Isotope Tracer Measures of Meal Fatty Acid Metabolism: Reproducibility and Effects of the Menstrual Cycle

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Running Title: Menstrual cycle and meal fatty acid metabolism

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ABSTRACT

Adding isotopic tracers of fatty acids to meals allows investigators to measure the oxidation and adipose tissue uptake of dietary fat. The present studies were conducted to measure the between-study variability of these types of experiments and to assess whether dietary fatty acids are handled differently in the follicular vs. luteal phase of the menstrual cycle. Healthy, normal weight men (n=12) and women (n=12) participated in these studies, which block randomized to control for study order, isotope (\(^{3}\)H triolein vs. \(^{14}\)C triolein), and menstrual cycle. Energy expenditure (indirect calorimetry), meal fatty acid oxidation, and meal fatty acid uptake into upper body and lower body subcutaneous fat (biopsies) 24 hours after the experimental meal were measured. Results: A greater portion of meal fatty acids were stored in upper body subcutaneous adipose tissue (24±2% vs. 16±2%, p<0.005) and in lower body fat (12±1% vs. 7±1%, p<0.005) in women than men. Meal fatty acid oxidation (\(^{3}\)H\(_{2}\)O generation) was greater in men than women (52±3% vs. 45±2%, p=0.04). Leg adipose tissue uptake of meal fatty acids was 15±2% in the follicular phase of the menstrual cycle and 10±1% in the luteal phase (P = NS). The variance and skewness of leg fatty acid uptake was somewhat greater in women than in men (P = NS); the variance in upper body meal fatty acid uptake did not differ between men and women. We conclude that leg uptake of dietary fat is slightly more variable in women than in men, but that there are no major effects of the menstrual cycle on meal fatty acid disposal.

Key Words: Triglyceride, adipose tissue biopsy, isotope dilution, indirect calorimetry
INTRODUCTION

Understanding the storage and oxidation of dietary macronutrients has a number of implications for obesity, body fat distribution and insulin resistance. Björntorp et al (1) and Mårin et al (20) helped pioneer the use of adding tracers to meals to assess the uptake of dietary nutrients into different tissue beds in vivo in humans, including the uptake of dietary fat into intra-abdominal adipose tissue (18). Other investigators have used meal fatty acid tracers to assess the oxidation of dietary fat (17,27). We have combined these approaches in attempts to account for the fate of meal fatty acids in their entirety (16,23,24). Our goal is to use this approach to uncover the relative contribution of regional variations in fatty acid uptake as a determinant of inter-individual differences in body fat distribution.

In addition to describing differences in meal fatty acid disposal between obese and lean or between men and women, these techniques can be used to determine whether hormonal (18), activity (27) or perhaps dietary interventions alter meal fatty acid disposal. Using the meal fatty acid tracer technique should permit investigators to assess whether there are means to alter meal fat disposal in a way that could shunt dietary fat away from visceral fat and into a depot less associated with adverse health consequences. Performing studies to assess the effect of an intervention on meal fatty acid uptake into regional fat is best done using a paired study design (studies pre-and post-treatment). By using a $^{14}$C-labelled fatty acid tracer for one study and a $^{3}$H-labeled fatty acid for the other study it is possible to avoid having the residual adipose tracer (20) invalidate the adipose tissue fatty acid uptake calculations.

The design of such paired studies to test the effect of an intervention on adipose tissue uptake of meal fatty acids is enhanced if one has foreknowledge of the degree of intra-individual variability, as well as whether there are other confounding factors. For example, in women,
menstrual cycle is considered to be a potential confounding variable for some metabolic parameters (2,3,10,26,29). Slight differences in the resting metabolic rate and the thermic effect of food have been reported in women in different phases of the menstrual cycle (10,26), although this has not been verified in other studies (21). Because of the possibility that energy metabolism may be affected by the menstrual cycle, we studied meal fatty acid metabolism in women only in the follicular phase of their menstrual cycle (23); this precaution is not necessary for studies of resting FFA (9) or glucose (29) metabolism. The present study was done largely to assess whether controlling for the phase of the menstrual cycle is necessary in future studies of meal fatty acid metabolism. We also assessed the intra-individual variability in meal fatty acid metabolism in men using the same study design as women. Herein, we report the uptake and oxidation of meal fatty acids in both men and women and the variability in the uptake in these two groups.
MATERIALS AND METHODS

Subjects. This protocol was approved by the Mayo Institutional Review Board. Written, informed consent was obtained from 24 healthy, non-obese volunteers (12 males and 12 premenopausal females). The subjects were taking no medications, including oral contraceptives. A complete blood count, chemistry group, and lipid profile were documented to be within normal limits before the study. All volunteers were weight stable for at least 2 months prior to the study and refrained from vigorous exercise for 2 days prior to the study.

Protocol

The study was designed to assess meal fatty acid metabolism in the follicular and luteal phases of the menstrual cycle in women as well as to assess the reproducibility of the measures. In order to measure adipose tissue meal fatty acid uptake on two occasions it was necessary to use $^{14}$C labeled triolein for one study and $^3$H labeled triolein for the other study. Because tracers can differ with regards to purity (24) the studies were designed to randomize for meal tracer administration. Half of the subjects received the $^{14}$C labeled triolein for the first study and $^3$H labeled triolein for the second study and half received the opposite schedule. For the women participants a similar allocation was used and, in addition, the studies were block randomized such that half of the women had their luteal phase study as their first study and half had the follicular as the first study. In this way there was an even distribution of meal tracers across first and second studies and follicular and luteal phase studies. In order to assess meal fatty acid oxidation using comparable methodology on both study days we included a carbon labeled isotope $[1-^{13}$C]triolein ($798 \pm 62$ mg for men and $590 \pm 68$ mg for women) on the $[^3$H]triolein meal day. Breath was collected for $^{13}$CO$_2$ enrichment on both study days; the samples from the
[1-14C]triolein study day served as the background enrichment for each individual for their [1-13C]triolein/[3H]triolein study day.

Subjects consumed all of their meals in the Mayo Clinic General Clinical Research Center (GCRC) for one week before the study to ensure consistent macronutrient intake (50% carbohydrate, 35% fat and 15% protein). They were instructed not to eat anything except what was provided for them through the study, and food intake was adjusted to maintain a stable weight if necessary. Each volunteer’s energy requirement was estimated using the Harris-Benedict formula (8) and usual daily activity. The macronutrient intake the week before the tracer studies for the men and women, respectively, was as follows: energy intake: 2,929 ± 100 and 2,080 ± 48 kcal/day; protein intake: 114 ± 4 and 81 ± 2 g/day; carbohydrate intake: 376 ± 13 and 267 ± 13 g/day (193 ± 8 and 136 ± 5 g/day simple carbohydrate); fat intake: 117 ± 4 and 83 ± 2 g/day (44 ± 7 and 30 ± 8 g/day saturated fat).

The volunteers underwent all body composition measurements prior to the study. Ten of the women were studied in the follicular and luteal phases of their menstrual cycle whereas two women underwent both studies in the follicular phase of the menstrual cycle. The studies were two weeks apart for both men and women, except for the two women in whom we conducted two follicular phase studies. All female volunteers had a negative pregnancy test prior to participating in the study.

The volunteers were admitted to the Mayo Clinic GCRC the evening before the study. The morning of the study after an overnight fast, a catheter was placed in a forearm vein and used to collect blood samples. Prior to consuming the test meal baseline breath and urine samples were collected for measurement of background 14CO2 (7) and 3H2O specific activity (SA).
The participant’s basal metabolic rate was measured at 0700 hr, after a 12 hr overnight fast, before arising from bed. At 0800 h the volunteers consumed a meal providing one third of their resting energy expenditure as determined by indirect calorimetry (586 ± 20 Kcal for men and 436 ± 22 Kcal for women). The meal consisted of a liquid formula (Ensure Plus, Ross Laboratories, Abbott Park, IL) containing 57% carbohydrate, 27% fat (16 % saturated fat, 27% monounsaturated fat, 57 % polyunsaturated fat) and 15% protein to which either 20 µCi of [l-14C] triolein or 60 µCi of [3H] triolein had been added as previously described (13). The mean ± SD triglyceride content of the morning meals with the tracer was 21.3 ± 4.0 gm. The volunteers were also provided with normal (solid food) meals at 1300 and 1800 hours consistent with their previously determined pattern. These meals provided the remainder of the daily energy content with the same distribution of protein, carbohydrate (complex and simple) and fat (saturated and unsaturated) as the diets provided during the week prior to the study. To determine the exact amount of [14C] triolein and [3H] triolein consumed, quadruplicate 50 µL samples of the meal were counted using dual channel liquid scintillation counting; the radioactivity per 50 µL was multiplied by meal volume to determine the total amount of tracer consumed. The meal was weighed to the nearest 0.1 gm. Aliquots of the meal were also saved for measurement of meal lipid (14C) and (3H) SA (see below), which allowed us to assure that the calculated meal fatty acid SA (total meal dpm / total mg meal fat) was not different from the directly assayed meal SA (dpm/mg).

After consuming the test meal, blood and breath samples (for 14CO2 SA and 13CO2 enrichment) were obtained hourly for 8 hours, then every 2 hours for an additional 4 hours, then every 4 hours until the next morning. The blood samples were analyzed for plasma triglyceride (TG) (3H) and (14C) SA. Indirect calorimetry was performed hourly for 8 hours, at the 10th hour
and the next morning (24 hr. after the test meal). Urine was collected for 24 hours after the test meal to measure nitrogen excretion, to calculate $^{3}$H$_{2}$O losses and to assess the concentration of $^{3}$H$_{2}$O in body water after 24 hrs. Because the intravenous saline infusion was continued to maintain venous access, the physical activity of the volunteers was necessarily limited.

Twenty-four hours after the test meal consumption, adipose tissue biopsies were obtained using sterile technique under local anesthesia. Biopsies were taken from the left or right abdominal subcutaneous, gluteal, and femoral regions. After the adipose tissue biopsies the intravenous catheter was removed, the volunteers ate breakfast, and were dismissed.

Materials

$[l-^{14}C]$ triolein and $[9,10-^{3}H]$triolein were purchased from NEN Life Science Products, PerkinElmer, Boston, MA. $[l-^{13}C]$ triolein and $^{2}$H$_{2}$O (Isotech, Miamisburg, OH) were also used in these studies.

Assays and Methods

Adipose tissue and meal lipids were extracted using standard (5) procedures and the TG SA was measured as previously described (24). The lipid was extracted from the tissues, accurately weighed and counted on the scintillation counter to less than 2% counting error. The adipose tissue triglyceride SA ($^{3}$H and $^{14}$C - dpm/mg lipid) was calculated for each site.

Oxygen consumption and CO$_{2}$ expiration were measured by indirect calorimetry using a DeltaTrac Metabolic Cart (Sensor Medics, Yorba Linda, CA). Plasma glucose concentrations were measured with a Beckman glucose analyzer (Beckman Instruments, Fulton, CA). Plasma insulin concentrations were measured using a chemiluminescence method with the Access Ultrasensitive Immunoenzymatic assay systems (Beckman, Chaska, MN). The plasma TG
concentrations were measured (11) and a 1.0 mL plasma sample was subjected to a Dole extraction (4) to measure TG radioactivity.

Body fat and fat free mass (FFM) was measured using dual energy x-ray absorptiometry (DEXA; DPX-IQ; Lunar Radiation, Madison, WI) (15). Intra-abdominal adipose tissue area was measured using a single sliced computed tomography (CT) at the L2-3 level (14). Total body water was measured with $^2$H$_2$O (25). Urine water was assayed for $^3$H$_2$O concentration using liquid scintillation counting. The triolein tracer was assayed for radiochemical purity by measuring the radioactivity in the TG and the non-TG fractions by HPLC (13). The 24 hour urine sample was also assayed for nitrogen content to allow for calculation of substrate oxidation.

**Calculations**

Visceral fat mass was predicted using the CT measures of intra-abdominal and subcutaneous adipose tissue combined with DEXA measured abdominal fat as previously described (14). Upper body subcutaneous fat was taken as upper body fat (DEXA) minus visceral fat. Leg fat was measured using the region of interest program with the DEXA instrument.

Substrate oxidation at each point in time was calculated using indirect calorimetry and urinary nitrogen excretion rates (6). Integrated substrate oxidation over the first 10 hours of the study was calculated using the area under the curve of fatty acid and carbohydrate oxidation for each individual. Meal fatty acid oxidation for the 24 hr. after the test meal was calculated with either the $^3$H$_2$O or $^{14}$CO$_2$ production to determine the percent of meal fatty acids oxidized using the $^3$H or $^{14}$C tracers, respectively. The production of $^3$H$_2$O was calculated by multiplying the concentration of $^3$H$_2$O in body water (using a urine sample obtained 24 hr. after the test meal) by
total body water as measured by the $^2$H$_2$O space, and adding the $^3$H$_2$O lost in the urine over the 24 h. This value (total $^3$H$_2$O dpm produced) was divided by the total $^3$H triolein consumed to calculate the fraction of meal fatty acids oxidized in the first 24 hr. after the meal. The $^{14}$CO$_2$ production was determined by multiplying the $^{14}$CO$_2$ SA by the CO$_2$ production rate, as measured by indirect calorimetry, at each time point. The nocturnal CO$_2$ production rate time points were not measured in this study, although the $^{14}$CO$_2$ SA was measured. Nocturnal CO$_2$ production rates were estimated as previously described (24). To calculate meal fatty acid oxidation using $^{14}$C triolein, the area under the $^{14}$CO$_2$ curve was divided by the amount of $^{14}$C triolein consumed. A similar approach was used to calculate $[1-^{13}$C]triolein oxidation, except that each $^{13}$CO$_2$ value on the $[1-^{13}$C]triolein day was corrected for the background level from the control study day.

Meal fatty acid uptake into adipose tissue was calculated as follows. The adipose tissue TG SA (dpm/gm) was multiplied by the site-specific (lower body and upper body subcutaneous) adipose TG mass to estimate the amount of tracer (which reflects the amount of meal TG) stored in that body fat region. The regional adipose tracer uptake was divided by the total meal tracer content to determine the fraction of the tracer, and thus the meal fat, that was stored in the different adipose tissue depots (19). The gluteal values are given only for comparison with femoral values and were not used to calculate lower body meal fatty acid uptake. This is because the two sites are quite similar and because the body composition techniques we use do not allow us to quantitate the gluteal depot in isolation.

Statistics

All data are presented as mean ± SEM unless otherwise stated. Comparisons of plasma insulin, glucose and triglyceride results over time between the different study days was done
using a repeated measures analysis of variance with factors for time of sample and study day (day 1 vs. day 2, $^3$H vs. $^{14}$C, follicular vs. luteal) and between group (men vs. women). A similar approach was used to compare meal fatty acid uptake and oxidation between groups and study days, except that the time factor was not used because these values were integrated 24 hr measures. In addition, a repeated measures factor for between site (upper body subcutaneous fat vs. lower body fat) differences in meal fatty acid uptake was included. If significant differences were found using the repeated measures ANOVA, comparisons of results between follicular and luteal or study 1 and study 2 for women and between study 1 and study 2 for men were done using a paired t test. Comparisons between men and women were done using a non-paired t-test. A Bonferroni correction was used when multiple statistical tests were performed on data that were not part of the a priori hypothesis testing. Part of the statistical analysis included tests for normality and constant variance; only the data for leg uptake of meal fatty acids in women did not pass the test for normal distribution and the constant variance (see Results). To determine whether the variance in leg uptake data for women was significantly different than the variance in other sites and in men a modified Levine test was used. In brief, the absolute difference between the median value and each individual’s value for a site and study day was calculated. A rank sum test is then applied to assess whether there are significant differences between the two sets of observations.
RESULTS

Subject Characteristics

Men and women participating in this study were well-matched for age (Table 1), but otherwise displayed the typical sex differences. Men were taller and heavier than women, with less body fat and with more FFM. Men had more visceral fat than women, whereas women had more leg fat. Women had somewhat more upper body non-visceral (subcutaneous) fat. Total body water was $76 \pm 1\%$ of FFM.

The plasma cholesterol concentrations were greater in men than women ($174 \pm 6$ vs. $153 \pm 6, P = 0.02$) and HDL cholesterol concentrations were less in men that women ($48 \pm 3$ vs. $65 \pm 4, P = 0.002$). The mean fasting plasma triglyceride concentrations were greater in men than women ($909 \pm 130$ vs. $602 \pm 64 \mu\text{mol}/\text{L}, P = 0.049$) and not different ($P = 0.81$) in women between the follicular phase and luteal phase studies.

Plasma progesterone concentrations in the follicular and luteal phase of the menstrual cycle were $2.4 \pm 0.4$ and $16.1 \pm 3.5$ ng/ml ($n = 10$), respectively. The plasma progesterone concentrations for the two women studied only in the follicular phase of their cycles averaged $2.8$ ng/ml (average between study difference = 0.45 ng/ml).

Tracer Determined Meal Fatty Acid Metabolism (Figure 1)

Overall analysis of the percent of the meal taken up in upper body subcutaneous and lower body subcutaneous fat between men and women was performed. There was no effect of the study (study 1 and study 2) or of the isotope ($^3\text{H}$ versus $^{14}\text{C}$) when the pooled analysis was performed. Thus, subsequent analysis was able to focus on mean values of study 1 and study 2 between genders and comparisons between follicular and luteal phase of the menstrual cycle with the knowledge that isotope effects and study order were not statistical confounders.
Regional subcutaneous meal fatty acid uptake. Meal fatty acid uptake into adipose tissue is presented two ways. The first is the mg of meal fatty acids stored in each gram of adipose tissue lipid. This is calculated by dividing adipose tissue lipid SA (dpm/gm) by the meal fatty acid SA (dpm/mg). The second approach is the percent of meal fatty acids (as assessed by the percent of the meal fatty acid tracer) stored in the different depots.

The meal fatty acid uptake in abdominal, femoral and gluteal fat in men averaged 0.66 ± 0.11, 0.40 ± 0.06 and 0.45 ± 0.08 mg meal fat/gm adipose tissue lipid. The uptake in abdominal fat was greater (P = 0.02) than in femoral fat, but not significantly greater (P = 0.14) than gluteal fat. The uptake of meal fatty acids in femoral and gluteal fat was not significantly different, nor were there differences between study 1 and study 2 or the uptake measured with $^{14}$C vs. $^3$H triolein. For women the meal fatty acid uptake in abdominal, femoral and gluteal fat averaged 0.41 ± 0.04, 0.28 ± 0.03 and 0.28 ± 0.03 mg meal fat/gm adipose tissue lipid. The uptake in abdominal fat was greater than in femoral fat (P = 0.01) and gluteal fat (P = 0.002). The uptake of meal fatty acids in femoral and gluteal fat was not significantly different. Abdominal subcutaneous meal fatty acid uptake averaged 0.38 ± 0.05 and 0.40 ± 0.06 mg meal fat/gm adipose tissue lipid in the follicular and luteal phase of the menstrual cycle (n = 10, P = 0.77). Femoral adipose tissue uptake of meal fatty acids in the follicular and luteal phases were 0.31 ± 0.06 and 0.23 ± 0.02 (P = 0.25) and gluteal uptake was 0.26 ± 0.03 and 0.26 ± 0.04 (P = 0.97).

Expressing the fate of meal fatty acids as the percent of the total consumed, we found that 24 ± 2% of meal fatty acids were taken up into upper body subcutaneous fat in women and 16 ± 2% in men (P <0.005, women vs. men). There was no difference in the uptake between the first study and the second study in men or women and no difference between the follicular and luteal phase of the menstrual cycle in women. There was no difference between study 1 and study 2 for
lower body subcutaneous meal fatty acid uptake in men, with an average of $7 \pm 1\%$ of meal fat taken up in lower body fat. For women, the average uptake (both studies) of meal fatty acids in lower body fat was $12 \pm 1\%$ ($P < 0.005$ vs. men) and there was no difference between study 1 and study 2. In the follicular phase of the menstrual cycle, $15 \pm 2\%$ of meal fatty acids were stored in the lower body subcutaneous fat, whereas in the luteal phase, $10 \pm 1\%$ was stored in lower body subcutaneous fat. The constancy of the variance and the skewness of femoral meal fatty acid uptake data was such that we analyzed the menstrual cycle effects a rank sum test. Still, the difference between the follicular and luteal phase leg uptake data was not statistically significant ($P \approx 0.08$). This statistical/analytical issue was not encountered with other aspects of the meal tracer data. The variance in the leg data for women was not significantly ($P = 0.10 - 0.20$) greater than the variance in the other uptake data, however, according to the modified Levine test.

*Meal fatty acid oxidation.* Meal fatty acid oxidation over 24 hours in men was $21 \pm 3\%$ with the $^{14}$C-triolein and $22 \pm 1\%$ with $^{13}$C-triolein (without a carbon fixation correction factor). For women, these values were $21 \pm 2\%$ and $25 \pm 2\%$ ($p = NS$ for both $^{14}$C vs. $^{13}$C and men vs. women). For all subjects, $^{14}$C triolein oxidation was estimated to be $21 \pm 2\%$ over 24 hours, and $^{13}$C triolein oxidation was $24 \pm 1\%$ over 24 hours ($p = 0.12$). The lack of between-study differences in $^{14}$C vs. $^{13}$C meal fatty acid oxidation implies that any observed differences in non-oxidative meal fatty acid disposal are not downstream effects related to major differences in oxidative metabolism of meal fat. As calculated using the $^{3}$H$_2$O generation from the $^{3}$H-triolein study, 24 hour meal fatty acid oxidation in men was $52 \pm 3\%$ and in women was $45 \pm 2\%$ ($P = 0.04$ men vs. women). If we used the acetate correction factor of $50\%$ (28) the $^{14}$C triolein and $^{13}$C triolein oxidation was $42 \pm 3\%$ and $47 \pm 2\%$ over 24 hours for all 24 subjects ($P = NS$ vs. the $^{3}$H$_2$O estimated 24 hr meal fatty acid oxidation of $47 \pm 2\%$).
Unaccounted for meal fatty acids. Using data from the $^3$H-triolein study day, the percentage of meal fatty acids that could not be accounted for was greater in men than women (29 ± 2% vs. 21 ± 4%, respectively) but the difference was not statistically significantly (P = 0.10).

Reproducibility of meal fatty acid uptake into adipose tissue. We assessed the reproducibility of meal fatty acid uptake data between the first and second study independent of isotope ($^3$H-triolein vs. $^{14}$C-triolein) because the initial analysis indicated no isotope effect. To assess the intra-individual variation we calculated the absolute difference between the fatty acid uptake during the first study vs. the mean of both studies. The absolute difference between the percent of meal fatty acids stored upper body subcutaneous fat in first study and the mean of both studies was 4 ± 4 % (mean ± SD, range 0 – 13%) for women and 4 ± 3 % (mean ± SD, range 0 – 10%) for men. The extreme example is a woman in whom 15 % of meal fatty acids were estimated to be stored in upper body subcutaneous fat in the first study and 41% in the second study (average uptake of 28%); there was a 13% difference in meal fatty acid uptake between either of her two studies and the mean of both studies. To put this in perspective, the inter-individual variation can be appreciated by recalling that 24 ± 9 % (mean ± SD, range 9 – 42%) and 16 ± 9 % (mean ± SD, range 3 – 42%) of meal fatty acid uptake was in upper body subcutaneous fat in women and men, respectively. The intra-individual variation lower body subcutaneous meal fatty acid uptake, assessed using the same approach, was 4 ± 4 % (mean ± SD, range 0 – 13%) for women and 4 ± 3 % (mean ± SD, range 0 – 5%) for men. The inter-individual variation in meal fatty acid uptake is reflected by the 12 ± 6 % of meal fatty acids stored in lower body subcutaneous fat (mean ± SD, range 7 – 35%) for women and 7 ± 5 % (mean ± SD, range 2 – 20%) for men. There was a correlation between the uptake of meal fatty acids in leg adipose tissue in study 1 and study 2 (r = 0.58, P = 0.048) for men, however, the
correlation between the uptake of meal fatty acids in abdominal adipose tissue between study 1 and study 2 was not statistically significantly ($r = 0.41$, $P = 0.18$). There was not a significant correlation between either upper body ($r = 0.27$, $P = 0.39$) or lower body ($r = 0.01$, $P = 0.98$) meal fatty acid uptake values between the two studies in women.

The individual values for the percent of meal fatty acids taken up into abdominal and leg fat for Study 1 and Study 2 for men are depicted in Figure 2; the same approach is used to depict this data for the follicular and luteal phase studies in women in Figure 3.

The interpretation of the data we gathered on regional meal fatty acid uptake, meal fatty acid oxidation and intra-individual variability is potentially impacted by the substrate, hormonal and energy expenditure milieu in which the measurements are made. Thus, below are presented these study outcomes.

**Plasma Insulin and Glucose Responses**

There were no significant differences between the glucose and insulin concentrations on study day 1 vs. study day 2, the $[^{3}\text{H}]$ vs. the $[^{14}\text{C}]$triolein study days, or the follicular vs. luteal study days. The lower panel of Figure 4 depicts the average plasma glucose responses in men and women for both study days and the upper panel shows the average plasma insulin responses.

**Plasma Triglyceride Responses**

There were no significant differences between the plasma triglyceride concentrations between study day 1 vs. study day 2 or the $[^{3}\text{H}]$ vs. the $[^{14}\text{C}]$triolein study days for either men or women. For women, plasma triglyceride concentrations were not different between the follicular vs. luteal study days (upper panel, Figure 5). Men had consistently greater plasma triglyceride concentrations than women ($p<0.05$); the average 24 hour plasma triglyceride concentrations in men and women are shown in the lower panel of Figure 5. In addition, the increase in plasma
triglyceride concentrations above fasting was greater in men than women (peak increase $1037 \pm 285$ vs. $262 \pm 45$ µmol/L, respectively, $P = 0.02$).

The concentration of the meal tracer in plasma triglycerides was examined as an indicator of the entry/removal of chylomicron triglycerides from the circulation. Figure 6 shows the plasma concentration (dpm/ml) of $^{14}$C triglyceride and $^3$H triglyceride from the two study days in men and women. The amount of the tracer incorporated in the meals was not different between men and women ($57 \pm 5$ µCi vs. $56 \pm 8$ µCi, respectively of $^3$H triolein and $21 \pm 4$ µCi vs. $21 \pm 6$ µCi, respectively of $^{14}$C triolein). The pattern of tracer presence in plasma was similar to that we previously observed: a gradual increase in $^3$H or $^{14}$C triglyceride occurred in the first 4 hours after the ingestion of the meal with the tracer followed by a peak at 6 hours (one hour after the ingestion of lunch). The plasma $^{14}$C triglyceride and $^3$H triglyceride concentrations were consistently greater in men than women on both study days, consistent with lesser clearance of meal-derived triglycerides in men.

**Energy Metabolism**

The basal metabolic rate for men and women in study 1 and study 2 and in women in the follicular versus luteal phase of the menstrual cycle are provided in Table 2. As expected, basal metabolic rates were higher in men than women, but there was no difference between the different study days in men and women. The 24 hour respiratory exchange ratio (RER) (averages of study 1 and study 2) for men and women are depicted in the lower panel of Figure 7. As assessed by repeated measures ANOVA with factors for time (minutes) and sex, men had a lower ($P < 0.05$) RER than women at several time points throughout the day (180, 300, 360 and 420). The RER throughout the day was not different in women in the follicular and luteal phase of the menstrual cycle (top panel of Figure 7). The estimated energy expenditure from fatty acid
oxidation over the 10 hours during which frequent indirect calorimetry measures were done is also provided in Table 2.
DISCUSSION

We assessed whether the metabolic fate of meal fatty acids is different in the follicular versus luteal phases of the menstrual cycle and at the same time measured the reproducibility of these types of experiments. Twenty-four healthy, non-obese volunteers participated in two studies of meal fatty acid metabolism ~ 2 weeks apart. The studies were block randomized to avoid possible confounding effects of study order or meal tracer ($^3$H or $^{14}$C triolein) effects and were scheduled to coincide with the follicular and luteal phases of the menstrual cycle for 10 of the 12 women. There were no significant differences in energy expenditure, total or meal fatty acid oxidation between the follicular and luteal phases of menstrual cycle in lean, healthy women. The variability in meal fatty acid uptake into leg fat in women was somewhat greater than in abdominal fat in women and abdominal and leg fat in men, although the difference was not statistically significant ($P = 0.10 – 0.20$). To the extent that the issue of variability in this parameter influences the design of future studies we should consider that it could be a sex-related difference in meal fatty acid disposal that is independent of or related to the menstrual cycle.

Surprisingly little research has been done to examine whether regional adipose tissue lipid uptake is affected by the menstrual cycle in women. Rebuffé-Scrive et al (22) measured both in vitro lipolysis and lipoprotein lipase (LPL) activity in femoral and abdominal adipose tissue samples taken from women in the follicular and luteal phases of their menstrual cycle. She found no differences in either of these fat cell parameters as a function of the menstrual cycle, whereas important differences were seen when fat cells were taken from women during pregnancy or lactation (22). LPL activity is thought to be a major determinant of triglyceride uptake by adipose tissue, although it has not been possible to detect strong correlations between LPL activity and triglyceride uptake into adipose tissue in vivo (19,23). Nevertheless, the data
presented in the report of Rebuffé-Scrive et al. (22) does not hint at differences in LPL activity or differences in the variability of LPL activity during the follicular phase of the menstrual cycle phase. Unfortunately we did not measure LPL activity in this study and thus cannot assess whether the variations in uptake of meal fat by adipose tissue was associated with difference in LPL activity.

We assessed whether substrate oxidation by indirect calorimetry and isotopic techniques differed between the two study days – (study one vs. study two, follicular vs. luteal) and whether the insulin and triglyceride responses were comparable. If we had discovered differences in one or more of these factors it would have introduced confounding variables as regards the meal fatty acid oxidation and uptake data. Because none of these factors differed significantly between study days, either in average values or in the variance, it is easier to draw conclusions regarding the metabolic fate of dietary fat. The somewhat greater variability of leg adipose tissue meal fatty acid uptake in women could be a reflection of a confounding effect of the menstrual cycle phase; no such variability was noted in men. This variability was not seen in other factors that could influence the results: energy expenditure, triglyceride or insulin responses. Intra-individual differences in resting energy expenditure averaged only 2% and 7% in men and women, respectively, the thermic effect of food after breakfast differed by only an average of 3-4 Kcal/5 hours between the two studies, the intra-individual differences in average plasma triglyceride concentrations were only 6 and 8% in men and women, and the intra-individual differences in average plasma insulin concentration was only 11% and 8% in men and women. The outlying value of leg meal fatty acid uptake values in women in the follicular phase of the menstrual cycle is unlikely to be due to analytical/sampling error. We have previously assessed the intra-study
analytical/sampling error (24) for femoral meal fatty acid uptake and found it to be <1/2 of the between study variation we observed in women in the present experiments.

Using the carbon labeled triolein tracers there were no detectable difference in meal fatty acid oxidation between studies. This implies that meal fatty acid oxidation did not differ between the two study days. When we corrected the $^{13}$C or $^{14}$C meal fatty acid oxidation data for carbon fixation using the acetate correction factor (28) of 50%, the carbon labeled meal fatty acid oxidation was comparable to the $^3$H meal fatty acid oxidation.

In this study, men had somewhat greater proportional fat oxidation throughout the day than did women, as assessed by the lower RQ at several time points. They also had slightly greater meal fatty acid oxidation as measured by the generation of tritiated water from the $^3$H triolein labeled meal. Although this could be due to a true sex differences in substrate oxidation under the conditions of these experiments, a more likely cause is a slightly negative energy intake relative to energy expenditure in men compared with women; energy deficits tend to be accounted for by fat oxidation (12). Unfortunately, because we did not have true 24 hr energy expenditure measurements (such as are possible with whole room calorimeters) we cannot be certain of the exact energy balance in our volunteers.

Consistent with previous studies (23), women stored a greater proportion of meal fatty acids in subcutaneous fat than men (Figure 1). There was also a slightly lesser portion of meal fatty acids that could not be accounted for in women than men. We hypothesized (23) that the meal fatty acids we could not account for after 24 hour had been stored in visceral adipose tissue and subsequently reported that by accounting for the meal fatty acid uptake into visceral fat it is possible to account for 100% of meal fatty acid disposal (16). The data from the present study
are consistent with a greater uptake of meal fatty acids into visceral fat in non-obese men compared with women.

In this report we provide reproducibility data for the study of meal fatty acid uptake in adipose tissue using isotopic tracers. In order to present this data we begin by assuming that, for an individual, the mean adipose tissue uptake of dietary fat for two studies is a better reflection of that person’s true average than the value from either study alone. The difference between the value from one study and the mean of two studies provides an estimate of how much the result from any given study will vary from a given individual’s average response. Using this approach we previously reported the side to side differences for abdominal and leg meal fatty acid uptake for a single experiment (~4% for abdominal and 10% for femoral). In the present study we found that between experiment differences for abdominal meal fatty acid uptake averaged 16% for women and 26% for men (4% \div 24% and 4% \div 16%, respectively). The between experiment differences were similar for leg uptake of meal fatty acids. The range of uptake values we observed are also reported. This data will help investigators to perform power calculations in order to better design studies, especially for paired experiments to test the effects of an intervention on meal fatty acid uptake into adipose tissue. For example, detecting a 5% change in meal fatty acid uptake into either lower body or upper body adipose tissue beds using a paired study design would require 7 subjects (80% power, alpha of 0.05, two-sided t-test). In contrast, detecting a 5% difference between groups (non-paired t-test) in meal fatty acid uptake into upper body fat and lower body fat with the same power would require 52 and 24 subjects per group, respectively.

In summary, we found that more meal fatty acids were taken up in leg adipose tissue of women during the follicular phase of the menstrual cycle, but that this difference from uptake
during the luteal phase was not statistically significant. There was somewhat greater variance and skewing of the leg adipose tissue meal fatty acid uptake data in women relative to upper body uptake and meal fatty acid oxidation; this also contrasted with the consistency of the data variance in men. If there are differences in leg uptake of meal fatty acids between different phases of the menstrual cycle in women it will take large studies to detect. Examining and/or controlling for potential variables (LPL activity, adipose tissue blood flow, etc.) in future studies may help address whether there are biological explanations for the intra-individual variability we observed. For the present, the phase of the menstrual cycle does not appear to greatly affect the planning for studies of meal fatty acid metabolism in women.
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GRANTS

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REFERENCES


FIGURE LEGENDS

Figure 1. The mean ± sem of meal fatty acid uptake in upper body subcutaneous (UBSQ) and lower body subcutaneous (LBSQ) adipose tissue in men and women 24 hours following the ingestion of the experimental meals is depicted. The concentration of meal fatty acid tracer in adipose tissue was multiplied by regional adipose tissue mass to determine the percent of the meal fatty acid uptake that occurred in upper body and lower body adipose tissue in men and women. The percent of meal fatty acids oxidized is calculated using the generation of $^3$H$_2$O water from $^3$H triolein. The unaccounted for meal fatty acids was calculating by subtracting those stored in subcutaneous fat plus those oxidized from the amount ingested. * P<0.05 vs. men. ‡ P = 0.005 vs. men.

Figure 2. The individual, paired values for the percent of meal fatty acids taken up into abdominal fat (left panel) and leg fat (right panel) for Study 1 and Study 2 for men are depicted.

Figure 3. The individual, paired values for the percent of meal fatty acids taken up into abdominal fat (left panel) and leg fat (right panel) for the follicular and luteal phase studies in women are depicted.

Figure 4. Plasma insulin (top panel) and glucose (bottom panel) responses throughout the day are depicted for the 12 men and 12 women participating in both studies. Values are mean ± sem.
Figure 5. Plasma triglyceride concentrations in 10 women studied during both the follicular and luteal phases of the menstrual cycle are depicted in the upper panel. The average plasma triglyceride concentrations for men and women from both studies are depicted in the lower panel. Values are mean ± sem.

Figure 6. Plasma triglyceride $^{14}$C (upper panel) radioactivity and $^3$H (lower panel) triglyceride radioactivity (dpm/mL) throughout the day for the women and men participating studied are depicted. Men and women received the same amount of the tracer (see Results). Values are mean ± sem.

Figure 7. The respiratory exchange ratio (RER) from indirect calorimetry throughout the study days are depicted. The upper panel displays the RER for the 10 women who had studies in both the follicular and luteal phases of their menstrual cycle. The lower panel displays the average RER for both studies for men and for women. Values are mean ± sem.
<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>p-value</th>
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<tr>
<td>Age</td>
<td>29 ± 2</td>
<td>32 ± 2</td>
<td>NS</td>
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<tr>
<td>Height (m)</td>
<td>1.81 ± 0.03</td>
<td>1.64 ± 0.02</td>
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<tr>
<td>Weight (kg)</td>
<td>80.3 ± 2.2</td>
<td>59.3 ± 1.6</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 0.6</td>
<td>22.2 ± 0.4</td>
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<tr>
<td>% Body Fat</td>
<td>18.0 ± 1.5</td>
<td>30.1 ± 1.2</td>
<td>&lt;0.0001</td>
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<tr>
<td>Fat (kg)</td>
<td>13.36 ± 1.12</td>
<td>16.78 ± 0.91</td>
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<tr>
<td>FFM (kg)</td>
<td>65.9 ± 2.5</td>
<td>41.3 ± 1.1</td>
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<tr>
<td>Visceral fat (cm²)</td>
<td>61 ± 11</td>
<td>26 ± 3</td>
<td>&lt;0.001</td>
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<tr>
<td>Abdominal SQ (cm²)</td>
<td>89 ± 11</td>
<td>96 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Upper body non-visceral fat (kg)</td>
<td>6.90 ± 0.52</td>
<td>8.84 ± 0.51</td>
<td>&lt;0.05</td>
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<tr>
<td>Visceral fat (kg)</td>
<td>1.74 ± 0.27</td>
<td>1.15 ± 0.12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leg fat (kg)</td>
<td>4.72 ± 0.41</td>
<td>6.79 ± 0.37</td>
<td>= 0.001</td>
</tr>
</tbody>
</table>

Values are mean ± sem. Body fat and leg fat were measured by dual energy x-ray absorptiometry; CT abdominal subcutaneous and visceral fat areas were measured using a single slice CT at the L₂₋₃ interspace.
Table 2. Energy Expenditure and Substrate Oxidation

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>Women</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Study 1</td>
<td>Study 2</td>
<td>Study 1</td>
</tr>
<tr>
<td>BMR (Kcal)</td>
<td>1751 ± 63</td>
<td>1748 ± 78</td>
<td>1283 ± 51</td>
</tr>
<tr>
<td>TEF (Kcal/5hr)</td>
<td>46 ± 4</td>
<td>51 ± 4</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>TEF (%) AUC – breakfast</td>
<td>8 ± 1 %</td>
<td>9 ± 1 %</td>
<td>7 ± 1 %</td>
</tr>
<tr>
<td>Carbohydrate (Kcal/10 hr)</td>
<td>210 ± 44</td>
<td>237 ± 23</td>
<td>227 ± 16</td>
</tr>
<tr>
<td>Fat oxidation (Kcal/10 hr)</td>
<td>272 ± 39</td>
<td>298 ± 25</td>
<td>158 ± 18</td>
</tr>
</tbody>
</table>

Values are mean ± sem. Men meal size was 590 ± 23 Kcal, and women meal size was 435 ± 23 Kcal. The values for the follicular and luteal phase studies are from the 10 women who underwent a study during each phase whereas the Study 1 and Study 2 values are those from all 12 women. The thermic effect of food (TEF) is expressed as a percent of the in the energy in the meal expended as an increase in metabolic rate. There were no significant differences between Study 1 and Study 2 values for men and women or follicular and luteal values for women.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.