in LOW (main effect for time, $P < 0.001$). Performance was enhanced by a similar extent in both groups (10.2% in HIGH and 10.5% in LOW).

Insert Fig 2 here

Paragraph 26

Cycling at 70% VO$_2$max before and after training

VO$_2$, RER and whole body substrate metabolism

Subjects cycled at the same absolute workload before and after training (241 ± 13 W in HIGH and 247 ± 16 W in LOW). VO$_2$ was unaffected by training ($P > 0.05$, Table 2). RER was unaffected by training in HIGH but decreased after training in LOW (group x time interaction, $P < 0.001$, Table 2). Accordingly, there was a significant

decrease in the rate of CHO oxidation (from 220 ± 8 to 194 ± 10 μmol/kg/min) and a

significant increase in the rate of fat oxidation (from 26 ± 2 to 34 ± 2 μmol/kg/min)

after training in LOW ($P < 0.01$, Table 2).

Insert Table 2 here

Paragraph 27

Tracer data

Plasma glucose and palmitate kinetics are shown in Table 3 and rates of substrate

oxidation are shown in Table 2. Ra and Rd glucose were unaffected by training in

HIGH but decreased after training in LOW (group x time interaction, $P < 0.05$, Table

3). Since Rd glucose represents plasma glucose oxidation, there was a significant

decrease in plasma glucose oxidation (from 39 ± 3 to 33 ± 2 μmol/kg/min) after

training in LOW ($P < 0.05$, Table 2). Likewise, tracer estimated rates of muscle

glycogen oxidation decreased significantly (from 181 ±´8 to 161 ± 9 μmol/kg/min)

after training in LOW ($P < 0.05$, Table 2). Ra and Rd palmitate increased after

training (main effect for time, $P < 0.05$, Table 3), however, since Rox palmitate

remained unchanged, %Rd_{ox} decreased after training (main effect for time, $P < 0.05$,

Table 3). Estimated rates of plasma FFA oxidation were unaffected by training

(Table 2).   Oxidation of muscle-derived TG increased (from 16 ± 1 to 23 ± 1

μmol/kg/min) after training in LOW only (group x time interaction, $P < 0.05$, Table

2).

Insert Table 3 here

Paragraph 28

Relative contribution of substrates to energy expenditure

The relative contribution of fat to energy expenditure during exercise was unaffected

by training in HIGH but increased significantly (from 32 ± 2 to 40 ± 2%) after

training in LOW (group x time interaction, $P < 0.05$, Fig. 3). This increase was due to higher rates of muscle-derived TG oxidation (Table 2), which provided $20 \pm 1\%$ of energy expenditure before training and $28 \pm 2\%$ of energy expenditure after training ($P < 0.05$, Fig. 3). The relative contribution of muscle glycogen to energy expenditure during exercise was unaffected by training in HIGH but decreased (from 57 ± 2 to $50 \pm 2\%$) after training in LOW (group x time interaction, $P < 0.05$, Fig. 3). In LOW there was also a small, non-significant, decrease in the contribution of plasma glucose (from 12 ± 1 to $10 \pm 1\%$).

Insert Fig 3 here

Paragraph 29

Skeletal muscle adaptations

Muscle glycogen content

Resting muscle glycogen content increased after training by 18 and 36% in HIGH and LOW, respectively, with no difference between groups (main effect for time, $P < 0.001$, Fig. 4).

Insert Fig 4 here

Paragraph 30

Protein content

COX2 and COX5 protein content was unaffected by training ($P > 0.05$, data not shown). FAT/CD36 protein content increased after training (main effect for time, $P < 0.05$, Fig. 5B), and tended to be more pronounced after training in LOW than HIGH (41.4 vs. 11.5%) but this was not significant. β-HAD protein content increased by 43% after training in LOW but decreased by 20% after training in HIGH (group x time interaction, $P < 0.01$, Fig. 5C). In contrast to the beneficial effect on proteins involved in fatty acid uptake and oxidation, the glucose transporter GLUT4 tended not

to increase as much in the LOW group (LOW = 7.1 ± 8.4% vs. HIGH = 20.6 ± 9.7%, Fig. 5A).

Insert Fig 5 here

Discussion

Paragraph 31

This study determined the effects of training with low muscle glycogen on exercise performance, substrate metabolism, and molecular adaptations in skeletal muscle. In order to manipulate muscle glycogen content each group trained according to different schedules. HIGH trained once daily, alternating between AT on day 1 and HIT the following day, whereas LOW trained twice every second day, firstly performing AT and then 1 h later performing HIT. We therefore assume that HIGH began all training sessions with normal glycogen stores, whereas LOW commenced HIT with reduced muscle glycogen (see method section for further details).

Paragraph 32

One of the main findings of the present study was that whole-body fat oxidation, during moderate-intensity exercise, was increased after training with low muscle glycogen (Table 2). Similar findings have been reported recently elsewhere (49). In that study, however, measures of substrate metabolism were limited to indirect calorimetry, whereas we also applied stable isotope tracers for a more detailed investigation of changes in substrate metabolism. By infusing [U-^{13}C]palmitate we were able to determine plasma palmitate kinetics, plasma FFA oxidation and muscle-derived TG oxidation. We found that plasma FFA oxidation was similar before and after training in both groups, but that muscle-derived TG oxidation was significantly increased after training with low muscle glycogen (Table 2 and Fig. 3). There has been considerable debate regarding the role of muscle TG as a substrate during exercise (for reviews see (26, 45)). However, using three different methodologies (stable isotopes, biochemical extraction and immunofluorescence microscopy),

Stellingwerff et al. (41) clearly demonstrated significant muscle TG use during prolonged sub-maximal exercise. Endurance training has also been shown to increase muscle TG use during exercise in previously untrained men (32). In the present study, training with high muscle glycogen did not increase TG utilization during exercise, suggesting that several years of endurance training had already maximized the capacity for muscle TG use in these subjects. Therefore, it is interesting to find that training for just 3 weeks with low muscle glycogen can significantly increase muscle TG use in a group of similarly well-trained subjects. There are several possible mechanisms for increased muscle TG oxidation during exercise, including: elevated pre-exercise muscle TG concentrations, increased hormone sensitive lipase (HSL) activity, and/or increased HSL migration to lipid droplets.

Paragraph 33

A $[6,6\text{-}^2\text{H}_2]$glucose tracer was also infused in order to estimate plasma glucose and muscle glycogen oxidation. In the present study we have shown that the reduction in whole-body CHO oxidation during exercise, after training with low muscle glycogen, was due to reduced rates of muscle glycogenolysis, as the decrease in plasma glucose oxidation was only modest when compared to the decrease in muscle glycogen oxidation (Table 2 and Fig. 3). This reduction in muscle glycogenolysis occurred despite higher resting muscle glycogen levels (Fig. 4). Since substrate availability cannot explain the changes in CHO metabolism, we suggest that training with low muscle glycogen evokes chronic adaptations within the skeletal muscle that causes a down-regulation in muscle glycogenolysis. A likely candidate for this down-regulation is the pyruvate dehydrogenase (PDH) complex. PDH catalyzes the conversion of pyruvate to acetyl-CoA and thus regulates the entry of CHO substrates

into the mitochondria (34). Acute studies have shown that commencing endurance exercise with low muscle glycogen increases mRNA and protein content of pyruvate dehydrogenase kinase 4 (28, 33), an enzyme that phosphorylates and inactivates PDH. Training with reduced CHO availability may cause persistent decreases in PDH activity that reduces muscle glycogenolysis during exercise, as has been shown previously following high fat feeding in humans (42).

Paragraph 34

Changes in substrate selection could be the result of an increase in the capacity to oxidize fat, a decreased capacity to oxidize CHO, or a combination of both. The current study suggests the latter possibility. Fat oxidation during exercise can be regulated at several sites, including: adipose tissue lipolysis, delivery of fatty acids to the muscle, transport across the plasma membrane, lipolysis of muscle TG stores, transport within the cytosol, and transport into the mitochondria (for reviews see (26, 38)). Fatty acid transport is a highly regulated process involving several specialized proteins (4, 19, 31). Unfortunately, we were unable to measures most of these proteins. Nonetheless, we present data for FAT/CD36, which is known to play an important role in fatty acid transport across both the plasma and mitochondrial membranes (19) and contributes to the regulation of mitochondrial fatty acid oxidation (18). In the present study, FAT/CD36 increased with training (Fig. 5B) and, although not statistically significant, this increase tended to be more pronounced after training with low muscle glycogen (41.4 vs. 11.5%), which may explain higher rates of fat oxidation during exercise.

Paragraph 35

Fat oxidation is also regulated by the activity of mitochondrial enzymes such as β-HAD (25). We observed a 43% increase in β-HAD protein content after training with low muscle glycogen (Fig. 5C), which is consistent with the 27.7% increase in β-HAD activity measured by Yeo et al. (49). Studies have shown that β-HAD is highly sensitive to changes in substrate availability. For example, Helge and Kiens (16) reported a 25% increase in β-HAD activity after 7 wk adaptation to a high fat diet (62% energy from fat). This increase occurred irrespective of training and demonstrates that altered substrate availability (e.g. increased dietary fat) influences β-HAD activity. Although no clear molecular mechanism has been identified, it is possible that the flux through β-oxidation plays an important role in the regulation of β-HAD content and activity. We did not alter macronutrient intake; however, performing exercise in a glycogen depleted state increases circulating free fatty acids (40), and increasing the availability of free fatty acids is known to increase their oxidation during high-intensity exercise (37). Therefore, it is reasonable to assume that rates of fat oxidation would have been elevated to a greater extent during training sessions performed with low muscle glycogen, and that this may have contributed to the changes in β-HAD content that we observed.

Paragraph 36

This study was the first to look at the effect of training with low muscle glycogen on the glucose transporter GLUT4. GLUT4 is the rate-limiting enzyme in muscle glucose utilization (29) and GLUT4 levels increase with training in animals (35), and both disease-free and type II diabetic humans (8). Furthermore, GLUT4 is significantly higher in endurance athletes than sedentary controls (13). Consistent with these results, GLUT4 levels tended to increase in the HIGH group (20.6 ± 9.7%).

However, the change in GLUT4 levels tended to be smaller in the LOW group (7.1 ± 8.4%). Together with the β-HAD and FAT/CD36 data, this suggests that the capacity for fatty acid oxidation increased more in the LOW group and the capacity for CHO oxidation increased less resulting in the changes in substrate metabolism that we observed during steady-state exercise. This also suggests that training with low muscle glycogen may be counterproductive for athletes who compete in high intensity events where CHO oxidation plays a significant role in performance, and that this type of training may be more suited to preparation for ultra-endurance activities.

Paragraph 37

Another major finding of the present study is that despite reduced power outputs during HIT (Fig. 1), training with low muscle glycogen enhanced time trial cycling performance by a similar amount to that of training with high muscle glycogen (Fig. 2). To date, only two previous studies have investigated the effect of training with low muscle glycogen (12, 49). Hansen et al. (12) had untrained subjects perform single-leg knee extensor exercise (1 h at 75% maximal power output) for 10 weeks using a similar twice every second day (LOW) or once daily (HIGH) design. Time to fatigue at 90% maximal power output increased after training in both legs; however, time to fatigue was greater in the LOW leg compared with the HIGH leg (19.7 ± 2.4 vs. 11.9 ± 1.3 min). In that study, untrained subjects performed a fixed (submaximal) amount of work in each training session, even though higher glycogen stores would normally allow for exercise at higher intensities and/or longer durations. In the present study, well-trained cyclists worked at self-selected intensities, just as they would in their normal training sessions. As expected, training with low muscle glycogen resulted in lower power outputs in HIT sessions. The finding that time trial

performance was still improved by a similar extent in both groups after the 3 wk training period is in direct agreement with the recent work of Yeo et al. (49), and suggests that the amount of work performed during training may not be critical. In this regard, we suggest that the additional "stress" of training with low glycogen compensates for a slight reduction in physical performance during training. Nonetheless, under the specific test conditions of the present study, training with low muscle glycogen did not translate into better performance and does not appear to offer a worthwhile benefit for already well-trained athletes. Whether the increased capacity for fat oxidation translates into better performance during a longer duration test of exercise performance or following a longer period of adaptation remains to be seen.

Paragraph 38

An important practical question is whether additional strategies could be employed to further maximize the muscle "signaling state" during exercise with low glycogen. Glycogen is known to affect the activity of a number of important signaling proteins (30) but whether the amount of glycogen or the localization of glycogen particles is the important factor in mediating this response is unknown. Further, it may be that nutritional interventions in a low glycogen state could either enhance the adaptive response, or reduce the drop-off in training intensity without affecting signaling. With regard to the latter, one technique that may allow for greater exercise intensity during low glycogen training is the use of a CHO mouth rinse. We have previously shown that simply rinsing the mouth with a 6% CHO solution was sufficient to improve performance in a 60 minute time trial by ~3% (5), approximately half of the difference seen between the HIGH and LOW training groups in the current study. Importantly, Chambers et al. (6) have recently shown that the benefit of the CHO

mouth rinse is due to brain receptor modulation, and as a result this intervention would not be expected to alter the metabolic state of skeletal muscle.

Paragraph 39

In conclusion, training with low muscle glycogen reduced self-selected training intensity and, in terms of performance, was no more effective than training with high muscle glycogen. However, training with low muscle glycogen increased muscle TG oxidation during moderate intensity exercise, which may have been due to enhanced metabolic adaptations in skeletal muscle. Future studies should examine the signaling pathways and regulatory mechanisms that govern metabolic adaptations to endurance training with low muscle glycogen.

Acknowledgements

Figure legends

Figure 1. Mean power output during each HIT session performed with either HIGH or LOW muscle glycogen. Values are means ± SE (n = 7 HIGH and n = 7 LOW). [#]Significant difference between HIGH and LOW groups, $P < 0.05$.

Figure 2. Time trial performance before and after training with HIGH or LOW muscle glycogen. Values are means ± SE (n = 7 HIGH and n = 7 LOW). *Significant difference between pre-training and post-training, $P < 0.05$.

Figure 3. Relative contribution of substrates to energy expenditure, during the 30-60 min exercise period, before and after training with HIGH or LOW muscle glycogen. Values are means ± SE (n = 7 HIGH and n = 7 LOW). *Significant difference between pre-training and post-training, $P < 0.05$.

Figure 4. Resting muscle glycogen content before and after training with HIGH or LOW muscle glycogen. Values are means ± SE (n = 7 HIGH and n = 7 LOW). *Significant difference between pre-training and post-training, $P < 0.05$.

Figure 5. Effect of training with HIGH or LOW muscle glycogen on resting protein content for GLUT4 (A), FAT/CD36 (B) and β-HAD (C). Values are expressed as percentage change relative to pre-training levels. Data shown are expressed as means ± SE (n = 7 HIGH and n = 7 LOW). *Significant difference between pre-training and post-training, $P < 0.05$. [#]Significant difference between HIGH and LOW groups, $P < 0.05$.

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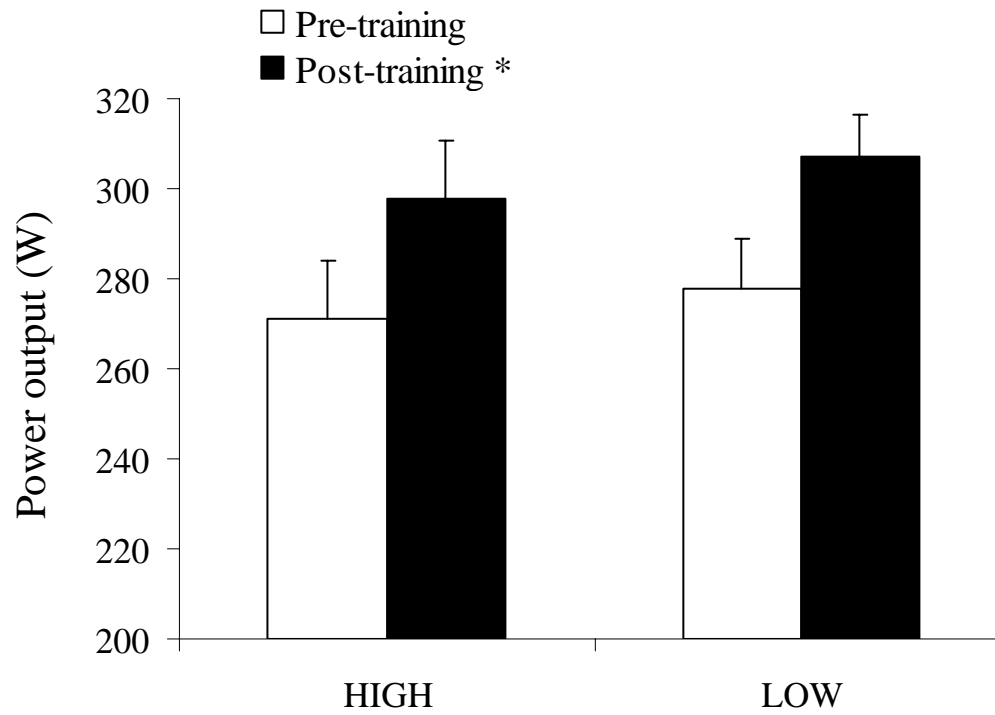
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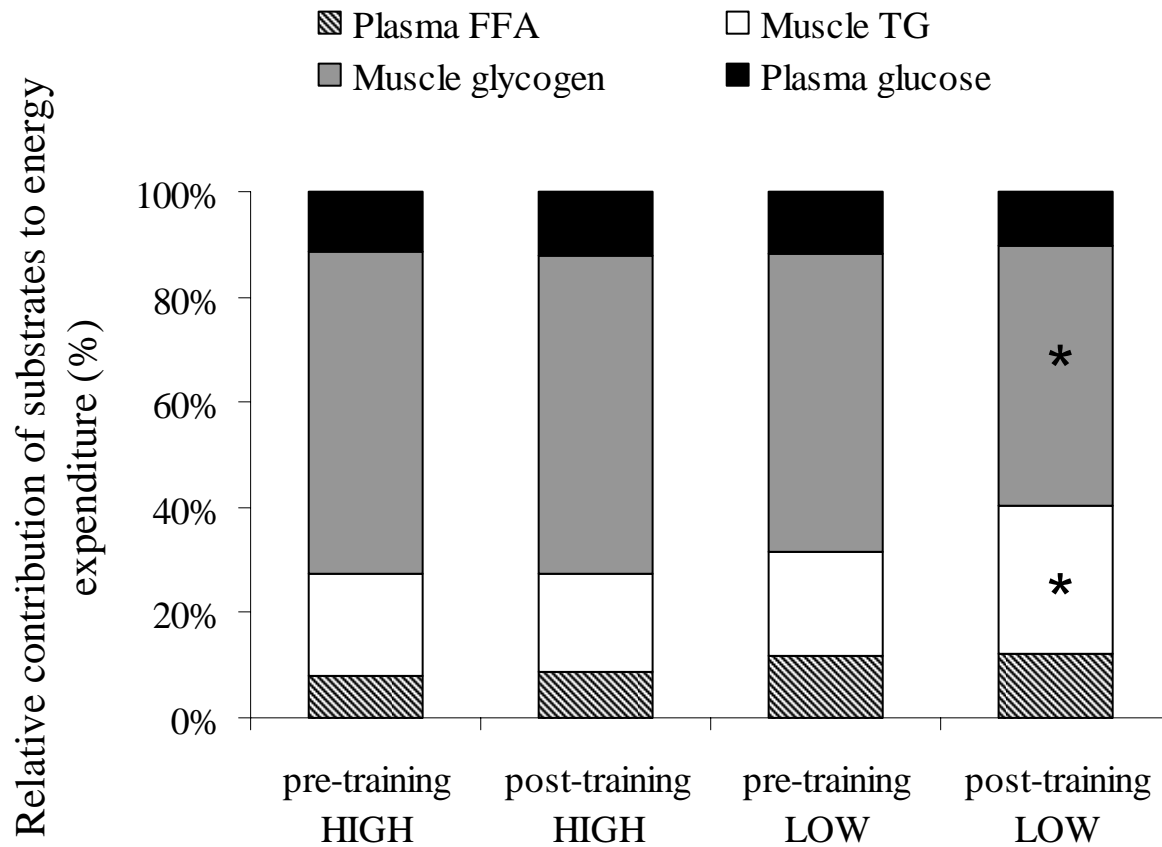
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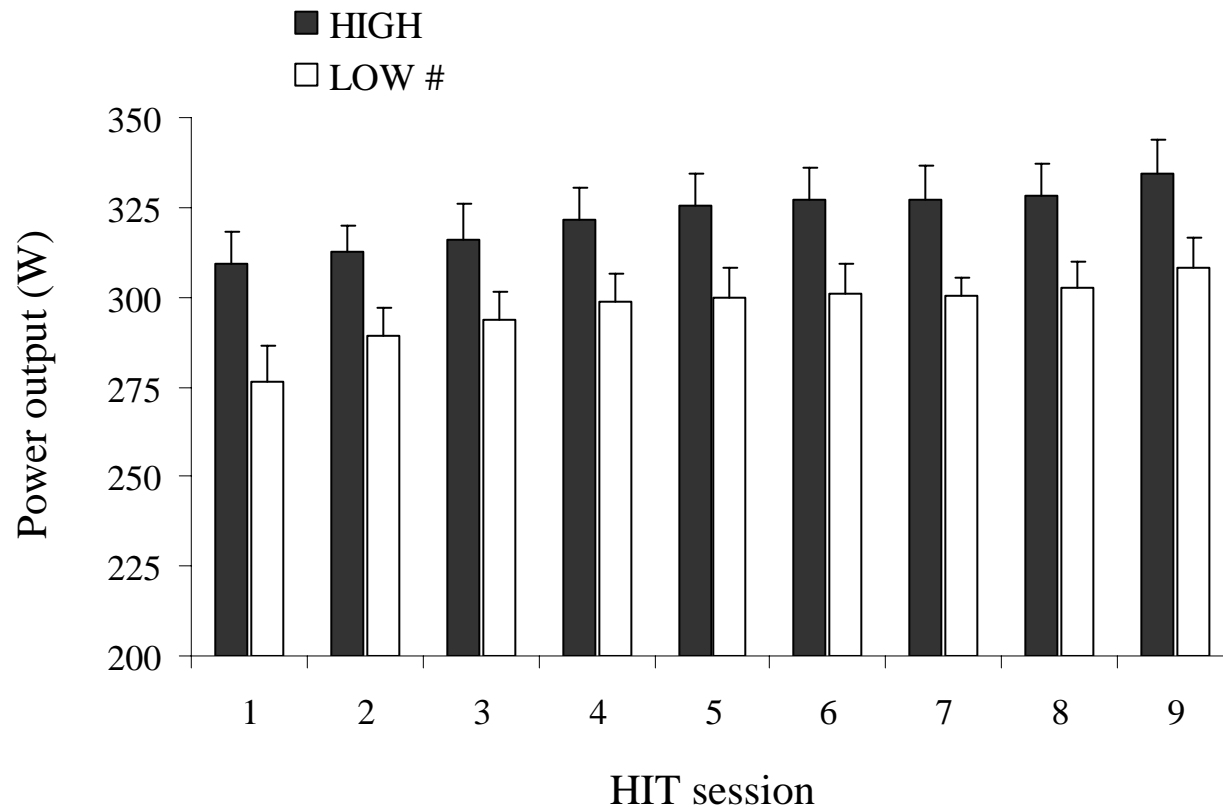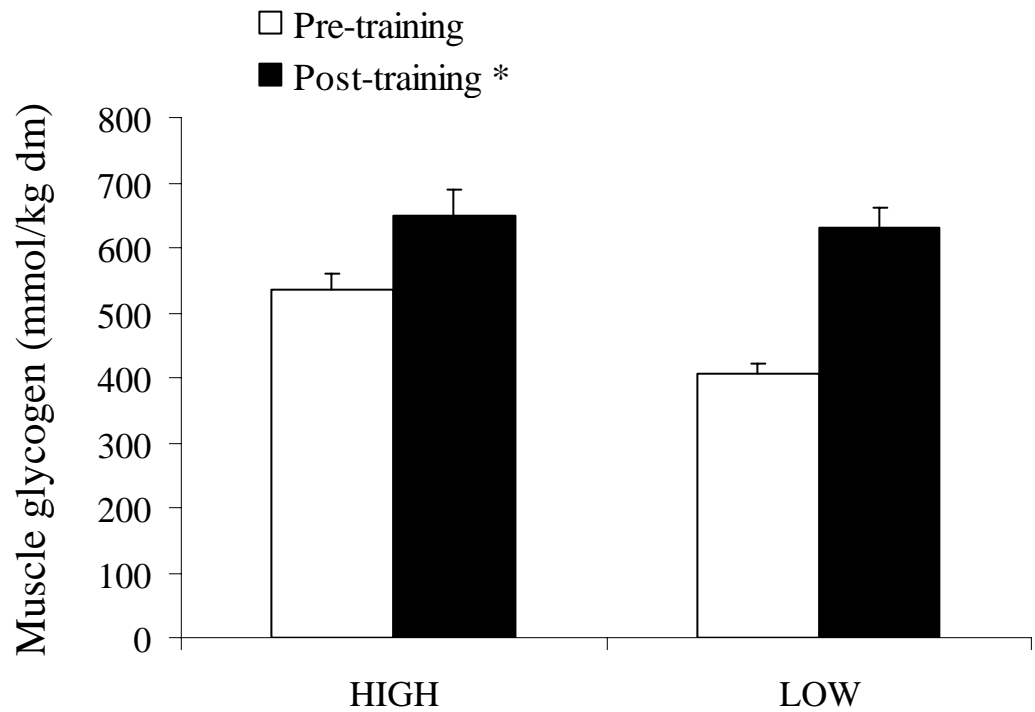
Figure 1

Figure 2

Figure 3

Figure 4