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Determination of the exercise intensity that elicits maximal fat oxidation in individuals with obesity

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Abstract

Maximal fat oxidation (MFO) and the exercise intensity that elicits MFO (Fat_{Max}) are commonly determined by indirect calorimetry during graded exercise tests in both obese and normal weight individuals. However, no protocol has been validated in individuals with obesity. Thus, the aims were to develop a graded exercise protocol for determination of Fat_{Max} in individuals with obesity, and to test validity and inter-method reliability. Fat oxidation was assessed over a range of exercise intensities in 16 individuals (Age: 28 (26-29) years, BMI: 36 (35-38) kg m^{-2}) (95%CI) on a cycle ergometer. The graded exercise protocol was validated against a short continuous exercise (SCE) protocol, in which Fat_{Max} was determined from fat oxidation at rest and during 10-min continuous exercise at 35, 50 and 65% of maximal oxygen uptake ($\text{VO}_{2\text{max}}$). Intraclass and Pearson correlation coefficients between the protocols were 0.75 and 0.72 and within subject coefficient of variation (CV) was 5 (3-7)%. A Bland Altman plot revealed a bias of -3% points of $\text{VO}_{2\text{max}}$ (Limits of Agreement: -12 to 7). A tendency towards a systematic difference ($p=0.06$) was observed, where Fat_{Max} occurred at 42 (40-44) and 45 (43-47)% of $\text{VO}_{2\text{max}}$ with the graded and the SCE protocol, respectively. In conclusion, there was a high-excellent correlation and a low CV between the two protocols, suggesting that the graded exercise protocol has a high inter-method reliability. However, considerable intra-individual variation and a trend towards systematic difference between the protocols reveal that further optimization of the graded exercise protocol is needed to improve validity.

Keywords

Obesity, fat metabolism, exercise metabolism, lipid metabolism, exercise intensity

Introduction

Maximal fat oxidation (MFO) and the exercise intensity eliciting MFO (Fat_{Max}) varies with training status; well-trained individuals reach MFO at 45-65% of $\text{VO}_{2\text{max}}$ (Achten et al. 2002; Achten and Jeukendrup 2003; Venables et al. 2005), whereas sedentary individuals reach MFO around 30-50% of $\text{VO}_{2\text{max}}$ (Venables et al. 2005; Venables and Jeukendrup 2008; Ara et al. 2011; Croci et al. 2013; Croci et al. 2014b). The concept of Fat_{Max} as determined by indirect calorimetry during whole-body exercise was established in 2001 by Achten and colleagues (Achten et al. 2002), using a graded exercise protocol. Prior studies had measured fat oxidation during continuous exercise at two (Sidossis et al. 1997; Thompson et al. 1998), three (Romijn et al. 1993; Romijn et al. 2000) or four (Bergman and Brooks 1999) submaximal exercise intensities, hence Fat_{Max} had until then not been accurately determined. Achten et al. concluded that their graded exercise protocol, commencing at 95 watt with increments of 35 watt every 3rd minute, could be used for valid and reliable assessment of Fat_{Max} in well-trained individuals (Achten et al. 2002; Achten and Jeukendrup 2003). Day-to-day reliability of various graded exercise protocols for determination of Fat_{Max} was subsequently tested (Meyer et al. 2009; De Souza Silveira et al. 2016; Stoa et al. 2016) and excellent Pearson's (0.84-0.97) and intraclass correlations coefficients (ICC) (0.82-0.98) were reported. However, Bland-Altman plots revealed large intra-individual variation between test days (Meyer et al. 2009; Croci et al. 2014a).

The graded exercise protocol developed by Achten et al. was not designed for individuals with obesity and relatively poor aerobic capacity, and most likely this group of subjects will only complete 2-3 steps of the original test, hence Fat_{Max} will not be accurately determined. Consequently, various graded exercise protocols commencing at lower exercise intensities (30-50 watt) have been employed to determine Fat_{Max} in obese (Van Aggel-Leijssen et al. 2002; Mogensen et al. 2009; Ara et al. 2011;

Ipavec-Levasseur et al. 2015; Tan et al. 2016a). However, no protocol developed specifically for obese has to our knowledge been validated against Fat_{Max} determined from continuous exercise at different intensities.

Thus, the purpose of the present study was to develop, validate and inter-method reliability test a graded exercise protocol for determination of Fat_{Max} in individual with obesity. The hypothesis was that the protocol could accurately determine Fat_{Max} .

Methods

Subjects

Sixteen individuals with obesity (8/8 women/men, age: 28 (26-29) years, BMI: 36 (35-38) kg m^{-2}) (95 % CI) participated in the study (**Table 1**), which was performed in accordance with the Helsinki declaration and approved by the local Research Ethics Committee, Copenhagen, Denmark (H-3-2013-146). The subjects were informed orally and in writing about the experiments and potential risks before written consent were obtained. Exclusion criteria were cardiovascular and/or metabolic disease. One subject with obesity was excluded from the analyses as the subject was unable to complete the exercise tests. Menstrual cycle and use of oral contraceptives were not controlled for.

Experimental design

Graded exercise protocol

The 16 subjects with obesity performed a graded exercise protocol on an electronically braked cycle ergometer (Monark 834E, Vansbro, Sweden). The protocol started with a resting period of five min and was followed by six min warm-up at 30 watt. Then the intensity was increased every 3rd min by 20 or 25 watt for women and men, respectively until the respiratory exchange ratio (RER) >1.0. After a short break (~2 min) an incremental exercise protocol (2 min at 100 watt followed by increments of 20/25

watt for women/men every min) to voluntary exhaustion was initiated. A plateau in $\text{VO}_{2\text{max}}$ (defined as a change of no more than $2 \text{ ml kg}^{-1} \text{ min}^{-1}$ with increasing work load) and $\text{RER} > 1.15$ were used as criteria for achieving $\text{VO}_{2\text{max}}$. Criteria for attainment of $\text{VO}_{2\text{max}}$ in obese is discussed in Wood et al. (Wood et al. 2010). Heart rate (HR) was measured continuously over the test (RS400, Polar Electro OY, Kempele, Finland). Total duration of the protocol was ~ 35 min. including rest, warm-up, exercise periods and breaks.

Short continuous exercise protocol (SCE)

To verify if the results from the graded exercise protocol could be used to accurately determine Fat_{Max} in obese, fat oxidation at rest and during continuous exercise at 35, 50 and 65% of $\text{VO}_{2\text{max}}$ was determined during 10-min exercise periods on a separate day (2-7 days in between). A resting period of 10 min was followed by 10 min at 35% of $\text{VO}_{2\text{max}}$, which also served as warm-up. The order of the two other exercise intensities (50 and 65% of $\text{VO}_{2\text{max}}$) were randomized and the exercise periods were separated by rest periods of 45 min (subjects rested in the supine position). Ad libitum water, but no food was served in the breaks. Total duration was $2\frac{1}{2}$ hours including rest and exercise periods.

Day-to-day reliability of the graded exercise protocol

Additionally, day-to-day reliability of the graded exercise protocol was tested in eight normal weight moderately trained subjects (3/5 women/men, age: 32 (30-34) years, BMI: 23 (22-24) kg m^{-2}), who performed the test on two different days (2-7 days in between). The graded exercise protocol was performed exactly as described for the individuals with obesity. No protocol has so far been validated and reliability tested in obese. The purpose of these additional tests was to allow comparison with graded exercise protocols developed for normal weight individuals.

Measurements

The subjects reported to the laboratory in the morning (between 8 and 10 a.m.) after an overnight fast (12h) on two different days (same time on both days). Body weight, height and waist circumference were measured. On the day of the graded exercise protocol a Dual-energy X-ray absorptiometry (DXA) scan (Lunar Prodigy Advance, Lunar, Madison, WI, USA) was performed to assess body composition. Resting blood pressure was measured in the seated position three times (interspersed by two min breaks) on the right upper arm (A&D Medical, Tokyo, Japan). A resting blood sample was drawn from the antecubital vein on both test days, and additional blood samples were obtained right before initiation of the 10-min continuous exercise periods at 35, 50 and 65% of $\text{VO}_{2\text{max}}$, respectively. The additional blood samples were taken in order to retrospectively check plasma concentrations of glucose, insulin, lactate, free fatty acids (FFA) and glycerol. Oxygen uptake (VO_2), expired carbon dioxide (VCO_2) and pulmonary ventilation (V_E) were measured continuously at rest and during exercise (Oxycon Pro, Jaeger, Würzburg, Germany).

Control of diet and physical activity

The subjects were told to eat identical diets the day before the two test days, and to abstain from any physical activity 48 hours before the tests. In line with previous studies determining Fat_{max} (Achten et al. 2002; Tan et al. 2016b) a standardized 24-hour dietary questionnaire was used to evaluate the diet before the first test day, and the result was provided to the subjects hence they could repeat the diet before their second test day.

Blood analysis

Blood was sampled in iced glass tubes and immediately centrifuged at 2500 g at 4°C for 10 min. The plasma fraction was collected and stored at -80°C prior to analysis. Plasma insulin concentration was measured using an ELISA kit (Alpco, Salem, NH, USA) and plasma concentrations of glucose, lactate,

FFA and glycerol were measured on an automated analyzer (COBAS 501, Roche, Mannheim, Germany) using standard assays. Hemoglobin concentration and hematocrit were analyzed immediately (Hemo Control Hemoglobin Analyzer, EKF Diagnostics, Magdeburg, Germany). HbA1c was analyzed on a DCA Vantage Analyzer (Siemens Healthcare, NY, USA).

Data analysis

VO_2 , VCO_2 and V_E data (sampling intervals of 10 sec) were averaged over the last 30 sec of each step of the graded exercise protocol. To check whether steady-state was reached at each 3-min step of the graded exercise protocol VO_2 and VCO_2 obtained from 2.00-2.30 min was compared with VO_2 and VCO_2 obtained from 2.30-3.00 min (Supplementary Table S1). VO_2 , VCO_2 and V_E were averaged over the last five min during rest and of each 10-min continuous exercise period. Fat oxidation rates were calculated using the stoichiometric equations described by Frayn (Frayn 1983), with the assumption that urinary nitrogen excretion was negligible. Fat oxidation rates at rest and during the graded exercise protocol and continuous exercise periods, respectively, were plotted against the relative exercise intensity (% $\text{VO}_{2\text{max}}$) and a 2nd degree polynomial regression was used to determine Fat_{Max} and MFO, VO_2 , V_E , HR and watt at Fat_{Max} for each subject individually. A 3rd degree polynomial regression was applied on fat oxidation data from the graded exercise protocol to control that Fat_{Max} and MFO were similar with this data analysis method.

Statistics

Data are presented as means $\pm$ 95% CI with significance set at an α -level of 0.05. Data were checked for normal distribution and equal variance. The inter-method reliability between the graded and the SCE protocol were analyzed with Pearson and intraclass correlation coefficients (ICC), and coefficient of variation (CV). Pearson correlation coefficient's and ICC's were interpreted as follows: >0.75 as

excellent, 0.40-0.74 as fair to high and <0.40 as poor (Fleiss 1986). Gender differences were analyzed with an unpaired t-test and systematic differences in Fat_{Max} and MFO, VO_2 , VCO_2 , V_E , HR and Watt at Fat_{Max} between the graded and the SCE protocol were analyzed with a paired t-test. The agreement (validity) in Fat_{Max} and MFO between the graded and the SCE protocol were assessed by a Bland-Altman plot establishing mean differences (bias) and $\pm 95\%$ limits of agreement (LoA). Systematic differences in fasting levels of plasma FFA, glycerol, glucose, insulin and lactate were analyzed with a paired t-test, and possible differences in plasma concentrations of FFA, glycerol, glucose, insulin and lactate before initiation of each continuous exercise period was analyzed by a One-Way ANOVA with adjustment for multiple comparisons.

Additionally, day-to-day reliability of the graded exercise protocol was assessed in normal weight subjects (Pearson correlation coefficient and ICC, CV, systematic differences, bias and $\pm 95\%$ LoA). Statistical analyses were performed in SAS Enterprise 4.3 (SAS Institutes, Cary, NC, USA) and figures were made in Graphpad Prism 6.01 Software Inc. (La Jolla, CA, USA).

Results

Subject characteristics

Obese women reached Fat_{Max} at a higher % of $\text{VO}_{2\text{max}}$ than obese males ($P < 0.05$), during both the graded (45 (43-47) vs. 39 (37-40), respectively) and the SCE protocol (48 (47-50) vs. 40 (38-42), respectively). Obese women also reached a higher MFO (g min^{-1}) compared to males during both the graded (0.36 (0.29-0.44) vs. 0.29 (37-40), respectively) and the SCE protocol (0.32 (0.27-0.37) vs. 0.26 (0.21-0.31), respectively), albeit the latter was only borderline statistically significant ($P < 0.10$). From Figure 1f and 2f it appears that the intra-individual variation in Fat_{Max} and MFO in women and men seem similar.

Validity and inter-method reliability of the graded exercise protocol for determination of Fat_{Max} in individuals with obesity

Fat oxidation, VO_2 , VCO_2 and V_E at each step of the graded and the SCE protocol are depicted in **Figure 1a-d**. Pearson's correlation coefficients and ICC's for Fat_{Max} and VO_2 , VCO_2 , V_E , HR and Watt at Fat_{Max} between the protocols were high-excellent for all variables (0.72-0.91) except from MFO, which was fair (0.48 and 0.56) (**Table 2**). Accordingly, CV's were $\leq 7\%$ for all variables investigated except for MFO, which was 11%. A Bland-Altman analysis of agreement between the protocols revealed a bias of -3% points of $\text{VO}_{2\text{max}}$ (95% LoA -12 to 7%) for Fat_{Max} and 0.04 g min^{-1} (95% LoA -0.09 to 0.17 g min^{-1}) for MFO, respectively (**Figure 1e-f**). A trend ($p=0.06$) towards systematic difference between the graded and the SCE protocol in determination of Fat_{Max} was observed (Table 2). No systematic differences in fasting levels of plasma FFA, glycerol, glucose, insulin and lactate between the two test days were observed, and there was also no difference in the plasma milieu before initiation of exercise at 35, 50 and 65% of $\text{VO}_{2\text{max}}$, respectively, in the SCE protocol (**Table 3**). Large intra-individual variation was, however, observed between the two test days in FFA and glycerol (**Figure 1g-h**), which was also illustrated in within subjects CV's (Table 3).

Day-to-day reliability of graded exercise protocol for determination of Fat_{Max} in normal weight subjects

Fat oxidation, VO_2 , VCO_2 and V_E at each step of the two graded exercise tests are shown in **Figure 2a-d**. Pearson correlation coefficient and ICC's were excellent (0.81-0.99) for all variables investigated except from MFO, which was fair (0.57-0.58). Within subjects CV's were low ($\leq 5\%$) for all variables except for MFO, which was high (11%). A Bland-Altman analysis of the agreement between the two tests revealed a bias of 1% points of $\text{VO}_{2\text{max}}$ (95% LoA -8 to 11% points of $\text{VO}_{2\text{max}}$) for Fat_{Max} and 0.00 g min^{-1} (95 % LoA

-0.17 to 0.17 g min⁻¹) for MFO (**Figure 2e, f**). No systematic differences in fasting levels of plasma FFA, glycerol, glucose, insulin and lactate between the two graded exercise tests were observed. Within subject CV's between the days were high (>14%) for all variables except glucose, which was low (3%) (Table 3).

Discussion

For the first time Fat_{Max} determined with a graded exercise protocol developed specifically for individuals with obesity was validated and inter-method reliability tested against Fat_{Max} determined from a SCE protocol. The main findings were a high to excellent ICC and Pearson correlation coefficient (0.72 and 0.75) and a low within subject CV (≤5%) between the two protocols, which suggest that the graded exercise protocol has a high inter-method reliability. However, despite a small bias (-3% points of VO_{2max}) between the two protocols in determination of Fat_{Max}, considerable intra-individual variation (95% LoA -12 to 7% points of VO_{2max}) and a trend (p=0.06) towards systematic difference reveal that further optimization of the graded exercise protocol is needed to improve validity.

Validity and inter-method reliability of the graded exercise protocol for determination of Fat_{Max} in individuals with obesity

The overall aim was to develop a graded exercise protocol, which could accurately determine Fat_{Max} in individuals with obesity. Importantly, the validity of the graded exercise protocol should be judged in the context of existing protocols for determination of Fat_{Max}. Achten et al. (Achten et al. 2002; Achten and Jeukendrup 2003) conducted the only other study validating a graded exercise protocol against a continuous exercise protocol. In contrast to the present study, the protocol validated by Achten et al. was for well-trained, and differences in the results may relate to differences in e.g. aerobic capacity and degree of adiposity. Based on the fact that there was no systematic difference between the graded

and the continuous protocol, Achten et al. concluded that their graded exercise protocol could accurately determine Fat_{Max} . In contrast, despite the fact that the bias between the protocols appeared similar or smaller in the present study (42 ± 2 vs $45 \pm 2\%$ point of $\text{VO}_{2\text{max}}$)(SEM) compared to Achten et al. (56 ± 3 vs $58 \pm 3\%$ and $64 \pm 3\%$ point of $\text{VO}_{2\text{max}}$) we observed a tendency towards a systematic difference in Fat_{Max} . A possible explanation for the divergent findings is lower measurement variation in the present study, which makes it easier to detect a difference. It is likely that also a difference in the number of comparisons made in the statistical analysis contribute to the different result (2 vs. 3 comparisons). Albeit not significant, Achten et al. also observed Fat_{Max} at a higher relative intensity with the continuous compared to the graded exercise protocol. In addition to assessing systematic differences between the two protocols in the present study, the inter-method reliability was determined by Pearson correlation coefficients, ICC and within subject CV. A strong relationship and a low CV was observed, which indicate that the graded exercise protocol has a high inter-method reliability.

To investigate the validity and to evaluate the practical use of the graded exercise protocol intra-individual variation was visualized (Bland Altman plot) and calculated. The improvements (3-15% points of $\text{VO}_{2\text{max}}$) reported in Fat_{Max} after aerobic training periods of 10 to 12 weeks (Mogensen et al. 2009; Nordby et al. 2015; Rosenkilde et al. 2015) are generally smaller than the 95% LoA (-12 to 7% point of $\text{VO}_{2\text{max}}$) reported in the present study, and by others (Meyer et al. 2009; Croci et al. 2014a). Thus, the graded exercise protocol is not suitable for assessment of individual changes in Fat_{Max} after e.g. a shorter intervention. However, the graded exercise protocol might be used for comparisons of means across larger groups, where the intra-individual variation in Fat_{Max} is accounted for in a power-calculation.

Day-to-day reliability was only determined in normal weight individuals and not in obese, which is a limitation. However, in line with other studies determining Fat_{Max} in normal weight subjects (Gmada et al. 2012; De Souza Silveira et al. 2016; Stoa et al. 2016) ICC and Pearson correlation coefficients between the two tests were excellent (0.81 and 0.82) for Fat_{Max} , within subject CV were low (3%), and no systematic differences were observed. Furthermore, the 95% LoA (-8 and 11 % point of $\text{VO}_{2\text{max}}$) between the two graded tests as determined by Bland Altman plot was also similar to what has been reported by others (Meyer et al. 2009; Croci et al. 2014a). Thus, the graded exercise protocol appears as reliable (day-to-day) as other protocols in determining Fat_{Max} in normal weight subjects (Meyer et al. 2009; Croci et al. 2014b). It should, however, be noted, that the magnitude of the 95% LoA between the graded and the SCE protocol in the obese in the present study was similar to the 95% LoA in the normal weight individuals performing the graded exercise protocol at two different occasions.

Factors contributing to variation in Fat_{Max}

Several factors may contribute to intra-individual variation in Fat_{Max} . Achten et al (Achten et al. 2002) concluded that 3-min. steps and increments of 35 watts were appropriate for well-trained subjects in order to reach steady-state, and that 4-6 steps was necessary to construct a fat oxidation curve with a good fit. Bordenave et al. recommended steps longer than 3-min in sedentary individuals as fat oxidation could otherwise be overestimated (Bordenave et al. 2007). In the present study, it could be speculated that the 3-min steps in the graded exercise protocol were too short for the obese to reach steady-state. To control this, data on VO_2 and VCO_2 from 2.00-2.30 and from 2.30-3.00 at each step were compared (Supplementary Table S1). No difference was observed for VO_2 . Although, the maximal mean difference in VCO_2 was only 4 ml, a trend towards higher VCO_2 from 2.30-3.00 compared to 2.00-2.30 was observed indicating that the obese subjects were only approaching steady-state. The optimal

way to control, whether the subjects were in steady-state from 2.30-3.00 would have been to compare two graded exercise protocols with 3-min and 4-min steps, respectively. The study was, however, not designed to do that, and that is a limitation and consequently fat oxidation may have been overestimated. However, adding one extra min to each step would have extended the total length of the protocol with 5-6 min, which in these subjects could also have affected VO_2 and VCO_2 , especially at the last steps.

The graded exercise protocol was verified against a SCE protocol, in which Fat_{Max} was determined from rest and continuous exercise at 35, 50 and 65 % of $\text{VO}_{2\text{max}}$ (interspersed by 45 min breaks). The 10-min exercise periods were distributed over 2½ hours on the same day. An alternative would have been to distribute the exercise periods on different days as it was done in the original study by Achten et al. The advantage of our approach was a minimized day-to-day variation in Fat_{Max} (experiments on two days only). It was recently shown that standardizing the diet and physical activity several days prior to the test is highly important for reliable measurements of Fat_{Max} (Stoa et al. 2016), and that would not have been easy with continuous exercise on 3-4 different days. With 2-3 days between each test day, the test period would disperse over a total of 10-15 days. On the other hand it cannot be excluded that fat oxidation during the two last 10-min exercise periods during the SCE protocol was affected by the prior steps. Blood samples were taken before initiation of exercise at 35, 50 and 65 % of $\text{VO}_{2\text{max}}$ to retrospectively check availability of plasma substrates. No systematic differences in the blood parameters investigated were observed (Table 3). However, there were large intra-individual differences in especially plasma concentrations of FFA and glycerol (Table 3, Figure 1g, h and Figure 2g, h), which probably influences the intra-individual variation observed in Fat_{Max} and MFO.

Fat oxidation was measured at rest and during exercise at three different intensities during the SCE protocol, which gives four points for the 2nd degree polynomial regression used to determine MFO and Fat_{Max}. The SCE protocol was designed to obtain measurements of fat oxidation at both sides of MFO (35 and 65% of VO_{2max}) and a measurement close to MFO (50% of VO_{2max}). The average fit of the curve was $r^2=0.93$ for the SCE protocol, which is a very good fit. It is not clear how many different exercise intensities Achten et al. used in their continuous protocol. However, increasing the number of continuous exercise intensities in order to possibly attain a better fit and a more accurate determination of Fat_{Max} and MFO is indeed relevant. A 3rd degree polynomial fit was also analyzed for each subject on the graded exercise protocol, and this resulted in slightly better fit ($r^2=0.93$) compared to a 2nd degree polynomial regression ($r^2=0.90$). However, no difference in Fat_{Max} and MFO was observed between the 2nd and 3rd degree polynomial regression (Fat_{Max}: 42 (39-46) vs. 42 (37-47)% of VO_{2max}, MFO: 0.33 (0.29-0.37) vs. 0.34 (0.30-0.39) g min⁻¹).

Albeit weak, resting concentration of plasma FFA ($r=-0.53$, $p<0.05$) (Rosenkilde et al. 2010), and levels of plasma FFA and glycerol during exercise at Fat_{Max} ($r=0.27$, $p=0.03$, $r=0.39$, $p=0.03$) correlated with MFO (Robinson et al. 2016). Thus, measuring availability of plasma metabolites such as plasma FFA and glycerol seem relevant in studies investigating validity and reliability of Fat_{Max} and MFO. Within subject CV's between the two test days in fasting concentrations of FFA and glycerol were high (9-22%) for both obese and normal weight individuals and the intra-individual variation in Fat_{Max} and MFO might reflect that. In line with the present study Lanzi et al. (Lanzi et al. 2014) observed higher availability of FFA in obese compared to lean, and also suggested that obese might have an impaired muscular capacity to oxidize FFA. On the other hand it was also shown that levels of FFA, fat mass and insulin resistance does not follow hand in hand as e.g. women have higher levels of FFA compared to men,

and at the same time they are more insulin sensitive (Karpe et al. 2011). Regulation of fat oxidation is complex; however, a step towards finding an optimal graded exercise test for determination of Fat_{Max} and MFO may involve measurement of fasting and exercise levels of plasma substrates.

In summary, for the first time a graded exercise protocol for determination of Fat_{Max} in individuals with obesity was developed, validated and inter-method reliability tested against Fat_{Max} determined from control measurements of fat oxidation at rest and during continuous exercise. There was a high to excellent correlation and a low CV between the two protocols in determination of Fat_{Max} , which suggest that the graded exercise protocol has a high inter-method reliability. However, considerable intra-individual variation and a trend towards systematic difference reveal that further optimization of the graded exercise protocol is needed to improve validity.

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Author contribution

JWH, SD and SL designed the study. SD, SL, CBP, SDS, CS, FD and JWH contributed to data acquisition, analyses and interpretation. SD wrote the manuscript. All authors revised the manuscript and approved the final article.

Conflicts of interests

The authors declare no conflict of interests

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Draft

Tables

Table 1 Subject characteristics

	Individuals with obesity	Individuals with normal weight
Gender (w/m)	8/8	3/5
Age (years)	28 (26-29)	32 (30-34)
Height (cm)	173 (172-175)	179 (160-199)
Weight (kg)	109 (105-113)	76 (73-79)
Fat (%)	46 (45-48)	24 (22-26)
Fat (kg)	49 (46-52)	17 (15-20)
FFM (kg)	60 (57-63)	59 (56-62)
Waist circumference (cm)	109 (106-112)	85 (83-87)
HbA1c (%)	5.1 (5.0-5.2)	4.9 (4.6-5.2)
Hematocrit (%)	42 (42-42)	43 (41-44)
Hemoglobin (mmol mol ⁻¹)	8.9 (8.4-9.3)	9.0 (8-10)
Systolic blood pressure (mmHg)	120 (117-122)	128 (126-131)
Diastolic blood pressure (mmHg)	82 (80-83)	80 (78-81)
Resting heart rate (beats min ⁻¹)	70 (69-72)	60 (59-61)
VO _{2max} (l min ⁻¹)	3.2 (3.2-3.2)	3.4 (3.4-3.4)
VO _{2max} (ml min ⁻¹ kg ⁻¹)	29.8 (28.2-31.4)	45.2 (44-47)
VO _{2max} (ml min ⁻¹ FFM ⁻¹)	54.2 (52.8-55.5)	58.4 (57-60)
HR _{max} (beats min ⁻¹)	184 (178-191)	174 (164-184)

Descriptive data of subjects included in the study. Data are presented as means with 95% confidence intervals.

Table 2 Determination of Fat_{Max} in individuals with obesity and normal weight

Individuals with obesity	Graded protocol	SCE protocol	Pearson correlation coefficient (r, p-value)	Intraclass correlation coefficient	CV (%)	Paired t-test (p-value)
MFO (g min ⁻¹)	0.33 (0.29-0.37)	0.29 (0.26-0.32)	0.56, 0.03	0.48	11 (8-15)	0.03
Fat _{Max} (% VO _{2max})	42.1 (38.5-45.7)	44.6 (42.5-46.7)	0.75, <0.001	0.72	5 (3-7)	0.06
Watt at Fat _{Max} (Watt)	69.8 (65.4-74.2)	78.8 (74.3-83.3)	0.91, <0.001	0.81	6 (3-10)	<0.01
VO ₂ at Fat _{Max} (l min ⁻¹)	1.34 (1.33-1.34)	1.43 (1.42-1.44)	0.83, <0.001	0.79	5 (3-7)	0.03
VCO ₂ at Fat _{Max} (l min ⁻¹)	1.19 (1.18-1.20)	1.30 (1.29-1.31)	0.83, <0.001	0.74	5 (3-8)	0.01
V _E at Fat _{Max} (l min ⁻¹)	33.4 (30.8-36.0)	37.6 (35.3-39.9)	0.84, <0.001	0.74	7 (3-10)	<0.01
HR at Fat _{Max} (beat min ⁻¹)	117 (114-119)	121 (119-123)	0.72 <0.01	0.70	4 (2-6)	0.14
Normal weight individuals	Graded protocol 1	Graded protocol 2	Pearson correlation coefficient (r, p-value)	Intraclass correlation coefficient	CV (%)	Paired t-test (p-value)
MFO (g min ⁻¹)	0.31 (0.25-0.38)	0.31 (0.23-0.40)	0.57, 0.01	0.58	11 (6-17)	0.98
Fat _{Max} (% VO _{2max})	42.3 (40.1-44.4)	40.9 (38.7-43.1)	0.81, <0.001	0.82	3 (0-7)	0.44
Watt at Fat _{Max} (Watt)	65.2 (57.4-73.1)	64.7 (56.4-73.1)	0.94, <0.001	0.92	5 (1-9)	0.94
VO ₂ at Fat _{Max} (l min ⁻¹)	1.40 (1.38-1.42)	1.39 (1.37-1.41)	0.87, <0.001	0.83	4 (0-9)	0.85
VCO ₂ at Fat _{Max} (l min ⁻¹)	1.09 (1.07-1.12)	1.09 (1.06-1.12)	0.87, <0.001	0.94	4 (1-8)	0.97
V _E at Fat _{Max} (l min ⁻¹)	34.7 (32.5-36.9)	35.0 (32.2-37.9)	0.87, <0.001	0.86	5 (1-9)	0.93
HR at Fat _{Max} (beat min ⁻¹)	105 (101-108)	101 (98-104)	0.97, <0.001	0.94	2 (0-3)	0.18
VO _{2max} (l min ⁻¹)	3.42 (3.40-3.44)	3.40 (3.38-3.42)	0.98, <0.001	0.99	1 (0-2)	0.62
HR _{max}	175 (173-176)	173 (171-175)	0.97, <0.001	0.97	1 (0-2)	0.21

SCE: Short continuous exercise, VO₂: oxygen uptake, VCO₂: expired carbon dioxide, V_E: lung ventilation, HR: heart rate. Individuals with obesity completed a graded exercise protocol, and 10-min rest and continuous exercise periods on two different days. Normal weight individuals completed the graded exercise protocol at two different days. Fat oxidations were plotted against exercise intensity, and a 2nd degree polynomial fit was used to determine Fat_{Max}, and MFO, VO₂, VCO₂, watt and V_E at Fat_{Max}. Inter-method (obese subjects) or day-to-day (normal weights subjects) reliability, variability and systematic differences were analyzed by Pearson correlation coefficient, intraclass correlation coefficient, within subject coefficient of variation (CV) and paired t-test. Data are means ±95% confidence intervals.

Table 3 Levels of plasma lipids, substrates and hormones in individuals with obesity and normal weight

Individuals with obesity	Graded protocol (pre)	SCE protocol (pre 35% of VO_{2max})	CV (Graded vs SCE protocol) (%)	Paired t-test (Graded vs SCE protocol) (p-value)	SCE protocol (pre 50% of VO_{2max})	SCE protocol (pre 65% of VO_{2max})	CV (Pre 35%, 50% and 65% in SCE protocol) (%)	One-Way ANOVA (Pre 35%, 50% and 65% in SCE protocol) (p-value)
FFA ($\mu\text{mol l}^{-1}$)	473 (387-559)	465 (370-560)	13 (8-19)	0.81	465 (381-548)	510 (410-611)	12 (11-20)	0.70
Glycerol ($\mu\text{mol l}^{-1}$)	79 (68-90)	76 (67-84)	9 (5-13)	0.70	62 (57-67)	65 (61-70)	14 (12-20)	0.29
Glucose (mmol l^{-1})	5.4 (5.1-5.8)	5.3 (4.9-5.8)	3 (1-4)	0.18	5.3 (4.9-5.7)	5.3 (5.1-5.6)	3 (2-4)	0.98
Insulin (pmol l^{-1})	74 (60-87)	88 (74-102)	14 (7-20)	0.07	80 (64-95)	73 (61-85)	14 (12-24)	0.93
Lactate (mmol l^{-1})	0.9 (0.7-1.0)	0.9 (0.8-1.1)	12 (7-17)	0.60	0.9 (0.8-1.1)	0.8 (0.7-1.0)	12 (12-19)	0.44
Normal weight individuals	Graded protocol 1(pre)	Graded protocol 2 (pre)	CV (Graded vs SCE protocol) (%)	Paired t-test (Graded vs SCE protocol) (p-value)				
FFA ($\mu\text{mol l}^{-1}$)	246 (237-255)	239 (224-254)	22 (11-32)	0.88	-	-	-	-
Glycerol ($\mu\text{mol l}^{-1}$)	38 (35-41)	45 (41-49)	14 (5-23)	0.24	-	-	-	-
Glucose (mmol l^{-1})	5.5 (5.0-5.9)	5.3 (4.7-5.9)	3 (0-7)	0.58	-	-	-	-
Insulin (pmol l^{-1})	16 (11-21)	17 (11-22)	22 (7-37)	0.87	-	-	-	-
Lactate (mmol l^{-1})	0.9 (0.8-1.0)	0.7 (0.6-0.9)	21 (9-32)	0.83	-	-	-	-

Plasma concentrations of lipids, metabolites and hormones before initiation of the graded exercise protocol and 10-min continuous exercise at 35, 50 and 65% of VO_{2max} . The order of the continuous exercise periods were randomized, except that the first intensity always was 35% of VO_{2max} . The 10-min continuous exercise periods were separated by 45 min. breaks in order to return to resting conditions before initiation of the next exercise period. SCE: Short continuous exercise, FFA: free fatty acids, CV: within subject coefficient of variation. Data are presented as means $\pm$ 95% confidence intervals.

Figure legends

Figure 1

Fat oxidation (**a**), oxygen uptake (VO_2) (**b**), expired carbon dioxide (VCO_2) (**c**) and lung ventilation (V_E) (**d**) are plotted against exercise intensity ($\% \text{VO}_{2\text{max}}$) for the graded and the short continuous exercise (SCE) protocols, respectively. Bland-Altman plot of the agreement in maximal fat oxidation (MFO) determined with the graded and the SCE protocol (**e**). Bland-Altman plot of the agreement in the intensity that elicits maximal fat oxidation (Fat_{Max}) determined with the graded and the SCE protocol (**f**). Women are indicated by squares and men by circles. Individual plasma concentrations of free fatty acids (FFA) (**g**) and glycerol (**h**). $n=15$ individuals with obesity. Data are mean with $\pm 95\%$ confidence intervals.

Figure 2

Fat oxidation (**a**), oxygen uptake (VO_2) (**b**), expired carbon dioxide (VCO_2) (**c**) and lung ventilation (V_E) (**d**) are plotted against exercise intensity ($\% \text{VO}_{2\text{max}}$) for the two graded exercise tests. Bland-Altman plot of the agreement in maximal fat oxidation (MFO) determined with the two graded exercise tests (**e**). Bland-Altman plot of the agreement in the intensity that elicits maximal fat oxidation (Fat_{Max}) determined with the two graded exercise test (**f**). Women are indicated by squares and men by circles. Individual plasma concentrations of free fatty acids (FFA) (**g**) and glycerol (**h**). $n=8$ individuals with normal weight. Data are mean with $\pm 95\%$ confidence intervals.

Figure 1

134x69mm (300 x 300 DPI)

Figure 2

134x69mm (300 x 300 DPI)