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Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme (ISSN: 1715-5320)

Citation for the published paper:
Hall, U. ; Edin, F. ; Pedersen, A. et al. (2016) "Whole-body fat oxidation increases more by prior exercise than overnight fasting in elite endurance athletes.". Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme, vol. 41(4), pp. 430-7.

http://dx.doi.org/10.1139/apnm-2015-0452

Whole-body fat oxidation increases more by prior exercise than overnight fasting in elite endurance athletes

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Running Head: Fat oxidation and diet manipulation in athletes

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Abstract

The purpose of this study was to compare whole body fat oxidation kinetics after prior exercise versus overnight fasting in elite endurance athletes. Thirteen highly trained athletes (nine men and four women; VO_{2max} 66 ± 1 ml · min^{-1} · kg^{-1}) performed 3 identical submaximal incremental tests on a cycle ergometer using a cross-over design. A control test (CON) was performed 3h after a standardized breakfast, a fasting test (FAST) 12 hours after a standardized evening meal, and a post-exercise test (EXER) after standardized breakfast, endurance exercise and 2h fasting recovery. The test consisted of 3 min each at 30, 40, 50, 60, 70, and 80% of VO_{2max} and fat oxidation rates were measured through indirect calorimetry. During CON, maximal fat oxidation rate was 0.51 ± 0.04 g · min^{-1} compared to 0.69 ± 0.04 g · min^{-1} in FAST ($P<0.01$), and 0.89 ± 0.05 g · min^{-1} in EXER ($P<0.01$). Also comparing across all intensities, EXER was significantly higher than FAST and FAST was higher than CON ($P<0.01$). Blood insulin levels were lower and free fatty acid and cortisol levels were higher at the start of EXER compared to CON and FAST ($P<0.05$). Plasma NMR-metabolomics showed similar changes in both EXER and FAST including increased levels of fatty acids and succinate. In conclusion, prior exercise significantly increases whole-body fat oxidation during submaximal exercise compared to overnight fasting. Already high rates of maximal fat oxidation in elite endurance athletes were increased approximately 75% after prior exercise and fasting recovery.

Key words: maximal fat oxidation capacity, fasting, prior exercise, endurance athletes, metabolomics

Introduction

Endurance capacity is highly related to maximal cardiac output and to the ability of skeletal muscle to oxidize fat and carbohydrate (Weibel and Hoppeler 2005; Levine 2008). In terms of skeletal muscle metabolism, glycogen stores in the body are small and depletion may occur towards the end of long duration exercise. If the rate of fat utilization is insufficient to maintain the energy demand at this time, fatigue occurs (Gollnick and Saltin 1988; Hermansen et al. 1967; Pernow and Saltin 1971; Gollnick et al. 1972). Important metabolic training adaptations include increased glycogen stores and increased fat oxidation capacity, two parameters that are crucial for athletes competing in events lasting more than 2-3 hours (Gollnick and Saltin 1988; Coggan and Williams 1995). Strategies for increasing glycogen stores are fairly well characterized whereas strategies for adapting to higher fat oxidation rate have gained increasing interest over the past decade from scientists, coaches and athletes (Bartlett et al. 2015). In order to enhance fat oxidation during prolonged exercise and to gain a better fat oxidation capacity over time, different acute diet and training strategies that can be periodized into the training schedule have been suggested (Cheneviere et al. 2012; Hansen et al. 2005; Morton et al. 2009; Yeo et al. 2008)

During an acute bout of endurance exercise, the amount of fat oxidized is partly a matter of skeletal muscle capacity to oxidize fat, but also a complex regulation of substrate availability. Important regulators are availability of exogenous and endogenous glucose, mobilization of triglycerides from adipose tissue and skeletal muscle, delivery of FFA via the blood stream, as well as a complex intramuscular regulation where the Acetyl-CoA /CoA ratio plays a central role (van Loon et al. 2001; Constantin-Teodosiu et al. 1991; Spriet 2011; Kiens et al. 2011). These metabolites are highly influenced by various hormones, including insulin, glucagon, cortisol, growth hormone, epinephrine, and

norepinephrine (McMurray and Hackney 2005). Changes in both metabolites and hormones can occur very quickly due to changes in activity level, nutritional intake or fasting. This enables using training and diet strategies to influence the rate of fat oxidation during exercise and recovery. For example, exercise in a fasted state is commonly used amongst athletes and has been shown to promote metabolic adaptations to fat utilization (Van Proeyen et al. 2011). Another method to increase fat oxidation takes advantage of the post-exercise period when resting fat oxidation is high. Increased fat oxidation following fasting or post-exercise is presumably partly due to circulating low levels of insulin and high levels of FFA, which activates fat oxidation and inactivates PDHa, thereby decreasing carbohydrate oxidation (Wu et al. 1999; Harris et al. 2002). High levels of cortisol ensure adequate activity of key enzymes required in liver and adipose tissue for gluconeogenesis and lipolysis (Widmaier 2011). Determination of these metabolites pre-exercise could be good markers in order to evaluate the diet and training strategy and the following fat oxidation rates.
Study protocols of two training sessions separated by fasting recovery have shown increased fat oxidation acutely (Cheneviere et al. 2012) and adaptations towards higher fat metabolism in training studies (Hansen et al. 2005; Yeo et al. 2008; Morton et al. 2009). The two strategies of training fasted and training after prior exercise have, however, not been compared side by side.

The diet and training strategies before an acute training bout are highly relevant and often used by elite endurance athletes. However, no studies have investigated the above mentioned strategies in elite athletes who exhibit a general high fat oxidation capacity with reports of almost a doubling in fat oxidation rates compared to untrained (Bircher and Knechtle 2004; Stisen et al. 2006). It is therefore an open question if these high fat

oxidation rates can be pushed even higher with training during fasting or after prior exercise.

The aim of this study was to compare fat oxidation in athletes after overnight fasting versus after a prior exercise bout. We hypothesized that highly trained endurance athletes would have a marked increase in fat oxidation during exercise after an overnight fast, but even more following prior exercise. Further, prior exercise followed by two hours of fasting recovery would depress insulin and increase plasma FFA and cortisol even more than the fasting strategy.

Methods

Subjects

Thirteen elite cyclists and triathletes (four women and nine men, age 32 ± 2 yrs and BMI 21.0 ± 0.8 kg · m^{-2} for women and 23.2 ± 0.3 kg · m^{-2} for men) were recruited. All athletes had extensive experience in endurance events and had a minimum of 3 years cycling practice as part of their main training schedule. The subjects gave their written informed consent to participate in the present study. Test procedures were performed in accordance with the Declaration of Helsinki 2008 and approved by the local ethics committee of Gothenburg University (Dnr 121-15).

A VO_{2max} test was performed on an ergometer bike (SRM, Jülich, Welldorf, Germany) using a protocol of 5 min warm-up, two 4-min steady state work-loads, followed by incremental increase of load every minute until exhaustion. Heart rate and work load were continuously recorded, and VO_2 and VCO_2 were measured using Jaeger Oxycon Pro (Erich Jaeger, Viasys Healthcare, Germany). Power corresponding to VO_{2max} was extrapolated and used to calculate loads for fat oxidation tests. VO_{2max} was 66 ± 1 ml O_2 · min^{-1} · kg^{-1} (62± 2 ml O_2 · min^{-1} · kg^{-1} for women and 68 ± 1 ml O_2 · min^{-1} · kg^{-1} for men)

Test design

The subjects completed two test days (separated by 3-9 days) in a randomized cross-over design (Fig 1). The day before tests they were sedentary and consumed exactly the same dinner both times, containing a minimum of 145 g (for women) or 180 g (for men) carbohydrate (equating to approximately 2.4 $g \cdot kg^{-1}$ bw of carbohydrate) to ensure replenished glycogen stores.

On day A, the athletes ate a standardized breakfast (1.20 $g \cdot kg^{-1}$ bw carbohydrate, 0.22 $g \cdot kg^{-1}$ bw protein, and 0.15 $g \cdot kg^{-1}$ bw fat) 3h before performing a submaximal incremental fat oxidation test (control test – CON). After 10 min rest, a 60 min training session followed consisting of alternative cycling and rowing (two repetitions of 20 min cycling and 10 min rowing) at 75% of VO_{2max} as determined by heart rate. Water was consumed ad libitum. After exercise, a 120 min fasting recovery followed before another fat oxidation test was performed (EXER).

On day B, the athletes arrived after an overnight fast and performed the incremental fat oxidation test FAST.

Venous blood samples were collected 10 min before the three fat oxidation tests, and after the exercise. Only pre-test samples are presented in the results. Capillary blood was collected before and after tests and exercise as well as every 30 min during the recovery. Resting metabolism was determined measuring VO_2 and VCO_2 immediately preceding the two fat oxidation tests. This was done with the subject sitting still in a calm environment for at least 5 min prior to measurement, and expired VO_2 and VCO_2 measured for 5 min once the test leader had deemed the outputs stable. During recovery, subjects were weighed and asked to drink water corresponding to the weight

lost through expiration. Body weights before the three tests were 71.7 ± 3.1, 71.1 ± 3.0 and 71.5 ± 3.2 kg for CON, EXER and FAST respectively.

Fat oxidation test

Subjects cycled on an SRM bike ergometer; 5 min on 25% of VO_{2max} as warm-up, followed by load increases every 3 min corresponding to 30%, 40%, 50%, 60%, 70% and 80% of VO_{2max} as calculated from the pre-test. VO_2 and VCO_2 were recorded continuously using a Jaeger Oxycon Pro and fat oxidation calculated from the last 60s of every load using Frayn's equations (Frayn 1983). Heart rate was monitored throughout.

Biochemical analysis

Capillary blood was analyzed immediately for glucose and lactate using a Biosen analyser (Biosen C-line, EKF-diagnostic GmbH, Magdeburg, Germany). Venous blood was drawn in vacutainer EDTA tubes and serum separation tubes. EDTA tubes were centrifuged immediately at 4°C and plasma stored at -80°C until analysis. Blood in serum separation tubes was allowed to coagulate at room temperature, centrifuged and serum stored at -80°C until analysis. To determine Cortisol and Insulin concentrations in serum, Quantitative Sandwich Elisa was used (MyBioSource, San Diego, CA 92195-3308, USA, Cat. No. MBS043519, Human Hydrocortisone Elisa Kit). The Insulin concentrations were determined using Human Insulin Kit from Invitrogen Corporation (7335 Executive Way, Frederick, MD 21704, USA Cat. No. KAQ1251). Free fatty acid analysis of plasma was performed by the accredited Laboratory for Clinical Chemistry, Sahlgrenska University Hospital (SWEDAC ISO 15189) using an enzymatic colorimetric method (ACS-ACOD by Wako Chemicals, Germany).

NMR metabolomics

Plasma samples were thawed at 4°C, briefly spun down and 200 µl supernatant transferred to 1.8 ml cryo vials (Sarstedt Gmbh, Nümbrecht, Germany, cat no 72.694.007, with septum caps, cat no 65.3716). A SamplePro Tube L liquid handling robot (Bruker Gmbh, Rheinstetten, Germany) was used to mix 100 µl sample with 100 µl buffer (75 mM sodium phosphate pH 7.4, 20% D_2O, 0.02% NaN_3, 1 mM TSP-d4) in a 96-well deepwell plate (Sarstedt Gmbh, Nümbrecht, Germany, cat no 82.1971.002) before transfer to a SampleJet rack with 3 mm tubes. Samples were kept at 2°C through the whole procedure except for the brief mix of buffer and plasma in the deep well plate which was done at ambient temperature.

NMR data was acquired on an Oxford 800 MHz magnet equipped with a Bruker Avance III HD console and a 5 mm TCI cryoprobe, using a 1D CPMG perfect-echo experiment with excitation sculpting for water suppression. The total duration of the CPMG pulse train was 193 ms. A sweep width of 20 ppm and 64 scans per experiment were used with a relaxation delay of 1.3 s and a data acquisition period of 2.04 s. A cooled SampleJet automatic sample changer was used to maintain temperature at 6°C of samples before measurement. Temperature during data acquisition was 37°C. Spectra were processed using TopSpin 3.5pl1 (Bruker GmbH, Rheinstetten, Germany). Processed spectral data was imported into MatLab (MathWorks Inc., Natick, MA) using in-house written scripts. Alignment was performed using icoshift 1.2 (Savorani et al. 2010; Tomasi et al. 2011) and integration performed after linear baseline correction. Natural abundance $^{1}H,^{13}C$-HSQCs for pooled samples from the CON, EXER and FAST were acquired on the same 800 MHz spectrometer, using a standard Bruker pulse sequence “hsqcedetgpsisp2.2” with a 90° pulse, 44 ms acquisition time, a 3 s pulse delay, a $^{1}J_{C-H}$ of 145 Hz, 8 scans and acquisition of 1024 data points (for ^{1}H) and 512

increments (for ^{13}C). The ^{1}H and ^{13}C pulse widths were p1 = 7.37 µs and p3 = 9.3 µs, respectively. The ^{1}H and ^{13}C spectral widths were 20 ppm and 100.00 ppm, respectively (16025.6 and 20114 Hz). ^{1}H-^{1}H TOCSY spectra were acquired for the same pooled samples, using the Bruker pulse sequence ' mlevgpphw5' with the same proton pulse width as for the HSQC, spectral widths of 13.95 ppm (11160 Hz) in both dimensions. The acquisition time was 229 ms and the pulse delay 2 s. 8 scans was used and 4096 points and 1024 increments were acquired in the direct and indirect dimensions, respectively. HSQC and TOCSY data processing and plotting were performed with TopSpin 3.5pl1. Metabolite identification was accomplished by the combination of the HSQC and TOCSY spectra, use of the Chenomx 8.1 spectral line fitting software (Chenomx Inc., Edmonton, Canada) on the 1D data and metabolite databases (Wishart et al. 2013; Ludwig et al. 2012). Metabolite concentration fold changes between the CON, EXER and FAST sampling times were calculated by relating the respective metabolite integrals to each other, i.e. as EXER/CON, FAST/CON and EXER/FAST. For metabolites with more than one integral, an average was taken before calculating the fold change.

Statistical analysis and calculations

For each incremental fat oxidation test, a third degree polynomial regression analysis was performed using fat oxidation as a function of measured intensity (VO_2) including points for resting fat oxidation and origo (0,0) according to previously described methods (Stisen et al. 2006). This enabled determination of maximal fat oxidation (MFO) and the intensity at which MFO occurs (Fat_{max}).

Fat oxidation data from the three trials were compared using a two-way analysis of variance for repeated measures (ANOVA). MFO, Fat_{max}, RER and blood metabolites from the three trials were compared using a one-way ANOVA. When a significant main effect

and/or interaction occurred, the location of pairwise differences between mean values was identified by using a post hoc test.

Simple linear regression was used to investigate the correlation between MFO obtained during the three trials and resting fat oxidation.

P values < 0.05 were taken to indicate statistical significance. All values are expressed as mean values ± SEM with n= 13 unless otherwise stated. One subject was excluded in resting RER due to hyperventilation. One subject was excluded in blood insulin due to outliers. For capillary samples, 10 subjects had their lactate recorded.

Multivariate data analysis of NMR data

All multivariate analysis was performed with SIMCA 14 (MKS Umetrics, Umeå, Sweden). The unsupervised method principal component analysis (PCA) was used to search for separations and outliers in the CON, FAST and EXER raw data. The samples of the CON, FAST and EXER were selected, and each variable was normalized per person, by subtracting the average and dividing by the standard deviation of each person. OPLS-DA (Bylesjö et al. 2006) was used to build discriminating models FAST vs. CON and EXER vs. CON. One peak per metabolite was used for analysis unless multiple peaks showed non-consistent behaviour, in which case all variables were kept. Metabolites were considered discriminant if the 95% confidence interval of the pq-loadings of the respective OPLS-DA model did not cross zero.

Results

Incremental fat oxidation tests

Fat oxidation rate was increased significantly at all intensities for the exercise (EXER) protocol compared to fasting (FAST), and fat oxidation rate at FAST was higher than in

control (CON) tests (P<0.01, Fig 2). After polynomial regression analysis for each subject and determination of maximal fat oxidation (MFO) and work intensity at MFO (Fat_{max}) it was confirmed that MFO for EXER was higher than FAST, and FAST higher than CON (P<0.01, Table 1). Fat_{max} occurred at almost identical intensities for EXER and FAST, both significantly higher than for CON (P<0.01). Respiratory Exchange Ratios at respective intensities for maximal fat oxidation (RER_{Fatmax}) was significantly lower for EXER compared to FAST, which was significantly lower than CON (P<0.01).
Measured oxygen uptake (VO_2) was increased in both EXER and FAST compared to CON and for EXER compared to FAST (P<0.05). Mean VO_2 values over all intensities were 58 ± 2, 61± 2 and 59 ± 2 % VO_{2max} for CON, EXER, and FAST, respectively. Similarly, heart rate was significantly increased for EXER compared to FAST, and both EXER and FAST were significantly higher than CON (P<0.05). Mean heart rate values over all intensities were 126 ± 2, 137 ± 2 and 128 ± 2 bts · min^{-1} for CON, EXER, and FAST, respectively.

Resting metabolism before fat oxidation tests

RER was lower pre-EXER compared to pre-CON (P<0.01, Fig 3). In the morning of the fasting trial (pre-FAST), RER tended to be lower than pre-CON (P=0.08).

Biochemical parameters

Capillary glucose levels were similar before fat oxidation tests CON and FAST, but lower before EXER (4.67 ± 0.12 mM, 3.98 ± 0.14 mM and 4.68 ± 0.12 mM for pre-CON, pre-EXER and pre-FAST, respectively, P<0.01). During fat oxidation tests, glucose levels increased to the same level in all three tests (4.84 ± 0.11 mM, 4.86 ± 0.12 mM and 5.09 ± 0.15 mM post-CON, post-EXER and post-FAST, respectively). Capillary lactose was measured in ten subjects with values 1.2 ± 0.1mM, 1.6± 0.8mM, and 1.0 ± 0.3mM for

pre-CON, pre-EXER and pre-FAST respectively, and 2.4 ± 0.4mM, 2.8± 0.6mM, and 3.6 ± 0.6mM for post-CON, post-EXER and post-FAST, respectively. There were no significant differences in lactate levels between pre fat oxidation test samples or between post fat oxidation samples.

Plasma free fatty acids (p-FFA) were increased in pre-EXER compared to pre-FAST, and pre-FAST was increased compared to pre-CON ($P<0.01$, Fig 5). Serum cortisol was increased and insulin was decreased in pre-EXER compared to both pre-CON and pre-FAST, ($P<0.05$, Fig 4).

Normalized metabolomics data from plasma NMR analysis showed similar pattern for samples taken pre-EXER and pre-FAST fat oxidation tests. The samples were then compared in relation to pre-CON to display which metabolites were different in situations of high fat oxidation compared to the control test. This showed similar changes in metabolites in pre-EXER and pre-FAST when compared to pre-CON (Fig 5). Significant increase in both pre-EXER and pre-FAST were seen for fatty acids and succinate ($P<0.05$). Identified metabolite changes that were significant in only one of the models were glycerol, lactate and acetate. Two metabolite peaks that were significantly decreased in both tests could not be identified. There was also a tendency of changes in both pre-EXER and pre-FAST for 3-hydroxybutyrate and acetoacetate (increased), and the branched chained amino acids leucine, iso-leucine and valine (decreased), although not significant.

Discussion

Training after an overnight fast is commonly used amongst endurance athletes and has been shown to elevate fat oxidation and increase muscular oxidative capacity (Van Proeyen et al. 2011). Here we show for the first time that training after 2 hours of fasting

recovery from a previous bout will increase fat oxidation even more, and that highly trained endurance athletes can increase the maximal fat oxidation rates by approximately 75%. Some metabolic responses are very similar in the fasting and post-exercise conditions, such as fatty acids, ketone bodies, BCAA, lactate and succinate levels in the plasma, but prior exercise affects plasma insulin, FFA and cortisol significantly more than fasting. Exercise stimulates these important regulators of fat oxidation showing that prior exercise is a potent stimulator of fat oxidation in a following training bout.

When used in intervention studies, both fasted training and training twice with carbohydrate restriction in between have shown enzymatic adaptation to increased fat oxidation (Hansen et al. 2005; Hulston et al. 2010; Morton et al. 2009; Yeo et al. 2008). The two approaches have, however, not previously been compared, and the few studies available looking directly at fat oxidation changes from these methods are generally from moderately trained subjects. The current study shows that MFO was significantly higher with the repeated training protocol compared to both overnight fasting and fed control tests. Earlier reports on MFO and Fat_{max} during exercise, either in a fasting (Achten and Jeukendrup 2003) or an exercise-recovery setting (Cheneviere et al. 2012; Cheneviere et al. 2009) have used moderately trained male subjects. It is interesting to note that in our study, elite athletes with relatively high fat oxidation capacity in the fed control state could still increase their MFO 35% by fasting and 75% by using the exercise-recovery protocol. Cheneviere et al (Cheneviere et al. 2012) used a slightly different exercise protocol and started the morning control tests in a fasted state (giving a total fast of 14h before the post-recovery test). Despite the longer fast and a longer exercise protocol, they showed overall results similar to the current study albeit a lower MFO.

Previous studies investigating fat oxidation kinetics have reported a shift of Fat_{max} towards higher intensities when in a fasted state compared to carbohydrate fed (Achten and Jeukendrup 2003), or after exercise and recovery (Cheneviere et al. 2012). In line with this, the current study shows a Fat_{max} shift towards higher intensities with both overnight fast and exercise-recovery compared to control tests. The earlier studies with exercise-recovery protocols have reported conflicting results in changes of RER at Fat_{max} (Cheneviere et al. 2009; Cheneviere et al. 2012) where the first study showed an increased fat oxidation but no change in RER_{Fatmax} whereas the second study reported a reduction in RER_{Fatmax} . Our study show a clearly reduced RER at Fat_{max} after exercise-recovery, significantly different to both fasting and control values, indicating that a much larger proportion of energy is derived from fat even at the higher intensities.

The study included both men and women of comparative training experience and fitness. Due to limited numbers no meaningful statistical comparison can be made between sexes, although there was a tendency towards lower MFO and higher Fatmax for women compared to men. Most relevant to the current study, though, was that the same pattern of changes in fat oxidation was seen in all participating athletes.

There are some limitations to the fat calculations which is based on the assumption that protein oxidation remains constant. Likely there are some differences in protein oxidation between the treatments given the length of fasting and lower glycogen stores following exercise and fasting. Data from the literature indicate around 2% protein oxidation in the normal fed state and around 5-7% in the glycogen depleted state (Blomstrand and Saltin 1999; Rennie et al. 2006). If these assumptions are correct, we overestimate the fat oxidation in the EXER and FAST trials with 3-5% compared to the CON trial. However, compared to CON, MFO increased 35% in FAST and 75% in EXER, so

corrections for changes in protein oxidation will not have any influence on the conclusion.

The observed increase in VO_2 during the EXER test could be explained by the higher fat oxidation rates. Fat oxidation consumes roughly 7% more oxygen compared to carbohydrate oxidation and although small differences, it can be significant in the long run. Interestingly, heart rates were highest throughout EXER, and slightly higher in FAST compared to CON. It is known, that repeated bouts of exercise with the same intensity increase heart rate (do Nascimento et al. 2015; Weltman et al. 1998) although the mechanisms are not clear. We have not recorded rate of perceived exertion, but the fat oxidation test performed 2 hours after the exercise session seemed tougher at the highest intensity. This strategy would therefore not be optimal for high intensity training sessions but more as a tool to be used for training fat oxidation capacity.

There are numerous reports on increased lipolysis and fatty acid metabolism following exercise (for a meta-analysis, see Henderson and Alderman, 2014), which was confirmed during the 2h post-exercise recovery in the present study where resting fat oxidation was significantly higher than pre-exercise. Several factors could contribute to the high fat oxidation seen during the second incremental test post-exercise, such as increased post-exercise lipolysis and FFA availability, high plasma cortisol levels, low plasma glucose and insulin levels and low muscle glycogen levels at the start of the test (McMurray and Hackney 2005). Increased cortisol levels can contribute to keep normal glucose levels via gluconeogenesis and the depressed insulin levels together with high plasma FFA levels contribute to the higher fat oxidation in the second bout. Cortisol also mediates protein metabolism during exercise (Del Corral et al. 1998) and high cortisol values could result in protein breakdown, reducing the training effect on mitochondrial biogenesis. Interestingly, metabolomics data showed a tendency to reduced plasma

BCAA following exercise indicating a more significant role in oxidation during exercise and/or supporting gluconeogenesis. It could therefore be relevant to study the effect of protein supplementation in the recovery period, in order to keep high fat oxidation and optimizing protein synthesis in the recovery period.

We detected increases in several other metabolic species in plasma. A rise in fatty acids, ketone bodies and glycerol in FAST and EXER is to be expected indicating increased lipolysis, whereas the explanation for significantly increased levels of circulating succinate during FAST and EXER is less obvious. However, there has been interest in succinate over the last decade as a signaling molecule and ligand of the GPR91 receptor with suggestions that it promotes gluconeogenesis and reabsorbtion of glucose in the proximal tubule (He et al. 2004). Lactate was increased significantly only in pre-EXER venous samples, indicating that lactate accumulation from the previous exercise bout was not completely cleared before the second bout. This was also confirmed in capillary samples during the study, although the difference here did not reach statistical significance (P=0.06). We found it interesting that several unidentified metabolites increase/decrease significantly in both FAST and EXER compared to control, but also that there are differences in unidentified metabolites between FAST and EXER. However, further discussions would be too speculative.

Reduced glycogen levels are believed to increase lipolysis and fat oxidation through its effects on blood glucose and hormone responses as well as have more direct effects (Blomstrand and Saltin 1999; Weltan et al. 1998). Recent advances have for example shown reduced glycogen to activate several important signaling pathways involved in fat oxidation, such as the AMPK and p38MAPK and its downstream targets such as PCG1a (Hansen et al. 2005; Pilegaard et al. 2005), although a recent study found no effect on mRNA response of markers of mitochondrial biogenesis (Jensen et al. 2015).

Based on this effect of glycogen reduction, several studies have used a “train low” protocol with reduced exogenous and/or endogenous carbohydrate availability (Bartlett et al. 2013).

Perspective

These results indicate that optimization of fat oxidation during training is best achieved with repeated training bouts when compared to training in a fasted state. Long-term adaptations of increased fat oxidation has previously been shown for both methods, however, an intervention study comparing the methods would determine whether the acute effects shown in this study translate to meaningful training adaptations and increased performance during long-term events. It will also be interesting to see what training duration and intensity is needed to stimulate the high fat oxidation, and to see if the increased fat oxidation will enhance performance in long-term endurance activities. From a general health perspective there could be substantial advantages to optimizing such a strategy, both for athletes and for a growing population exercising for health purposes. For instance, one of the main benefits of exercise intervention in disease prevention and treatment is increased fat oxidation together with reduced blood lipids and increased glucose tolerance and insulin sensitivity. Overweight individuals display reduced whole body fat oxidation both in a resting and exercising state and this is coupled to several indicators of metabolic syndrome (Rosenkilde et al. 2010). A nutrition and exercise strategy to optimize fat oxidation would therefore be desirable to many individuals, athletes or not.

In conclusion, the study shows that prior exercise significantly increases whole-body fat oxidation at submaximal exercise intensities compared to overnight fasting. Already

high rates of maximal fat oxidation in elite endurance athletes were increased by approximately 75% after prior exercise and fasting recovery.

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to thank Rui Pinto supported by BILS (Bioinformatics Infrastructure for Life Sciences) for invaluable help with bioinformatics analysis, and Gitte Kaiser Hartvigsen for analyzing cortisol and insulin. This study was supported by Team Danmark Research Council, and the authors wish to acknowledge the support from the KAW foundation through the 'NMR for Life' project.

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Table 1. Fat oxidation parameters in control (CON), fasting (FAST) and following exercise training (EXER).

	CON	EXER	FAST
Maximal Fat Oxidation (g/min)	0.51 ± 0.04	0.89 ± 0.05*#	0.69 ± 0.04*
Fat_{max} (% of VO_{2Max})	55 ± 2	62 ± 1*	62 ± 2*
RER_{Fatmax}	0.88 ± 0.007	0.82 ± 0.004*#	0.86 ± 0.004*

Values are means ± SE, n=13. * significantly different from CON, # significantly different from FAST ($P<0.01$).

Figure 1. Overview of the study design. Fat oxidation tests were performed in three different conditions. CON: 3 hours after a standardized breakfast, EXER: 120 min after 60 min exercise training at 75% of VO_{2max}, and FAST: after an overnight fast. The test days (A and B) were randomized for all 13 subjects.

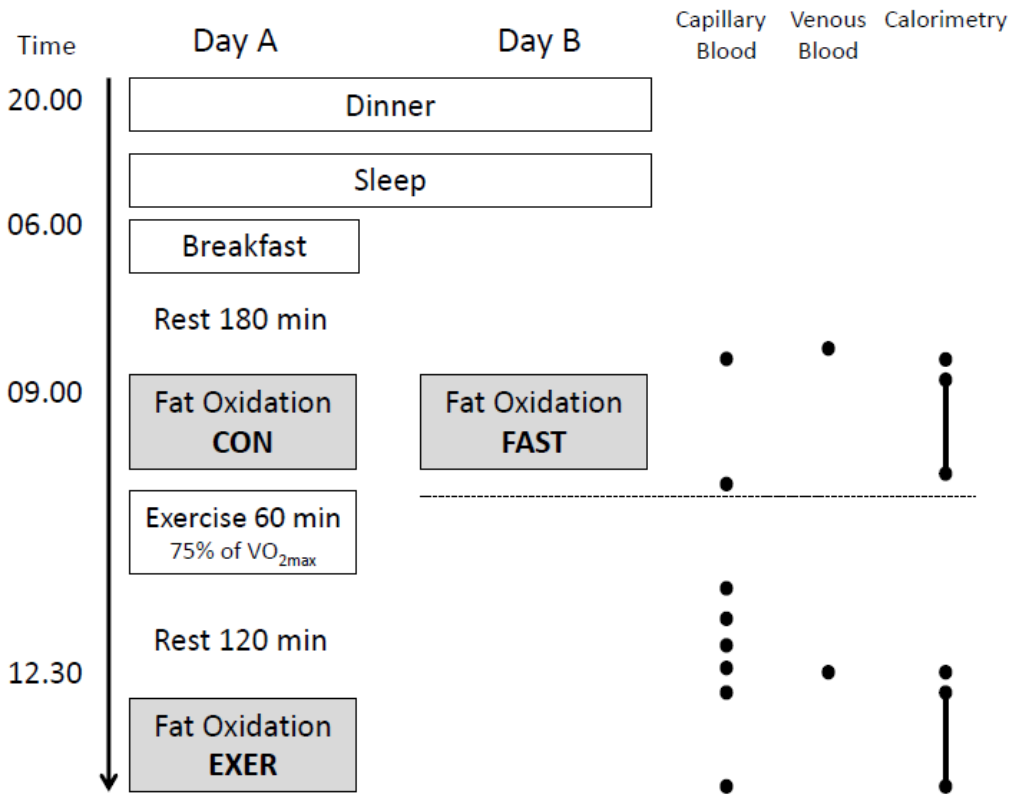


Figure 2. Fat oxidation rates at rest and at exercise intensities from 40 to 80% of VO_{2max} in three different conditions, CON, EXER and FAST. Oxidation rates were calculated from measured VO_2 and VCO_2 the last 60s of every exercise intensity using Frayn's equations (Frayn 1983).

Values are means ± SE; n=13. * Significantly different from CON (P<0.01); # Significantly different from FAST (P<0.01)

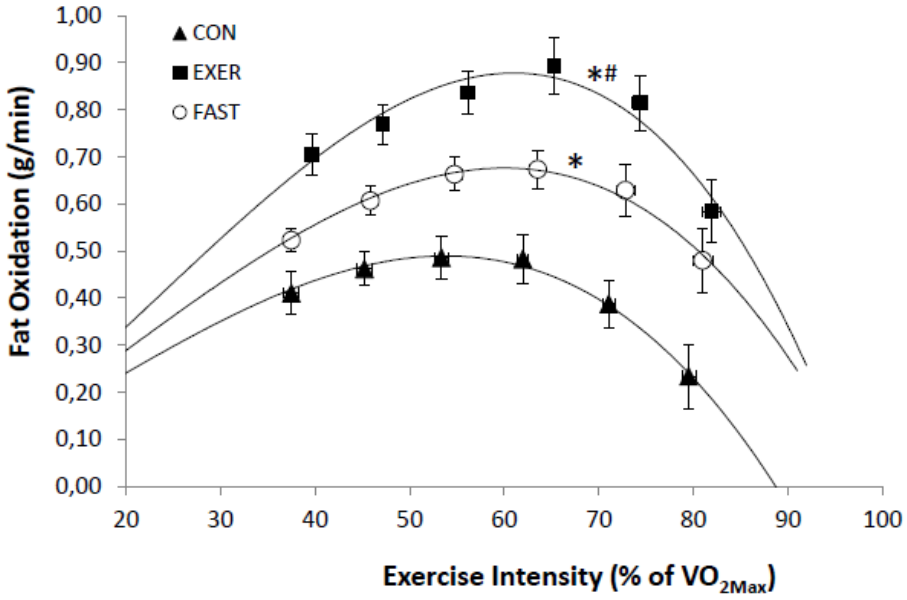


Figure 3. Resting RER values before fat oxidation tests, pre-CON, pre-EXER, and pre-FAST.

Values are means ± SE; n=12. * Significantly different from CON (P<0.01).

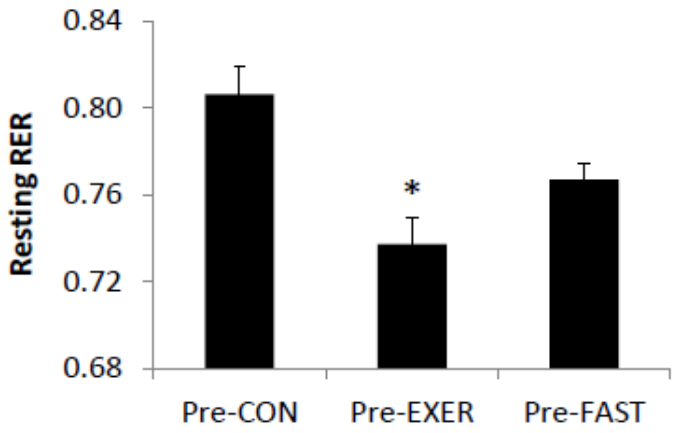


Figure 4. Plasma FFA, Cortisol and Insulin values measured immediately before fat oxidation tests, pre-CON, pre-EXER, and pre-FAST.

Values are means ± SE; n=13 (n=12 for insulin). * Significantly different from CON (P<0.01); # Significantly different from FAST (P<0.05)

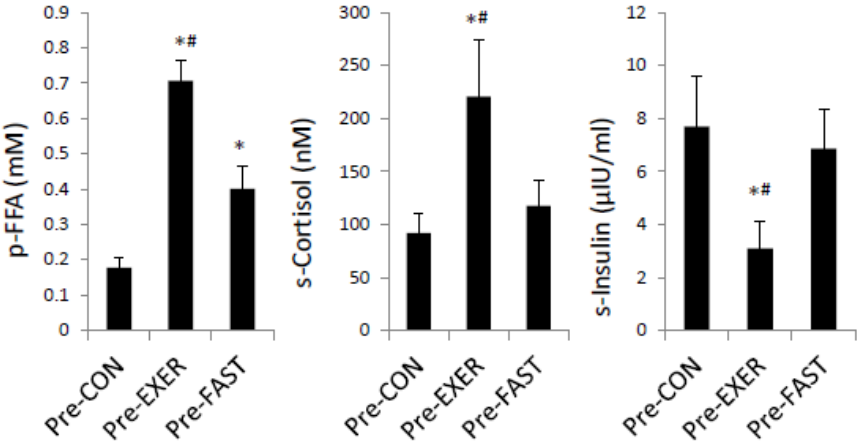
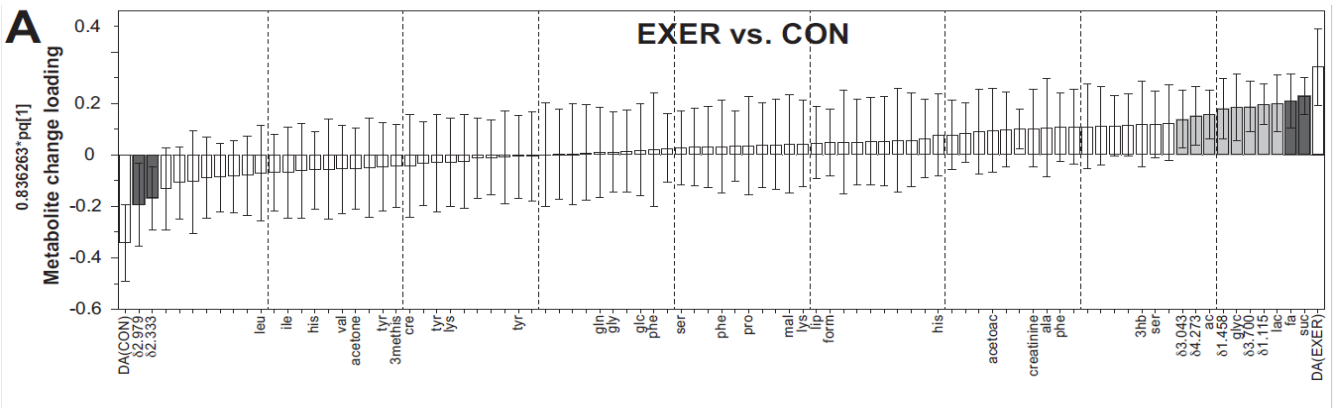
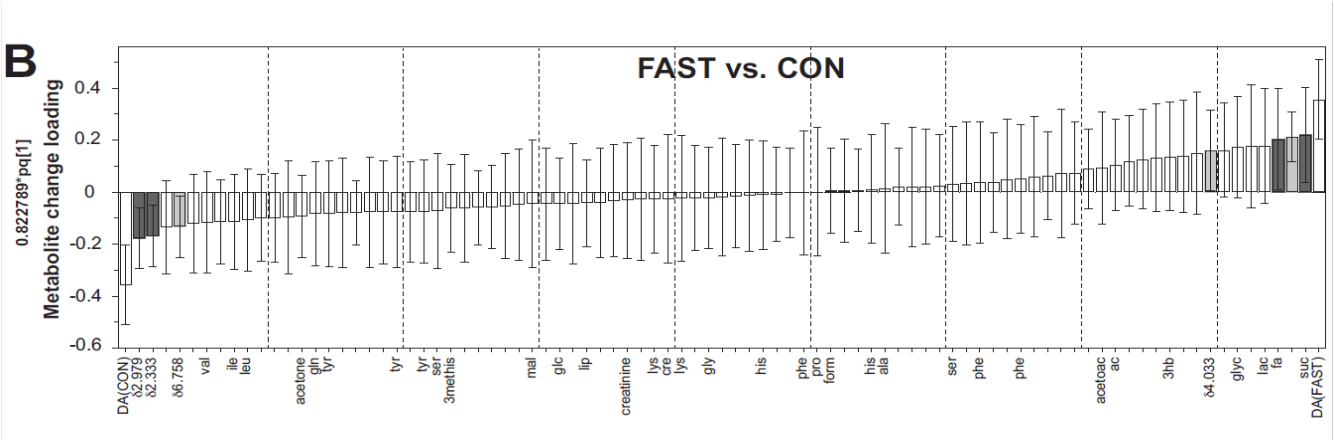
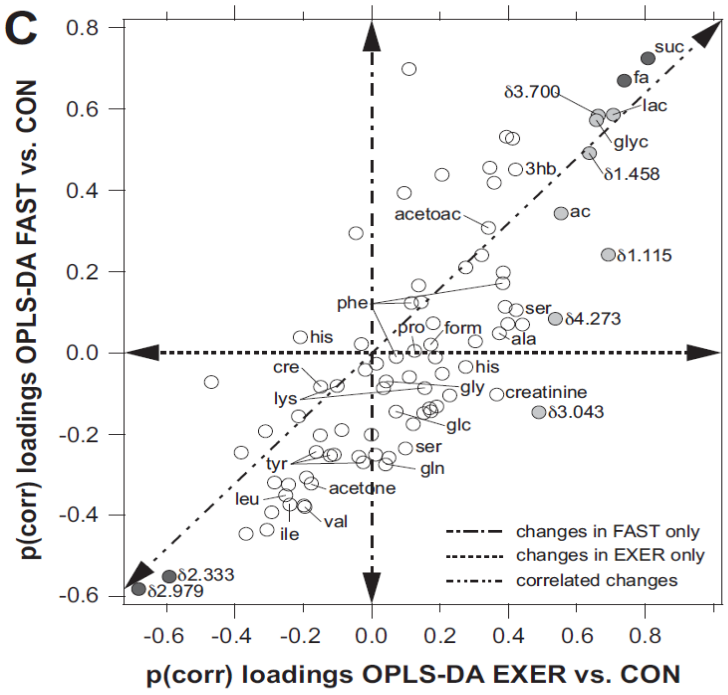
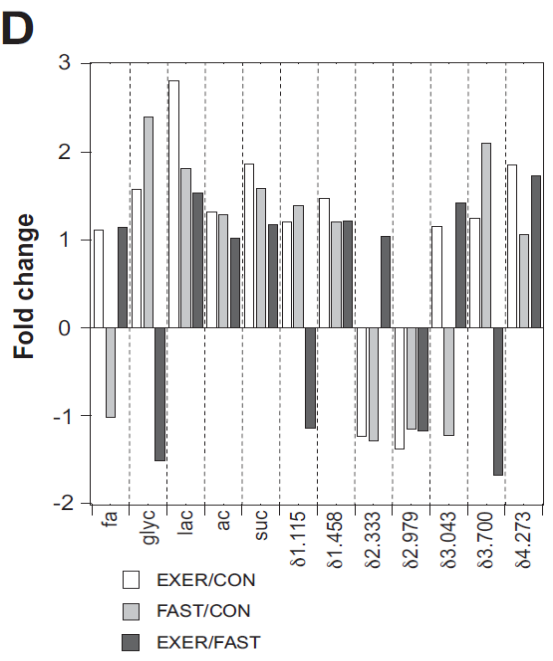


Figure 5. Changes in pre-EXER and pre-FAST plasma samples compared to pre-CON samples determined by metabolomics analysis. *A)* and *B)* pq-loadings plots for the EXER vs. CON and FAST vs. CON OPLS-DA models. The 95% confidence intervals are shown as bars. Metabolite columns are shaded light or dark grey depending on whether they are discriminating in either or both OPLS-DA models, respectively. Changes in metabolite levels are significant when the loading magnitude of a given metabolite exceeds the respective confidence interval. C) Shared and unique structure (SUS) plot displaying the OPLS-DA loadings of the CON vs. EXER model (ordinate) and CON vs. FAST model (abscissa). Filled dark grey circles denote metabolites that were significantly increased (positive pcorr) or decreased (negative pcorr) in both of the respective EXER or FAST samples. Light grey filled circles are metabolites which were discriminating in only one of the OPLS-DA models. Metabolites that are discussed in the text, but showing no significant changes are marked in the figure. D) Plot of concentration fold changes for the metabolites found significantly changed in either or both OPLS-DA models. Unidentified metabolites are denoted with the chemical shift (δ) of the respective signal. Metabolite abbreviations: 3hb, 3-hydroxybutyrate; 3methis, 3-methyl histidine; ac, acetate; acetoac, acetoacetate; ala, alanine; cre, creatine; fa, fatty acids; form, formate; glc, glucose; gln, glutamine; glyc, glycerol; gly, glycine; his; histidine; ile, isoleucine; leu, leucine; lip, lipid methyls; lys, lysine; mal, malonate; phe, phenylalanine; pro, proline; ser, serine; suc, succinate; tyr, tyrosine; val, valine


A
EXER vs. CON
0.836263*pq[1]
Metabolite change loading
0.4
0.2
0
-0.2
-0.4
-0.6
DA(CON)
δ2.979
δ2.333
leu
ile
his
val
acetone
tyr
3methis
cre
tyr
lys
tyr
gln
gly
glc
phe
ser
phe
pro
mal
lys
lip
form
his
acetoac
creatinine
ala
phe
3hb
ser
δ3.043
δ4.273
ac
δ1.458
glyc
δ3.700
δ1.115
lac
fa
suc
DA(EXER)


B
FAST vs. CON
0.822789*pq[1]
Metabolite change loading
0.4
0.2
0
-0.2
-0.4
-0.6
DA(CON)
δ2.979
δ2.333
δ6.758
val
ile
leu
acetone
gln
tyr
tyr
tyr
ser
3methis
mal
glc
lip
creatinine
lys
cre
lys
gly
his
phe
pro
form
his
ala
ser
phe
phe
acetoac
ac
3hb
δ4.033
glyc
lac
fa
suc
DA(FAST)


C
p(corr) loadings OPLS-DA FAST vs. CON
p(corr) loadings OPLS-DA EXER vs. CON
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
suc
fa
δ3.700
lac
glyc
3hb
δ1.458
acetoac
ac
δ1.115
phe
ser
δ4.273
his
pro
form
ala
his
cre
gly
creatinine
lys
δ3.043
glc
ser
tyr
gln
acetone
leu
val
ile
δ2.333
δ2.979
changes in FAST only
changes in EXER only
correlated changes


D
Fold change
3
2
1
0
-1
-2
fa
glyc
lac
ac
suc
δ1.115
δ1.458
δ2.333
δ2.979
δ3.043
δ3.700
δ4.273
EXER/CON
FAST/CON
EXER/FAST