Young women partition fatty acids towards ketone body production rather than VLDL-triacylglycerol synthesis, compared to young men

[1,2]Kyriakoula Marinou, [3]Martin Adiels, [1]Leanne Hodson, [1]Keith N Frayn, [1]Fredrik Karpe and [1]Barbara A Fielding

[1]Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, UK
[2]Department of experimental physiology, Athens University School of Medicine, Greece
[3]Wallenberg Laboratory/Sahlgrenska center for Cardiovascular and Metabolic Research, Göteborg University, Sweden

Address for correspondence;

Barbara A Fielding, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, OX3 7LJ, UK

email:barbara.fielding@ocdem.ox.ac.uk, tel: 01865 857289

Running head

Hepatic fatty acid partitioning

Word count: abstract: 247 words; Text;4203 words; References:28

ABSTRACT

Before the menopause, women are relatively protected against cardiovascular disease compared with men. The reasons for this are not completely understood but hepatic fatty acid metabolism may play a role. This study aimed to investigate the utilization of plasma non-esterified fatty acids by the liver and to determine whether they are partitioned differently into ketone bodies and VLDL-triacylglycerol (TG) in healthy lean young men and women. Volunteers were studied during a prolonged overnight fast (12-19h) using an intravenous infusion of [U-^{13}C]palmitate. After 12h fasting, the women had a more advantageous metabolic profile with lower plasma glucose ($P < 0.05$) and TG ($P < 0.05$) but higher plasma NEFA ($P < 0.05$) concentrations. Plasma 3-hydroxybutyrate (3-OHB) concentrations rose more in women than men, and the transfer of ^{13}C from [U-^{13}C]palmitate to plasma [^{13}C]3-OHB reached a plateau 6-7h after the start of the infusion in women but was still increasing at 6h in men. This implies a slower 3-OHB production rate and/or dilution by other precursor pools in men. In women, the high isotopic enrichment of plasma 3-OHB suggested that systemic plasma fatty acids were the major source of 3-OHB production. However, in men, this was not observed during the course of the study ($P < 0.01$). There were no sex differences for the incorporation of ^{13}C into $VLDL_1$- or $VLDL_2$-TG. The ability of young women to partition fatty acids towards ketone body production rather than VLDL-TG, may contribute to their more advantageous metabolic profile compared to young men.

Key words: 3-hydroxybutyrate; stable isotopes

INTRODUCTION

Pre-menopausal women are relatively protected against cardiovascular disease (CVD) compared with men [1] but the reasons for the gender difference are not completely understood. An understanding of the underlying mechanisms for this difference may help us to prevent worsening of cardiovascular risk factors in men and women in later life. There is evidence for some gender differences in the plasma metabolic profile in young adults. Short-term fasting reveals gender differences in people who are young and lean; women have lower plasma glucose and higher plasma non-esterified fatty acid (NEFA) concentrations than men after fasting, as summarised in Soeters et al[2]. The ketone body 3-hydroxybutyrate (3-OHB) is important energy source for the brain and other tissues during prolonged fasting. Under starving conditions, it is considered to act as a central signal and energy -providing substrate involved in the regulation of food intake[3]. Also it seems that 3-OHB has an anti-lipolytic effect, and is a ligand for the nicotinic acid receptor. In that way its effect is similar to nicotinic acid[4]. However, to the best of our knowledge, gender comparisons of plasma 3-OHB concentrations have rarely been reported in healthy young men and women. The difference in blood 3-OHB concentrations has been reported to be greater in response to fasting with significantly higher concentrations in women than men after 30 h fasting, but plasma insulin concentrations were similar[5]. After an overnight fast, plasma TG concentrations in women were lower as compared to men but this difference did not reach statistical significance and did not change during a further 10 h of fasting[6]. However, plasma VLDL-TG concentrations were lower in women[6] in response to the extended fasting. The results from that study suggested that the liver in women secretes fewer but TG-richer VLDL particles than the liver in men, and that clearance was faster in women. There are two major classes of VLDL secreted by the liver: $VLDL_1$ is larger and more TG-rich than $VLDL_2$. The latter can either be secreted directly from the liver, or formed by the peripheral hydrolysis of $VLDL_1$. These two classes of lipoproteins have different properties; in subjects with type 2 diabetes the secretion of $VLDL_1$ is associated with liver fat content, hypertriglyceridaemia and atherogenic risk [7]. VLDL-TG production represents the export of fatty acids partitioned towards esterification and secretion rather than storage or oxidation in the liver. Therefore, the balance between the pathways of esterification and oxidation in men and women may be important for the development of CVD risk factors.

Our aim was to investigate gender differences in plasma metabolites and hepatic fatty acid metabolic partitioning in the liver in response to an extended overnight fast. This enabled us to stress metabolic pathways in order to investigate gender differences in young people before the onset of traditional cardiovascular risk factors. Since systemic NEFA are the major substrate for VLDL-TG and 3-OHB production, we aimed to follow the transfer of a stable isotope label from plasma NEFA into the products of hepatic metabolism.

Materials and Methods

Subjects. Twelve healthy lean young men and women were studied, matched for age and BMI with no significant difference in waist circumference but the women had a greater percent body fat (Table 1). Subjects, recruited from the wider Oxford community via advertisement, were free from any disease, were weight stable and were not taking any lipid lowering medication or medication that would alter lipid metabolism. The studies were performed during the first week (follicular phase) of the menstrual cycle and conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the Oxfordshire Clinical Research Ethics Committee. Written informed consent was obtained from all subjects.

Study protocol. Before the study day, subjects were asked to avoid foodstuffs naturally enriched in ^{13}C for 48 h and refrain from strenuous exercise and alcohol for 24 h before the study. On the evening before the study, subjects were required to eat a low fat meal. On the day of the study, subjects arrived at the clinical research unit after an overnight fast. Fat mass was measured by bioelectrical impedance analysis and fat free mass was calculated as the difference compared with total body weight. A cannula was inserted into an antecubital vein, and a baseline blood sample was taken for background isotopic enrichment. At time 0, a continuous intravenous infusion (0.04 μmol kg^{-1} min^{-1}) of potassium [U-^{13}C]palmitate (isotope purity 98%; Cambridge Isotope Laboratories, Inc. Andover, MA) complexed to human albumin was started, to label the plasma NEFA pool. This continued until the end of the experiment (at 7 h).

Analyses. Whole blood was collected into heparinized syringes (Sarstedt, Leicester, UK), plasma was rapidly separated by centrifugation at 4°C, and plasma NEFA, VLDL-TG and 3-

OHB concentrations were determined enzymatically as previously described[8,9]. Plasma insulin concentrations were measured by radioimmunoassay[9].

In 10 subjects, $VLDL_1$ (Svedberg flotation rate (S_f) 60-400) and $VLDL_2$ (S_f 20-60) were isolated by sequential flotation using density gradient ultracentrifugation in a SW40Ti swinging bucket rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm at 15°C for 4 h for S_f 60-400 lipoproteins and for a further 16 h to float S_f 20-60 lipoproteins.

Fatty acid analysis and isotopic enrichment. Fatty acid methyl esters (FAMEs) were prepared from NEFA, $VLDL_1$- and $VLDL_2$-TG fractions and isotopic enrichment measured by gas chromatography (GC) and GC-mass-spectrometry (MS) respectively, as previously described[9]. Specific fatty acid concentrations were determined by multiplying the proportion of the specific fatty acid by the corresponding plasma concentration as determined enzymatically for plasma NEFA, $VLDL_1$- and $VLDL_2$-TG.

Isotopic enrichment from [U-^{13}C]fatty acids appearing in 3-OHB in deproteinised plasma was measured using a modified method of Beylot el al[10]. Solutions of [2,4-$^{13}C_2$]3-OHB (Cambridge Isotope Laboratories, Inc. Andover, MA) and unenriched 3-OHB were prepared in 4 % perchloric acid (PCA) and diluted in deionised water to make an enrichment standard curve (100 µmol/L). Plasma samples (0.5 ml) were deproteinised with 1 ml PCA (70 g/L). Duplicate 1 ml aliquots of the supernatant were neutralised with 500 µl neutralising reagent (0.5 mol/L $KHCO_3$ and K_2CO_3) on ice. After centrifugation, 500 µl aliquots of the deproteinised plasma were pipetted into 10 ml glass tubes and acidified to pH 1 with 1M HCl. 500 µl 3-OHB standards were acidified in the same manner, after prior addition of 50 µl neutralising reagent. The 3-OHB from samples and standards was extracted into 4.5 ml ethyl acetate/diethyl ether (1/1 v/v) in 10 mL glass tubes by mixing by hand for 2 min and then rotary mixing for 1 h. The aqueous and organic phases were separated by centrifugation and the upper, organic phase was evaporated to dryness under N_2 at room temperature. T-butyl dimethylsilyl derivatives of 3-OHB were prepared by the addition of 20 µl pyridine and 20 µl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) + 1% t-butyl dimethyl chlorsilane (TBDMCS). After incubation for at least 45 min at room temperature, the contents of the tubes were transferred to GC vials and analysed by GC-MS. The GC was equipped with a 30 m capillary column with a 5% diphenyl/95% dimethyl polysiloxane stationary phase (from Thames Restek, Saunderton, UK, i.d. 0.32 mm, film thickness 0.25

μm). The 5890 GC was coupled to a 5973N MSD (Agilent Technologies, Stockport, UK). Ions with mass-to-charge ratios (*m/z*) of 275 (M+0), 277 (M+2) were determined by selected ion monitoring. We assume that the latter corresponds with the formation of 3-OHB from one labelled [$^{13}C_2$]acetyl-CoA molecule, derived from [U-^{13}C]palmitic acid via ß-oxidation. Initially, we selected ions of *m/z* 279 (M+4) but the GC-MS was not sufficiently sensitive to consistently detect a peak.

The tracer/tracee ratio (TTR) of a baseline measurement (before administration of the stable isotope tracer) was subtracted from each sample TTR to account for natural abundance. The TTRs for [U-^{13}C]palmitate were multiplied by the corresponding palmitate concentration in plasma NEFA and $VLDL_1$ - and $VLDL_2$- TG to give tracer concentrations. Likewise, the TTRs for [^{13}C]3-OHB (M+2)/(M+0) were multiplied by the corresponding plasma 3-OHB concentrations to give tracer concentrations. Mole percent excess (MPEs) were calculated from TTRs using the formula MPE =1/(1+1/TTR).

Calculations. The proportion of plasma systemic NEFA contributing to VLDL-TG at 7 h were calculated as previously described[11]. The remainder are assumed to be fatty acids from splanchnic sources, including those from visceral fat lipolysis, liver fat and *de novo* lipogenesis.

Whole body rate of appearance (R_a) of NEFA was calculated using the isotopic enrichment in plasma after intravenous infusion of the [U-^{13}C]palmitate[12] during the last 5 h of the infusion. Rate of disappearance of NEFA (Rd NEFA) was calculated as Ra NEFA – dQ/dt, where dQ = the change in amount of tracee with time[12].

Classic mathematical modelling of 3-OHB production could not be performed because the level of enrichment in 3-OHB did not allow for calculation of the precursor pool enrichment. However, we wished to examine the extent to which the enrichment of [^{13}C] in plasma 3-OHB reflected synthesis from systemic NEFA. Therefore, we estimated the maximal enrichment (MPE) in 3-OHB for (M+2) at 7 h, assuming that the precursor for 3-OHB production was only from the plasma NEFA pool. Since we infused a palmitic acid tracer, we assumed that the enrichment in the mitochondrial acetyl-CoA pool would be diluted by (non-selective) contributions from other specific fatty acids. Therefore we accounted for the number of carbon atoms in each fatty acid species and their relative proportions in the NEFA

pool for each participant. The plasma enrichment of [U-^{13}C]palmitate multiplied with the dilution factor gave an estimation of the [^{13}C]acetyl-CoA enrichment in the mitochondria. The estimated maximal MPE was calculated from the formula for the probability of picking exactly one labelled and one unlabelled element, 2p(1-p) where p is the probability of an element to be labelled.

The M+4 isotopomer for 3-OHB in women at plateau enrichment was estimated from the calculated precursor enrichment (MPE) and binomial expansion [13].

In order to determine an approximation of the relative partitioning of systemic plasma NEFA into 3-OHB and VLDL-TG, we calculated the ratio of [^{13}C]3-OHB: [^{13}C]VLDL-TG in plasma, where [^{13}C] is expressed in μmol/L.

Statistical Analysis. Data were analysed using SPSS for Windows v16 (SPSS, UK, Chertsey, UK). Statistical significance was set at $P < 0.05$. All data are presented as means (SEM). Repeated measures ANOVA, with time and group as factors, was used to investigate the change between men and women over time whilst fasting. Comparisons between groups after the extended overnight fast (7 h) were made using a Mann-Whitney test.

Terminology. Conventional terminology for stable isotope tracer techniques is used in this paper [12]. The passage of a molecule through the GC-MS results in the production of a major ion of interest with mass M. An isotopomer is a molecule that has the same chemical composition but a different mass because it has an isotopic tracer incorporated somewhere in the molecule. Thus, the M isotopomer for 3-OHB refers to an ion that has no ^{13}C tracer incorporated and M+2 represents an ion that has a mass that is two atomic mass units greater than the M isotopomer; this is assumed to be due to the substitution of two ^{12}C atoms by two ^{13}C atoms.

Results

Differences in plasma variables between men and women after an overnight fast are given in Table 1. After 12 h fasting, the men had a less advantageous metabolic profile in terms of higher plasma glucose ($P < 0.05$) and TG ($P < 0.05$) concentrations but lower plasma NEFA ($P < 0.05$) concentrations. In order to check that the twelve subjects that we chose were representative of the wider community in this respect, we retrieved data from 266 men and

405 women, aged 30-50 years, from the Oxford Biobank[14]. All individuals were selected to have a BMI of less than 25. In exact accordance with the six men and six women in this study, the larger group of women had higher plasma NEFA concentrations (553 (13) v 475 (16) μmol/L, $P < 0.05$, the same plasma 3-OHB concentrations, lower plasma TG concentrations (870 (20) v 1090 (40) μmol/L, $P < 0.05$), lower plasma glucose concentrations (4.8 (0.0) v 5.2 (0.0) mmol/L $P < 0.05$) and similar plasma insulin concentrations. After extension of the postabsorptive period during the 7 h of the present experiment, expected changes in plasma variables occurred (Figure 1). Significant differences between men and women for glucose, TG and NEFA were maintained, and the marginally higher values for 3-OHB found in women after 12 h fasting ($P = 0.078$) became significant ($P = 0.035$). Plasma insulin concentrations decreased significantly with time ($P = 0.02$) but remained similar between men and women after prolonged fasting.

Concentrations of $VLDL_2$-TG but not $VLDL_1$-TG were significantly higher in men than women (Table 1). We observed that $VLDL_1$-TG concentrations were higher than $VLDL_2$-TG in both sexes combined (P = 0.028). During prolonged fasting the concentrations of $VLDL_1$-TG decreased significantly (Figure 1) but no further sex differences were revealed. There was a small decrease in $VLDL_2$-TG concentrations during the study, but this was not significant (females $P= 0.275$, males $P=0.663$).

The TTR of [U-^{13}C]palmitate from the intravenous infusion reached a plateau in the plasma NEFA pool by 1 h in men and women (Figure 2). Ra NEFA (μmol/min) was similar in men and women but lower in women when expressed per kg fat mass (Table 2). When expressed according to fat free mass there was no difference between men and women (Table 2).

The fatty acid tracer was rapidly incorporated into plasma 3-OHB and VLDL-TG. Whereas enrichment reached a plateau in plasma 3-OHB at 6-7 h after the start of the infusion in women, it was still increasing at 6 h in the men (Figure 2). Plasma concentrations of [^{13}C]3-OHB were correspondingly higher in women than men during the course of the experiment ($P = 0.007$ for effect of gender, $P = 0.004$ for time*gender interaction). In women, the observed isotopic enrichment of the fatty acid tracer in 3-OHB was close to that predicted, if systemic plasma fatty acids were the sole source of 3-OHB production (Table 2). In fact, the observed MPE reached 78% of the estimated maximal MPE and thus only 22% of

the 3-OHB could not be accounted for by the systemic NEFA sources. These values are in close agreement with the levels reached for the $VLDL_1$- and $VLDL_2$-TG (Table 3) in these lean women. However, in men the observed enrichment was less than half expected, implying either an alternative source of fatty acids, or a slower rate of production. The incorporation of [^{13}C] tracer into $VLDL_1$- and $VLDL_2$-TG was not significantly different between men and women, either in terms of TTR (Figure 2) or [^{13}C]VLDL-TG concentration (data not shown).

The ratio of [^{13}C]3-OHB: [^{13}C]VLDL-TG in plasma was significantly higher in women than men (2.73 (1.7) vs 0.66 (0.30), P = 0.027 for $VLDL_1$ and 3.24 (1.6) vs 0.97 (0.43), P = 0.014 for $VLDL_2$ respectively).

As we have reported previously in the postprandial period for healthy people for total VLDL-TG [11], fatty acids from systemic plasma NEFA contributed a greater proportion of fatty acids to $VLDL_1$-TG and $VLDL_2$-TG than splanchnic sources, in men and women (Table 3). However, the proportion of systemic fatty acids was lower in men than women for $VLDL_2$-TG, with a corresponding greater proportion from splanchnic sources.

Discussion

In the present study, we found significant differences in hepatic metabolism in healthy lean young men and women. Gender comparisons of plasma 3-OHB in very young men and women have rarely been reported, but we are in agreement with previous findings of higher concentrations in young women after a 30 h fast[5] and we found that a shorter period of fasting (12-19 h) revealed higher concentrations in women. We found no difference in plasma 3-OHB concentrations between men and women without the provocation of a prolonged fast, in either a young age group (n = 12) or in middle-aged individuals (n = 671). Since ketone bodies are only produced in the liver, systemic concentrations, usually of 3-OHB, can be taken to reflect hepatic ketone body production. Lower 3-OHB concentrations have been reported in subjects with hyperlipidaemia[15,16], obesity[17] and insulin resistance[11]. Therefore, lower 3-OHB concentrations seem to be associated with a less advantageous metabolic profile. These differences could be due to differences in production or clearance. Our findings suggest that in young lean women, a higher plasma concentration of 3-OHB, compared with men, is due to a greater production, although we did not measure production directly.

There were gender differences in the incorporation of carbon units from [U-^{13}C]palmitate, representing systemic fatty acids, into 3-OHB. The higher 3-OHB TTR ratio and attainment of plateau enrichment in women suggests that new 3-OHB substantially contributes to total 3-OHB. Thus it could be hypothesised that women rapidly switch on 3-OHB production from systemic plasma NEFA, allowing plasma concentrations of 3-OHB to increase rapidly and the TTR to flatten out. Men have a slower 3-OHB switch and at 7 h the contribution of plasma NEFA is still increasing as a source of 3-OHB. However, it should be noted that our findings are not necessarily representative of n-3 polyunsaturated fatty acids which may partition away from beta-oxidation in women[18].

The reason for a greater ability of women to turn on 3-OHB production is not clear. The production of ketone bodies is dependent, to a large extent, on the supply of plasma NEFA[19,20]. Plasma NEFA concentrations increased experimentally lead to increased plasma concentrations of 3-OHB[19,20]. Beylot et al found that the percentage conversion of plasma NEFA to 3-OHB increased in a linear fashion as the precursor concentration increased [21]. This is thought to be due to reduced malonyl-CoA and reduced inhibition of carnitine palmitoyl transferase I (CPT-1) and could possibly account for the gender differences that we observed. A greater percentage conversion of plasma NEFA to 3-OHB may have occurred in the women of the present study, whose mean plasma NEFA concentration was almost twice that of men. Glucagon is also an important stimulator of ketone body production, but it is not clear if there is a gender difference. Glucagon levels have been reported as similar in men and women[5], but in an older study by Merimee et al[22], plasma glucagon concentrations were significantly higher in premenopausal women than men after a prolonged fast (72 h). Insulin reduces 3-OHB production indirectly, via a direct effect on adipose tissue lipolysis, affecting supply of NEFA. A direct effect on ketone body production has been described in some[23] but not all studies[24,21].

Although plasma NEFA concentrations were higher in women, they had a similar delivery of fatty acids into the systemic circulation than men as determined by Ra NEFA. However, even though the women were lean, they had a significantly higher fat mass than the men and when Ra NEFA was expressed in relation to fat mass, the Ra NEFA was significantly lower. This is in agreement with the recent publication of Mittendorfer et al[25] who reported a down-regulation of Ra NEFA per unit of fat mass in obesity. So, even within lean individuals, we were able to observe an effect of fat mass on Ra NEFA. In contrast to

our findings (no difference between men and women), the study of Mittendorfer et al found a higher Ra NEFA (μmol/min) in women, and reported similar plasma NEFA concentrations. A possible explanation for the observed difference between the two studies may be because we enrolled leaner, younger volunteers.

After 12 h fasting, the men in our study had a more disadvantageous metabolic profile in terms of higher plasma glucose and TG concentrations, but lower plasma NEFA concentrations. Since the latter are generally thought of as an adverse CVD risk factor, especially in terms of obesity and diabetes[26], this was unexpected. However, we have shown that a metabolic disadvantage of a low plasma NEFA concentration is the reduced ability of the liver to switch to ketone body production during short-term fasting and in the long term this could possibly lead to the development of fatty liver. Plasma NEFA is also a substrate for VLDL-TG synthesis. In this study, more than three-quarters of VLDL-TG delivered from systemic plasma NEFA, in men and women. However, particularly in $VLDL_2$, the contribution of systemic plasma NEFA to VLDL-TG was lower in men than in women. This may have been due to a lower flux from systemic fatty acids, and/or a greater contribution from splanchnic sources. The latter would include *de novo* lipogenesis, fatty acids from the lipolysis of visceral fat, or from cytosolic storage pools.

We calculated an 'estimated' maximum ^{13}C enrichment in 3-OHB that would be achieved if systemic fatty acids were the sole precursor pool for 3-OHB production. This was similar for men and women. For women, the achieved value was 78 % of the value expected, implying that systemic fatty acids were the major precursor pool for 3-OHB production. This is in line with the hypothesis that cytosolic TG fatty acids may not provide substrate for ketone body production, suggesting compartmentalization of the precursor pool of ketone bodies [27]. It also suggests that fatty acids from visceral fat did not substantially contribute to ketone body production in these lean young women. However, in men, the enrichment of ^{13}C in 3-OHB was less than half the value expected. Consistent with this would be dilution of the precursor pool by non-systemic fatty acids, such as those from visceral fat. Alternatively, a lower 3-OHB production rate in men would mean that the maximum isotopic enrichment had not been reached during the course of the experiment. Parallel to these findings for 3-OHB isotopic enrichment, the VLDL isotopic enrichment in women was close to that of the plasma NEFA pool, thus over 85 % of VLDL was derived from this pool. This suggests that in women, the precursor fatty acids for both metabolic pathways over the duration of our study

was approximately 80 % from plasma NEFA, and implies that plasma NEFA was an immediate and major source of fatty acids for hepatic fatty acid metabolism, contributing equally to 3-OHB and VLDL-TG synthesis during a prolonged overnight fast. However, since there were no gender differences in VLDL isotopic enrichment, the implication is that women rapidly partition a greater proportion of systemic fatty acids to ketone bodies than men. In men, it cannot be ruled out that the same sources are used for 3-OHB and VLDL-TG, but since the 3-OHB enrichment is still rising at the end of the experiment, and the total 3-OHB pool is much lower than for women, it is clear either that the time-course is considerably slower or that other sources of fatty acids are involved.

Ketone bodies may be infused intravenously to give kinetic information on ketone body production [23,28,21], but the use of a fatty acid stable isotope tracer has not been utilised previously to investigate ketone body metabolism in humans. The disadvantage of the latter approach is that we were not able to do classic kinetic modelling, because the lack of detectable M+4 tracer labelling in 3-OHB did not allow for calculation of the precursor pool enrichment. Quantitatively, the M+4 isotopomer would not contribute significantly to the total appearance of the palmitate tracer in 3-OHB. We calculated that in women, the enrichment (MPE) of the 3-OHB M+4 isotopomer was less than 0.5 % of the M+2 isotopomer. However, the results were informative. In particular, we were able to show that in women, systemic plasma NEFA was the main source of fatty acids for 3-OHB production, and that hepatic production of 3-OHB followed different kinetics in men and women. We calculated the [^{13}C]3-OHB: [^{13}C]VLDL-TG ratio in plasma and found that mean values were greater than 2.5 in women. This illustrates the quantitative importance of ketone body production for NEFA turnover in women, in the early fasting period. Beylot and co-workers [21] calculated that for a plasma 3-OHB concentration of 464 µmol/l, 13 % of plasma NEFA are converted to 3-OHB in healthy young men. Our results would suggest an even higher rate of conversion in women.

The hepatic partitioning of fatty acids to ketone bodies or esterification is difficult to study in humans but may be an important metabolic regulatory point that warrants further study. We have shown that in young women, plasma fatty acids tend to be readily converted to ketone bodies after an extended overnight fast, possibly partly because of a higher systemic pool than in men. Moreover, there is evidence of preferential partitioning to ketone bodies rather than VLDL-TG, at least in early starvation. From an evolutionary point of view, women may therefore have been better adapted to cope with periods without food. In modern

times, the greater ability of women to oxidize plasma NEFA into ketone bodies may partly explain the fact that premenopausal women have a better metabolic profile compared with men and may help protect women against the accumulation of liver fat. Fatty acid partitioning could potentially be manipulated by lifestyle changes or pharmacological intervention.

ACKNOWLEDGEMENTS

This work was funded in part by the project 'Hepatic and adipose tissue and functions in the metabolic syndrome' (HEPADIP, http://www.hepadip.org/), which is supported by the European Commission as an Integrated Project under the 6th Framework Programme (Contract LSHM-CT-2005-018734). Funding is also gratefully acknowledged from the European Atherosclerosis Society, Novo Nordisk and the Sahlgrenska Center for cardiovascular and Metabolic Research funded by the Foundation for Strategic Research. We thank Jane Cheeseman, Louise Dennis and Siobhán McQuaid for expert help during the studies. We confirm no conflict of interest for the work reported here. KM carried out the studies and performed laboratory and data analysis. MA analysed data and helped with manuscript preparation. LH performed laboratory analysis, data analysis and helped with manuscript preparation. BF developed stable isotope methods, supervised lab work and took the lead on manuscript preparation. FK and FK supervised the studies and helped with manuscript preparation. KF, LH, BF and FK designed the study.

REFERENCES

1. Booth GL, Kapral MK, Fung K, et al. (2006) Relation between age and cardiovascular disease in men and women with diabetes compared with non-diabetic people: a population-based retrospective cohort study. Lancet 368, 29-36.
2. Soeters MR, Sauerwein HP, Groener JE, et al. (2007) Gender-related differences in the metabolic response to fasting. J Clin Endocrinol Metab 92, 3646-52.
3. Laeger T, Metges CC & Kuhla B (2010) Role of beta-hydroxybutyric acid in the central regulation of energy balance. Appetite 54, 450-5.
4. Taggart AK, Kero J, Gan X, et al. (2005) (D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. J Biol Chem 280, 26649-52.
5. Haymond MW, Karl IE, Clarke WL, et al. (1982) Differences in circulating gluconeogenic substrates during short-term fasting in men, women, and children. Metabolism 31, 33-42.
6. Magkos F, Patterson BW, Mohammed BS, et al. (2007) Women produce fewer but triglyceride-richer very low-density lipoproteins than men. J Clin Endocrinol Metab 92, 1311-8.
7. Adiels M, Taskinen MR, Packard C, et al. (2006) Overproduction of large VLDL particles is driven by increased liver fat content in man. Diabetologia 49, 755-65.
8. Humphreys SM & Frayn KN (1988) Micro-method for preparing perchloric extracts of blood. Clin Chem 34, 1657.
9. Bickerton AS, Roberts R, Fielding BA, et al. (2007) Preferential uptake of dietary fatty acids in adipose tissue and muscle in the postprandial period. Diabetes 56, 168-76.
10. Beylot M, Beaufrere B, Normand S, et al. (1986) Determination of human ketone body kinetics using stable-isotope labelled tracers. Diabetologia 29, 90-6.
11. Hodson L, Bickerton AS, McQuaid SE, et al. (2007) The contribution of splanchnic fat to VLDL-triglyceride is greater in insulin resistant than insulin sensitive men and women: studies in the postprandial state. Diabetes
12. Wolfe RR & Chinkes D. (2005). Isotope Tracers in Metabolic Research. Hoboken: John Wiley & Sons, Inc.
13. Hellerstein MK, Christiansen M, Kaempfer S, et al. (1991) Measurement of de novo hepatic lipogenesis in humans using stable isotopes. J Clin Invest 87, 1841-52.
14. Tan GD, Neville MJ, Liverani E, et al. (2006) The in vivo effects of the Pro12Ala PPARgamma2 polymorphism on adipose tissue NEFA metabolism: the first use of the Oxford Biobank. Diabetologia 49, 158-68.
15. Havel RJ, Kane JP, Balasse EO, et al. (1970) Splanchnic metabolism of free fatty acids and production of triglycerides of very low density lipoproteins in normotriglyceridemic and hypertriglyceridemic humans. J Clin Invest 49, 2017-35.
16. Evans K, Burdge GC, Wootton SA, et al. (2008) Tissue-specific stable isotope measurements of postprandial lipid metabolism in familial combined hyperlipidaemia. Atherosclerosis 197, 164-70.
17. Vice E, Privette JD, Hickner RC, et al. (2005) Ketone body metabolism in lean and obese women. Metabolism 54, 1542-5.
18. Burdge GC & Wootton SA (2003) Conversion of alpha-linolenic acid to palmitic, palmitoleic, stearic and oleic acids in men and women. Prostaglandins Leukot Essent Fatty Acids 69, 283-90.
19. Grey NJ, Karl I & Kipnis DM (1975) Physiologic mechanisms in the development of starvation ketosis in man. Diabetes 24, 10-6.
20. Johnston DG & Alberti KG (1982) Hormonal control of ketone body metabolism in the normal and diabetic state. Clin Endocrinol Metab 11, 329-61.
21. Beylot M, Picard S, Chambrier C, et al. (1991) Effect of physiological concentrations of insulin and glucagon on the relationship between nonesterified fatty acids availability and ketone body production in humans. Metabolism 40, 1138-46.
22. Merimee TJ & Fineberg SE (1973) Homeostasis during fasting. II. Hormone substrate differences between men and women. J Clin Endocrinol Metab 37, 698-702.

23. Keller U, Gerber PP & Stauffacher W (1988) Fatty acid-independent inhibition of hepatic ketone body production by insulin in humans. Am J Physiol 254, E694-9.
24. Miles JM, Haymond MW, Nissen SL, et al. (1983) Effects of free fatty acid availability, glucagon excess, and insulin deficiency on ketone body production in postabsorptive man. J Clin Invest 71, 1554-61.
25. Mittendorfer B, Magkos F, Fabbrini E, et al. (2009) Relationship between body fat mass and free fatty acid kinetics in men and women. Obesity (Silver Spring) 17, 1872-7.
26. Wilding JP (2007) The importance of free fatty acids in the development of Type 2 diabetes. Diabet Med 24, 934-45.
27. Gibbons GF, Islam K & Pease RJ (2000) Mobilisation of triacylglycerol stores. Biochim Biophys Acta 1483, 37-57.
28. Avogaro A, Nosadini R, Bier DM, et al. (1990) Ketone body kinetics in vivo using simultaneous administration of acetoacetate and 3-hydroxybutyrate labelled with stable isotopes. Acta Diabetol Lat 27, 41-51.

Table 1. Volunteer characteristics and baseline overnight fasting plasma metabolite concentrations

	Men	SE	Women	SE
Age (years)	25.5	4.4	22.5	2.2
BMI (kg/m^2)	21.5	2.5	21.0	1.6
Waist (cm)	77.3	6.7	72.8	4.1
Fat mass (% body weight)	12.6	2.1	22.5***	3.9
Body weight (kg)	70.65	3.7	61.63*	1.59
NEFA (µmol/L)	296	26	557*	88
3-OHB (µmol/L)	39	7	64	12
TG (µmol/L)	1145	146	870*	93
Glucose (mmol/L)	5.1	0.8	4.8*	0.13
Insulin (mU/L)	11.2	1.2	8.2	1.8
$VLDL_1$-TG (µmol/L)	388	113	255	65
$VLDL_2$-TG (µmol/L)	207	40	129*	15

Values are means and SE, n= 6 /group. For metabolite concentrations values are means and SE, n= 6 /group after an overnight fast (12h). *P<0.05; ***P<0.001 vs men.

Table 2. NEFA kinetics and 3-OHB ^{13}C enrichment arising from the systemic NEFA pool at 420 min

	Men	SE	Women	SE
Ra NEFA (µmol/min)	594	101	533	61.5
Ra NEFA (µmol/min per kg fat mass) *	68.7	12.5	38.1	2.59
Rd NEFA (µmol/min per kg fat free mass)	9.4	1.2	11.2	1.2
3-OHB estimated maximal MPE**	0.0082	0.0050	0.0075 [a]	0.0010
3-OHB observed MPE	0.0033†	0.00028	0.0062 [a]	0.0017
3-OHB observed/ maximal MPE (%)	42‡	5.3	78	10[a]

Values are means and SE, n= 6 /group.[a]:n=5. Effect of gender over period of extended fasting (14-20 h) † $P<0.05$, ‡ $P<0.01$ vs women. ** $P<0.01$ time by gender. MPE: mole percent excess.

Table 3. Contribution of splanchnic and systemic fatty acids to VLDL-TG at 7 h in men and women.

	$VLDL_1$				$VLDL_2$			
	Splanchnic		Systemic		Splanchnic		Systemic	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	Absolute concentration of different fatty acid sources to VLDL-TG (µmol/l)							
Men	61.5	22	*205* §	*53*	52.3	12	118 §	20
Women	27.6	22	155 §	56	12.7	7.4	81.3 §	22
	Relative contribution of different fatty acid sources to VLDL-TG (%)							
Men	25.0	6.4	75.0 §	6.4	30.0	2.7	70.0 §	2.7
Women	14.7	5.5	85.3 §	5.5	14.5	5.5	85.5 *	5.5

Significantly different from men * $P < 0.05$. Significantly different versus splanchnic § $P < 0.05$.

Figure legends

Figure 1

Plasma concentrations of metabolites in response to continued overnight fasting, and analysed by repeated measures ANOVA. Solid circles, men (n = 6), open circles women (n = 6). Data points are shown as mean and SEM. **A**: Glucose. There was a significant effect of time (P <0.05) and gender (P <0.05). B: Triacylglycerol (TG). There was a significant effect of time (P < 0.001) and a tendency for an effect of gender (P = 0.087). **C**: Non-esterified fatty acids (NEFA). There were significant effects of time (P < 0.01) and gender (P <0.01). D: 3-hydroxybutyrate. There were significant effects of time (P < 0.01) and gender (P <0.05). E**:** Plasma $VLDL_1$ concentrations, men (n = 5), women (n = 5), in response to continued overnight fasting. There was a significant effect of time (P <0.05). F: Plasma $VLDL_2$ concentrations, there was a tendency for an effect of gender (P = 0.066).

Figure 2

Plasma tracer to trace ratios (TTR) resulting from an intravenous infusion of [U-^{13}C] palmitate, starting at time 0. **A**: Non-esterified fatty acids (NEFA, n = 6). There were significant effects of time (P <0.05), time by sex interaction (P <0.05) and a tendency for an effect of gender (P = 0.086). B: 3-hydroxybutyrate, n=5. There was a significant effect of time (P <0.001) and gender (P <0.05). **C**: $VLDL_1$. There was a significant effect of time (P < 0.001). D: $VLDL_2$. There was a significant effect of time (P <0.001).